PART I

The Transcription of Simple and Complex DNAs by the RNA Polymerase of *Escherichia coli*

PART II

The Structure and Replication of Intracellular Bacteriophage Lambda DNA

Thesis by

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I wish to dedicate this thesis to

my mother

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ABSTRACT

Part I of this thesis is a study of the *in vitro* transcription of DNA by *Escherichia coli* RNA polymerase. Experiments on the transcription of phage lambda DNA form Chapter 1. The transcription of lambda DNA is partially asymmetric. When symmetric transcription occurs, RNA so transcribed is found in a double-stranded form which is resistant to digestion by ribonuclease. The temperature of synthesis and the presence of Mn^{++} in the synthetic mixture affect the frequency of symmetric transcription. RNA-DNA hybridization experiments indicate that no more than 50% of the lambda genome (25% of the DNA) is transcribed *in vitro*.

Experiments on the transcription of some eucaryote DNAs are presented in Chapter 2. Only a small fraction (15-20%) of the RNA transcribed from these DNAs is observed to form hybrid with complementary DNA under a variety of conditions. That RNA which does form hybrid,is transcribed asymmetrically from a limited portion (about 10%) of the DNA. Evidence is presented for heterogeneity in the rate at which various RNA and DNA

sequences form hybrid. This heterogeneity is believed to be of the same nature as the heterogeneity revealed by renaturation studies of eucaryote DNA.

Part II of this thesis is a study of the structure and replication of intracellular phage lambda DNA. Studies on purified non-replicating intracellular lambda DNA are presented in Chapter 1. Three species of lambda DNA are present following infection of immune bacteria by lambda phage: a closed-circular molecule, component I; an open-circular molecule containing one or more single-strand breaks, component II; and linear phage DNA, component III. The physical and infective properties of these molecules are studied. Component I in the native or denatured state is found to be almost equally infective to spheroplasts. Component II, however, is infective in the native state and shows a large increase in infectivity upon denaturation. This is due to the liberation of single-stranded rings which are more efficient in infecting spheroplasts than are native DNA molecules. Component III decreases greatly in infectivity following denaturation. Evidence is presented from sedimentation studies of components I, II, and III which suggests that the pitch of the Watson-Crick helix is variable in solution.

Experiments on the fractionation of replicating intracellular lambda DNA by chromatography on benzoylated-naphthoylated DEAE cellulose (BNC) are presented in Chapter 2. Intracellular lambda DNA is labeled following induction and mitomycin C treatment (to suppress host DNA synthesis) of lysogens and the purified DNA is adsorbed to BNC. Components II and III along with native phage DNA are eluted from BNC by a gradient of 0.3-1.0M

NaCl. A subsequent gradient of 0-2% caffeine elutes a heterogeneous species of intracellular DNA. This species is rapidly labeled by short pulses of ^{3}H-thymidine and is virtually non-infective in the native state to spheroplasts. Denaturation of this species renders it very infective to spheroplasts suggesting that it contains single-strand rings. Analysis of the single-strand composition of this species by alkaline sedimentation reveals material sedimenting up to 1.5 times the rate of single-strand phage DNA. A model for DNA replication, involving initiation of one daughter strand by covalent addition to the 3'-OH of the identical parent strand, is presented based on the single strand composition of the DNA eluted from BNC by caffeine.

Supporting data from pulse and pulse-chase experiments are presented in Chapter 3. Approximately half of the label incorporated in very short pulses into material sedimenting at neutral pH as intracellular lambda DNA sediments in alkali faster than phage DNA single strands.

Very short pulses have revealed the presence of a small, rapidly labeled component in induced cells which appears to be DNA and sediments at about 10S at neutral and alkaline pH. The nature of this component is obscure at present.

TABLE OF CONTENTS

PART I

The Transcription of Simple and Complex DNAs by the RNA Polymerase of *Escherichia coli*

INTRODUCTION

The literature on RNA polymerase has been reviewed several times in the last few years (16,22,38, see also 39). This introduction will focus on those studies which bear on the specificity of interaction of bacterial RNA polymerase with DNA. The most carefully studied RNA polymerase is that of E. coli and we shall be primarily concerned with that enzyme.

Physical Properties of RNA polymerase

Physical studies of RNA polymerase have centered largely on sedimentation analysis. The highly purified enzyme is observed to have an $S_{20,w}$ of 21-24 at low salt concentrations (0.1 M KCl). Measurements in the analytical ultracentrifuge give $S_{20,w} = 21$. The higher value is observed upon zonal sedimentation through sucrose gradients and may be due to concentration effects. At high salt concentrations (0.5 M KCl or more) the enzyme sediments with $S_{20,w} = 13$, suggesting a dissociation of the molecule into subunits. The dissociation is reversible by dialysis of the enzyme to a low salt concentration (34,35,47). Equilibrium sedimentation in 0.1 M KCl gives $M_W = 9.0 \pm 0.9 \times 10^5$ daltons and in 0.5 M KCl gives $M_W = 4.5 \pm 0.4 \times 10^5$ daltons, indicating that at low salt concentrations the enzyme is a dimer (35).

Treatment at high pH in 6M urea is reported to irreversibly dissociate 24S molecules to 3S subunits. Disc electrophoresis separates these subunits into two components (12,50).

An abrupt transition from 21S to 13S molecules as the salt concentration is raised is not observed. Sedimentation of enzymatic activity through sucrose gradients of increasing salt concentration shows a transition of 21S activity to an activity sedimenting rather heterogeneously between 15S to 18S and then a transition to the 13S activity observed at high salt concentrations. It has been suggested that this phenomenon is due to a rapid equilibrium between dimer and monomer at intermediate salt concentrations (47) or to a heterogeneity in the ability of 13S molecules to aggregate (34).

It has been noted that aged preparations of enzyme lose activity and concomitantly the ability to form 21S molecules at low salt concentration, forming instead heterogeneous material of 16-19S (34,35). Chromatography on hydroxylapatite has been observed to fractionate RNA polymerase into a fraction capable of forming 21S molecules at low salt concentrations and another fraction capable of forming only 17S molecules at low salt concentrations(34).

Surprisingly, the most highly purified enzyme yet obtained is reported not to aggregate. In 0.1 M KCl this enzyme has an $S_{20,w} = 11$ and a $M_W = 3.7 \times 10^5$ daltons as determined by equilibrium sedimentation (29). The relation between the 11S particles and the 13S particles has not been determined nor has the nature of the material sedimenting between 13S and 21S molecules.

Electron microscopy of 24S particles shows them to consist of a hexagonal array of subunits to form a particle 125Å in diameter by 95Å deep. These particles are calculated to have a maximum molecular

weight of 9 x 10^5 daltons, assuming a partial specific volume of 0.74 (12,41). 18S particles are likewise reported to be a hexagonal array of subunits, and at high protein concentrations long rods are observed which appear to be hexagonal particles aggregated face to face (9).

Direction of RNA Transcription

If ^{32}P-$\beta\gamma$-labeled ATP is used as a precursor for RNA synthesized in vitro an incorporation of ^{32}P into RNA is observed (31). A similar result is observed if ^{32}P-γ-labeled GTP is used as precursor. Significantly less incorporation of ^{32}P-γ-labeled UTP and CTP is observed (1,28,30,5). The ^{32}P is introduced at the end of an RNA chain. Alkaline hydrolysis of the enzymatically synthesized RNA yields ^{32}P-labeled adenosine tetraphosphate, pppAp, and guanosine tetraphosphate, pppGp, from the 5' ends of the molecules, as well as unlabeled 2'-3' nucleoside monophosphates from internal positions and nucleosides from the 3' ends of the molecules. Kinetic and pulse-chase studies of the incorporation of ^{32}P-γ-labeled ATP or GTP indicate that chain initiation begins with a triphosphate (ATP or GTP) and synthesis proceeds from the 5' end toward the 3' end of the RNA chain (5,28). Thus at least two types of initiation sites must exist: one at which initiation is with ATP and another at which it is with GTP.

Binding of RNA Polymerase to DNA

The binding of RNA polymerase to DNA is highly dependent upon ionic strength and the presence or absence of triphosphates, i.e. whether RNA synthesis is possible. In the absence of triphosphates and divalent cations the enzyme is capable of binding to DNA at ionic strengths below 0.25 as determined either by sedimentation of the complex through sucrose gradients (10,34,36,47,46), by trapping of the complex on nitrocellulose filters (24,44) or by competition for enzymatic activity between DNA and dAT copolymer (29).

Below ionic strength = 0.1 it is clear that different DNA molecules bind a large but variable amount of RNA polymerase. T7 DNA is found to bind 2-3 μg of enzyme protein/μg DNA or approximately 50 enzyme molecules/molecule of DNA (24,26). Similar values have been reported for lambda DNA (46) and polyoma DNA (10) although under slightly different conditions the only limit to saturation seems to be the space available along the DNA molecule (34). The complexes formed at low salt concentrations are at least partially dissociable upon dilution or upon addition of uncomplexed DNA or copolymer (24,29,34,36,44). Competition studies of uncomplexed DNA with saturated complexes suggest that there are at least two types of binding at low salt concentrations: a readily reversible binding and one that is virtually irreversible. The number of strong (irreversible) binding sites is estimated to be 7-8 for T7 DNA and 5-6 for lambda DNA (44). Further evidence for two types of binding is obtained from sedimentation studies of polyoma DNA-RNA

polymerase complexes. The amount of enzyme that can bind to polyoma DNA and be enzymatically active in the complex is approximately 2.5 μg protein/μg DNA. Upon progressive addition of more enzyme to such complexes the sedimentation rate of the complex is observed to increase progressively, but the bound enzyme activity does not increase above the activity observed at 2.5 μg protein/μg DNA unless additional DNA is added to the complex after sedimentation (10,34). Thus it has been suggested that a type of site exists to which an enzyme molecule can bind specifically and initiate RNA synthesis upon addition of divalent cation and triphosphates. Binding can also occur, it is suggested, to other regions of the DNA molecule in a non-specific manner which does not lead to initiation under conditions where RNA synthesis is permitted and in fact reversibly inactivates the enzyme (34). Inactivation of RNA polymerase by T4 DNA has also been observed (4) and this phenomenon may be responsible for the sigmoidal dependence of RNA synthesis upon enzyme concentration that has been reported (30).

Denaturation of DNA leads to an increase in the amount of RNA polymerase that can be bound per unit of DNA (24,29,44). For example denatured lambda DNA is estimated to contain 90 strong binding sites per genome compared to only 5-6 for native DNA.

RNA polymerase has been reported to bind strongly to the ends of native DNA molecules, perhaps due to single-stranded regions at the ends of molecules (2). Electron micrographs, however, indicate that the majority of the enzyme molecules bound are bound to internal sites (10, 41), and shearing of DNA to smaller pieces does not increase its capacity to synthesize RNA (4).

Complexes formed at low salt concentrations become markedly unstable as the ionic strength is increased and are completely dissociated between 0.2-0.3 M KCl (24,34,46). Interestingly, the enzyme is still quite active at KCl concentrations of 0.3-0.4 M indicating that when RNA synthesis is permitted the enzyme need not bind strongly for initiation to occur (34,43). Studies on RNA polymerase binding to polyoma DNA at ionic strengths between 0.20 and 0.225 indicate that only specific binding, i.e. binding leading to initiation upon addition of triphosphates, occurs even at a weight ratio of enzyme to DNA of 1000/1. DNA induced inactivation of RNA polymerase is not observed at these high ionic strengths, and a saturation of 0.6-1.2 μg enzyme protein/μg DNA is observed (34). This saturation value is only 25-50% of the specific saturation value obtained at low ionic strength. This fact, coupled with the fact that there is some uncertainty as to the number of active molecules in a preparation of RNA polymerase casts doubt on any attempt to equate the number of bound enzyme molecules with the number of transcriptional units in a DNA molecule.

Below an ionic strength of 0.1 a dimerization of the 13S RNA polymerase particles occurs (see above). Both 21S dimer and 13S monomer exhibit enzymatic activity. The possibility cannot be excluded that the 13S monomer exhibits activity only after a DNA-mediated dimerization occurs. However, there is no compelling evidence to indicate which if either of these forms is the "native" molecule (29,34). Under ionic conditions which allow both 13S and faster sedimenting enzyme molecules to exist (conditions which permit non-specific binding) the enzyme

molecules sedimenting faster than 13S complex preferentially with DNA. It has been suggested that 13S particles are heterogeneous with respect to their capacity to aggregate and that only those molecules which do aggregate may engage in non-specific binding (34).

In summary, binding studies suggest two classes of sites on the DNA molecule: sites at which initiation of RNA synthesis occurs and sites at which binding is possible but initiation is not. No strong evidence exists for or against binding heterogeneity within these two classes. Further, some binding studies suggest heterogeneity of the RNA polymerase.

Termination of RNA Synthesis

Once RNA synthesis has begun the complex of DNA-enzyme-nascent RNA is non-dissociable by all but very destructive means (3,17,29,36,37). The complex is stable in 0.5 M KCl. Treatment with ionic detergent or phenol is reported to dissociate the nascent RNA from the complex (3,37). The presence of an RNA-DNA hybrid at the growing end of the nascent RNA chain which is not dissociable by detergent or phenol has been demonstrated (17, see also 25), and it seems likely that the reports of detergent or phenol induced dissociation are due to partial degradation of the RNA.

Transcription *in vitro* under conditions where initiation is possible indicates that no more than one RNA molecule/enzyme molecule is synthesized, in agreement with the observation that the complex is non-dissociable (3). Prolonged synthesis at low salt concentration can

result in complexes containing RNA chains of molecular weight 2-3 x 10^6. Synthesis under conditions where RNA polymerase is in large excess and initiation is possible (low salt) can lead to synthesis of up to 50 RNA molecules/T7 DNA (3,37).

The formation of large complexes after extended RNA synthesis is believed to be responsible for the inhibition of RNA polymerase activity (product inhibition) that is observed. Normally RNA synthesis ceases after 1-2 hours of incubation. Removal of the synthesized RNA by addition of RNase allows pyrophosphate liberation to continue for many hours at a constant rate (29,26). Product inhibition is not observed if transcription is carried out in 0.2 M KCl (43) suggesting that release of the RNA product from the complex may occur. This point needs more direct confirmation.

There is evidence that *in vitro* polypeptide synthesis is much more efficient in a coupled DNA-RNA polymerase-protein synthesizing system than it is if synthetic RNA is first purified and then added to a polypeptide synthesizing system (51). This fact together with the observation that nascent RNA is not released from the complex has led to speculation that *in vivo* protein synthesis and RNA transcription are intimately coupled (45). This would seem to be born out by the observation that amber and ochre codons lead to RNA chain termination *in vivo* although it is evident that these codons cannot be the normal termination signal (23,49). Addition of 70S ribosomes to a DNA-RNA polymerase-RNA complex formed *in vitro* leads to dissociation of at least some of the complexes (25).

The Fidelity of Transcription

There is very good evidence to indicate that *in vivo* RNA synthesis is coded by only one strand of the DNA in any given unit of transcription (6,48,49, see 38 for review). This will be referred to as asymmetric transcription.

Virtually asymmetric transcription has been obtained *in vitro* using purified *E. coli* RNA polymerase and native (but broken) T2 or T4 DNA (13,15,27) ØX174 RF (18) and lambda DNA (33). Thus the interaction between purified enzyme and DNA is sufficient under proper conditions to give asymmetric transcription. The same enzyme preparations that asymmetrically transcribe T2 DNA *in vitro* give only partially asymmetric transcription of T7 DNA and SP01 DNA as judged by complementarity of the synthetic RNA. RNA polymerase purified from *M. lysodeikticus* gives symmetric transcription of all DNA templates tested (8). The achievement of asymmetric transcription *in vitro* is proof that some specific feature of the DNA secondary structure provides a site to which RNA polymerase can bind specifically and initiate transcription from the proper DNA strand. There is some evidence that these sites may be regions of the double-helix containing purine tracts on one strand and complementary pyrimidine tracts on the other (48,49). Denaturation of the template in all cases leads to symmetric transcription through the formation of a DNA-RNA hybrid molecule (8,14,15,18,52,53,54).

In view of the heterogeneity of binding interactions observed *in vitro* it is not surprising that both asymmetric and symmetric trans-

cription have been reported by different (and sometimes identical) investigators (see 8 and 13 for summary of the conflicting literature). This may indicate that different species of DNA molecules do not possess totally identical initiation sites. Partially asymmetric transcription could be due to a heterogeneity of sites or to a heterogeneity of possible interactions at identical sites.

Another type of specificity exhibited *in vitro* by *E. coli* RNA polymerase is the ability to preferentially transcribe those genes expressed early in infection by T2, T4 and lambda phage. T2 and T4 messenger RNA synthesized *in vivo* early in infection is able to compete with more than 90% of the RNA transcribed *in vitro* for complementary sites on denatured DNA. The converse experiment shows that RNA synthesized *in vitro* is able to compete with more than 90% of the *in vivo* messenger RNA suggesting that no species of early messenger RNA is not transcribed *in vitro* (13). Varying the conditions for *in vitro* transcription does not lead to increased synthesis of late messenger RNA. There is no indication that the relative abundances of RNA species synthesized *in vivo* and *in vitro* are identical for T2 or T4. A T4-specific factor has been identified in infected cell lysates which allows the transcription of late messenger *in vitro* but its nature is obscure at present (42,50).

Genetic studies (11,20) and hybridization studies with *in vivo* messenger RNA (6,33,40,49) have shown that early and late genes are clustered on the lambda genome. The lambda DNA molecule can be broken in half by shear and the two halves separated by means of their dif-

ferent base compositions (19,32). Messenger transcribed from the repressed prophage and early *in vivo* messenger is found by hybridization studies to be transcribed only from the AT rich half of the molecule (right half). Later in the course of infection transcription commences from the GC rich half of the molecule (left half) (6,33,49). RNA transcribed *in vitro* with purified *E. coli* RNA polymerase is found to preferentially form hybrid with DNA from the right half of the molecule. However, 24-38% of the total RNA synthesized can form hybrid with DNA from the left half of the molecule, the higher values being obtained when RNA is synthesized using a high ratio of enzyme/DNA (7).

Whole complementary strands of denatured lambda DNA can be separated by buoyant density after complexing with G or IG copolymer (21). Hybrid formation by messenger RNA with one strand or the other of the DNA has allowed the direction of synthesis and the coding strand of the DNA to be determined for individual genes. *In vivo* messenger RNA is found to be transcribed asymmetrically from both strands of the right half of the DNA molecule. Approximately 10% of the messenger is transcribed from the heavy strand and the remainder from the light strand (6,49). RNA transcribed *in vitro*, however, while transcribed preferentially from the right half of the molecule forms hybrid equally with both heavy and light strands (6,7). This observation may either mean that some regions transcribed *in vitro* are not transcribed *in vivo* or that *in vivo* many more copies of the same messenger are transcribed from the light strand than from the heavy strand while the rate of transcription *in vitro* is equal from both strands.

In summary, a great deal of the specificity of transcription observed *in vivo* is maintained *in vitro* by purified *E. coli* RNA polymerase. The basis for this specificity is not well understood nor are the reasons for its loss which is observed on occasion. Purification of RNA polymerase does not yield a unique entity and the possibility can be raised that the native molecule has yet to appear.

The first chapter, Part I, of this thesis investigates some of the properties of RNA transcribed from native lambda DNA and some of the factors which lead to asymmetry of transcription. Evidence is presented which indicates that only a portion of the genome is transcribed *in vitro* by *E. coli* RNA polymerase. This work was originally performed as a control for the work presented in chapter two.

In the second chapter the transcription of some eucaryote DNAs by *E. coli* RNA polymerase is examined by means of RNA-DNA hybrid formation. Evidence is presented to show that the RNA transcribed *in vitro* is heterogeneous with regard to the rate at which it anneals to complementary DNA. A small fraction of the RNA forms hybrid with denatured DNA at a rate comparable to the rate of formation of lambda RNA-DNA hybrid. This fraction is shown to be transcribed from a limited portion of the DNA.

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Chapter 1

Transcription of Lambda DNA

1. MATERIALS AND METHODS

(a) Solutions, chemicals and enzymes

All solutions are made with distilled, deionized water and are sterilized before use.

SSC is 0.15M NaCl, 0.015 M Na_3 citrate. 0.1x, 1x, 2x etc. indicates the degree of concentration of this solution.

Citrate buffer is 0.01M Na_3 citrate-HCl, pH 5.4, 0.02M NaCl.

Acetate-EDTA is 3.0M Na acetate, 0.001M EDTA pH 7.0.

^{3}H-ribonucleotide triphosphates are purchased from Schwartz Bioresearch, Inc. Unlabeled ribonucleotide triphosphates are purchased from either CalBiochem, Inc. or Sigma Chemical Co. and stored at -20°C. Biogel P-30 and P-100 are purchased from Bio-Rad Laboratories. Ribonuclease-A, (RNase) Type II-A, 90 Kunitz units/mg is purchased from Sigma Chemical Co., dissolved at 5mg/ml in 0.01M Na_3citrate-HCl, pH 5.0, boiled 10 minutes, quickly cooled and stored frozen at -20°C. Electrophoretically purified deoxyribonuclease (DNase) is from Worthington Biochemical Co.

^{32}P-MS2 RNA is a gift from Drs. R. Kelly and G.N. Godson.

(b) Preparation of Lambda b_2b_5c DNA

The growth and purification of lambda phage are described in Chapter 1, Part II of this thesis. Extraction of phage DNA is described

by Young and Sinsheimer (32). DNA preparations are dialysed against 0.1 x SSC. Preparations are estimated to be at least 95% whole molecules by boundary sedimentation in the analytical ultracentrifuge.

(c) Preparation of RNA Polymerase.

E. coli cells, strain B, 2% early log phase are purchased from General Biochemicals, Chagrin Falls, Ohio. The procedure of Chamberlin & Berg (3) is used to prepare RNA polymerase. Fraction 3 is purified by chromatography on DEAE cellulose (Brown & Co., Keene, New Hampshire) or on DEAE Sephadex A-50 as described by Bremer & Konrad (2). The purified enzyme is stored frozen at -70°C.or in liquid nitrogen in sealed ampules dissolved in glutathione buffer (13). Preparations vary in specific activity from 1000-3000 units/mg of protein. In the absence of added template the enzyme preparations incorporate 0-6% of the radioactivity incorporated in the presence of added template. The optimum ratio of RNA polymerase/DNA and the optimum length of incubation are usually determined for each preparation of RNA polymerase; optimum synthesis is considered to be the maximum synthesis of RNA for a minimum amount of RNA polymerase.

(d) In vitro Synthesis of RNA

The conditions used for synthesis of RNA are similar to those described by Wood & Berg (31) and contain in 1.0 ml: 0.08 ml, 1M Tris,

pH 7.9; 0.04 ml, 0.1M $MgCl_2$; 0.1 ml, 0.1M $MnCl_2$; 0.01 ml, 1M β-mercaptoethanol; 0.01 ml, 0.01M EDTA; 0.03 ml of each of the ribonucleoside triphosphates (25 μm/ml); a radioactively labeled triphosphate to give the desired specific activity; 50-160 μg of DNA; and 1.5-6.0 times the weight of the DNA of RNA polymerase. In some cases $MnCl_2$ is omitted and the $MgCl_2$ concentration increased to keep the concentration of divalent cation constant. The temperature of synthesis is 37°C unless stated otherwise.

After one to two hours of incubation the reaction is stopped by the addition of 100 μg/ml of DNase and incubation continued for 30 minutes. The mixture is then phenol extracted twice with an equal volume of re-distilled phenol equilibrated with 0.1 x SSC. The phenol layers are then re-extracted with 0.1 x SSC and the aqueous layers combined. A tenth volume of acetate-EDTA is added and the nucleic acid precipitated by the addition of 2 volumes of cold 95% ethanol. The precipitate is collected by centrifugation, dissolved in citrate buffer and passed through a column of Biogel P-100 (sometimes P-30 is used) (2 cm x 15 cm) equilibrated with citrate buffer layered over a column of Amberlite IRC-50 (2 cm x 2.5 cm) (previously washed and equilibrated with citrate buffer) (31). The material appearing as a symmetrical peak is precipitated as described above, dissolved in 0.1 x SSC and stored frozen. Generally the mass of RNA synthesized does not exceed the mass of the template DNA. RNA concentrations are calculated from the specific activity of the reaction mixture and agree with the value obtained by optical density at 2600Å.

(e) Hybrid Formation

RNA-DNA hybrid formation is carried out in 2 x SSC at 60°C. DNA is heat-denatured in 0.1 x SSC in a boiling water bath for 10 minutes followed by rapid cooling to 0°C. RNA-DNA hybrids are assayed by the procedure of Nygaard & Hall (21) using Schleicher and Schuell B6 nitrocellulose filters. The hybrid solution in 2 x SSC is passed through the filter and the filter washed with 60 ml of 2 x SSC at room temperature. Filters are dried and counted in toluene base scintillation fluid prepared with Liquifluor (Pilot Chemicals, Inc.). If RNase-resistant hybrid is to be assayed the hybrid is treated with RNase (5 μg/ml) in 2 x SSC at 37°C for 10 minutes prior to filtration.

The background of RNA trapped on the filter in the absence of DNA is determined for each experiment and subtracted from the data; this value is approximately 1% of the radioactivity passed through the filter.

DNA saturation experiments are performed according to the procedure of Gillespie & Spiegelman (11). Denatured DNA is applied to nitrocellulose filters in 6 x SSC and the subsequent incubation and washing of the filters is with 6 x SSC (11). Filters are 13 mm in diameter, punched to lay on the bottom of 1 dram shell vials. This permits incubation with RNA in a volume as small as 0.25 ml. Each vial always contains a filter with no DNA to measure the RNA bound non-specifically to the filter. RNase treatment of the filters is described by Gillespie & Spiegelman (11). After RNase treatment and washing the

RNA bound non-specifically to the filter is approximately 0.02% of the RNA in contact with the filter.

(f) Acid Precipitation of Nucleic Acids

Radioactively labelled nucleic acids are quantitated by precipitation in 5% trichloroacetic acid. 50-100 μg/ml of denatured calf thymus DNA (Sigma Chemical Co.) are used as carrier. Precipitates are collected on Whatman GFA glass fiber filters, dried, and counted as described above.

2. RESULTS

(a) Properties of Synthetic Lambda RNA

(i) Ribonuclease Sensitivity

Lambda RNA transcribed *in vitro* is only partially sensitive to digestion by ribonuclease compared to MS2 RNA. This partial sensitivity is evident only when digestion is carried out at a high salt concentration (2 x SSC). Figure 1 shows the rate of digestion of a mixture of ^{3}H-lambda RNA and ^{32}P-MS2 RNA at high and low salt concentrations (Figure 1, a&b). Such behavior is indicative of double-stranded RNA (9). Increasing the temperature of incubation with RNase to 60°C does not alter the observed fraction of ribonuclease-resistant RNA (Figure 1c) demonstrating that this structure persists

under the conditions used to form RNA-DNA hybrids. The presence of 35-40% double-stranded RNA suggests a rather high frequency of symmetric transcription.

(ii) Kinetics of Double-Stranded RNA Synthesis.

The kinetics of synthesis of total RNA and double-stranded RNA is shown in Figure 2. At the indicated times after the start of incubation 10 µl of the reaction mixture were diluted into 0.2 ml of 2 x SSC and frozen immediately on dry ice. The samples were thawed in a water bath at 62°C for 5 minutes to destroy the enzyme (22). An aliquot of each sample was precipitated to determine total RNA synthesis and the remainder of each sample was treated with RNase (46 µg/ml) and DNase (58 µg/ml) in the presence of 0.05 M $MgSO_4$ at 37°C for 30 minutes followed by precipitation. The action of the RNase was monitored by the addition of ^{32}P-MS2 RNA to each sample prior to digestion but is not shown in Figure 2 as digestion was complete (only approximately 2% of the ^{32}P-RNA remained acid-precipitable). It should be noted that the double-stranded RNA is synthesized right from the beginning of incubation and parallels total RNA synthesis rather closely.

(iii) The Effect of Temperature on the Synthesis of Double-Stranded RNA.

Identical reaction mixtures were prepared and incubated at temperatures between 20°C and 46°C for one hour. The relative amount of RNA synthesized at each temperature is shown in Figure 3a. Each

RNA sample was purified by treatment with DNase (160 μg/ml) for 30 minutes at the temperature of synthesis followed by phenol extraction, ether extraction of the phenol from the aqueous layer, and removal of the ether by nitrogen gas. The ribonuclease-resistance of each sample is shown in Figure 3b.

The optimum temperature for RNA synthesis is between 30°C and 37°C. The synthesis of double-stranded RNA increases sharply between 30°C and 37°C, however (see Chapter 2 for additional data).

Several explanations of these observations are permitted. Asymmetrically transcribed RNA may prime the synthesis of a complementary strand (17) in a reaction possessing a different temperature coefficient than the transcription of DNA. Or symmetric transcription may occur with a different temperature coefficient for initiation on each complementary strand. In the latter case since RNA is not released from the complex (see Introduction) annealing of complementary RNA molecules must occur either simultaneously with synthesis or subsequent to synthesis while bound in the complex.

If formation of double-stranded RNA results from annealing after synthesis of a strand is completed a temperature dependence for this annealing would be expected and might explain the observed temperature effect. Thus RNA synthesized at temperatures below 37°C may be the product of the same degree of symmetric transcription observed at 37°C but may not have had the same opportunity to anneal as the RNA synthesized at 37°C.

In order to test this last point the RNA synthesized at various temperatures was incubated at 60°C for 17 hours in 2 x SSC (conditions that allow annealing of complementary RNA strands, see below). No change in the relative fraction of ribonuclease-resistant RNA was observed. Thus the effect noted in Figure 3 must be upon the transcription mechanism of RNA polymerase. It is interesting to note that all of the complementary RNA synthesized (whatever the mechanism) appears as double-stranded RNA.

If Mn^{++} is omitted from the reaction mixture the amount of double-stranded RNA synthesized at a given temperature is further reduced (see Chapter 2 of this thesis).

(iv) Denaturation and Renaturation of Double-Stranded RNA

The majority (80% or more) of the double-stranded RNA can be made sensitive to RNase in 2 x SSC by prior treatment in a boiling water bath for 10 minutes in 0.005 M EDTA, pH 7.9, followed by rapid cooling to 0°C and dilution into 2 x SSC containing RNase.

The double-stranded RNA reforms upon return to higher salt concentrations. Reannealing of the complementary RNA is concentration dependent as demonstrated in Figure 4. Incubation in 2 x SSC at 60°C allows the ribonuclease-resistant RNA to reform. However, prolonged incubation never leads to the formation of a larger fraction of double-stranded RNA than existed subsequent to synthesis, in agreement with the experiments in the previous section. Thus transcription is only partially symmetric. The RNase-sensitive RNA appears to be the product of asymmetric transcription.

(v) Sedimentation of Total RNA and Double-Stranded RNA

The sucrose gradient sedimentation pattern of total RNA and ribonuclease-resistant RNA is shown in Figure 5. ^{32}P-MS2 RNA is used as a marker. The $S_{20,w}$ of MS2 RNA under these conditions is approximately 31 (27). Thus the average $S_{20,w}$ of the total RNA is about 6 and that of the double-stranded RNA about 5. The value of 6S is in agreement with that obtained by Geiduschek, Nakamoto & Weiss (10) for T4 RNA synthesized *in vitro* and by Chamberlin & Berg (3). This value is probably only an indication of the level of RNase contamination in the RNA polymerase preparation. Cohen, Maitra & Hurwitz (7) using RNA polymerase free of RNase (16) find that a large proportion of RNA transcribed *in vitro* from lambda DNA sediments at 23S or more. Such large lambda RNA molecules have also been detected *in vivo* (15).

(b) The Ability of Synthetic RNA to Form Hybrid with Complementary DNA

For reasons discussed more fully in Chapter 2 of this thesis it is of interest to determine how much of the RNA synthesized *in vitro* using lambda DNA as template can form hybrid with denatured lambda DNA. The kinetics of RNase-resistant hybrid formation in solution between lambda RNA and DNA is shown in Figure 6. A solution containing 1.8 μg/ml ^{3}H-RNA (35% double-stranded) and 15 μg/ml denatured DNA is incubated at 60°C in 3 x SSC and hybrid formation assayed at the indicated times. Five hours after the beginning of incubation the DNA concentration was doubled in a portion of the mixture and thereafter assayed for hybrid in parallel with the original mixture.

After 11 hours 35% of the RNA had formed RNase-resistant hybrid. This corresponds to 54% of the single-stranded RNA. As shown above double-stranded RNA is stable under the conditions used for hybrid formation and would not be expected for form hybrid. After one hour of incubation the rate of hybrid formation decreases abruptly and then remains constant for the duration of the experiment. Addition of more, freshly denatured, DNA after five hours of incubation increases the rate of hybrid formation but not to the degree observed during the first hour of incubation. A brief extrapolation of the curve in Figure 6 suggests that considerably more than 54% of the single-stranded RNA would form hybrid.

Similar kinetics of hybrid formation between synthetic T4 RNA and T4 DNA have been observed by Green (13) who finds that only 45% of the RNA will form hybrid upon prolonged incubation. However, if the unhybridized RNA is isolated from the hybrid by filtration through a nitrocellulose filter 45% of this RNA is again capable of hybrid formation. Thus the limited hybrid formation observed here (54%) is most probably a kinetic phenomenon and does not indicate that some fraction of the RNA is incapable of hybrid formation. Nor does it necessarily imply much heterogeneity of the RNA with regard to hybrid formation.

In order to determine if the double-stranded RNA synthesized in vitro can form hybrid with denatured DNA, RNA was heat denatured as described above and incubated with denatured DNA at an RNA concentration of 0.019 μg/ml. At this RNA concentration double-stranded RNA does not reform when incubated under conditions conducive to hybrid

formation (see above). As a control native RNA was incubated with denatured DNA at the same RNA concentration. As is evident in Figure 7 denaturation of the RNA increases the amount of hybrid formed with denatured DNA.

A similar experiment performed at an RNA concentration of 1.4 µg/ml showed no increase in the amount of hybrid formed by denatured RNA compared to the control. This indicates that annealing of complementary RNA molecules is preferred to hybrid formation between complementary RNA and DNA molecules as is indicated by the work of others (5,23).

(c) The Ability of Denatured DNA to Form Hybrid With Synthetic RNA

It is of interest to know how much of the lambda genome is transcribed *in vitro* by *E. coli* RNA polymerase. RNA synthesized under optimal conditions ($30^{\circ}C$ in the absence of Mn^{++} giving 16% ribonuclease-resistant RNA) is incubated with 100 mµg of denatured DNA bound to nitrocellulose filters at high ratios of RNA/DNA. Figure 8 shows the kinetics of hybrid formation under these conditions. It is evident that at very high RNA/DNA ratios a limit is quickly reached in the amount of hybrid that can form. This limit is reached more slowly at lower RNA/DNA ratios and not attained at still lower ratios. 100 mµg of DNA can form hybrid with no more than 25 mµg of RNA.

The above value represents a limitation in the amount of DNA that can form hybrid for the following reasons. First, incubation of a DNA containing filter, that has previously been saturated in its ability to form hybrid, with fresh, unhybridized RNA does not increase the

amount of hybrid formed. Second, RNA that has saturated the ability of one DNA containing filter to form hybrid is capable of subsequently saturating the same amount of DNA on another filter, indicating that RNA is not limiting.

The value of 25 mμg RNA/100 mμg DNA is a minimum value since some DNA may have been lost from the filters during incubation and subsequent washing. In another experiment similar to that of Figure 8, the loss of DNA is monitored using ^{32}P-labeled lambda DNA on the filters. Figure 9 shows that the loss of DNA is not serious. The average of the values in Figure 9, all of which should be at saturation according to the data of Figure 8, is 27 mμg RNA/100 mμg DNA.

3. DISCUSSION

The synthesis of ribonuclease-resistant RNA in vitro with lambda DNA as template has not been previously reported. Naono & Gros (20) report no more than a few percent of their enzymatically synthesized lambda RNA to be RNase-resistant, and the question apparently has not been investigated by others (6,7,12).

Synthesis of ribonuclease-resistant RNA employing polyoma DNA as template is reported by Winocour (30) who finds 26% of this RNA is insensitive to RNase. Chamberlin & Berg (3) have reported similar findings with other templates. Geiduschek, Moohr & Weiss (9) using T2 DNA as template for M. lysodeikticus RNA polymerase find 18% of the synthesized RNA to be ribonuclease-resistant immediately after

synthesis. However, this RNA upon annealing becomes more than 80% double-stranded in agreement with the findings of others that M. lysodeikticus RNA polymerase always gives symmetric transcription (8). Colvill et al. (8) find that E. coli RNA polymerase using T7 and SPO1 DNA as templates gives partially complementary RNA products as determined by annealing. It is not possible to determine if this complementary RNA is synthesized in a double-stranded form as the purification procedure employed (phenol extraction at 75°C) would be expected to denature double-stranded RNA (18).

The data presented here do not distinguish between the two following explanations for the synthesis of complementary lambda RNA. 1. Transcription of the lambda DNA is completely asymmetric. Subsequently, initiation occurs on some of the product RNA molecules followed by synthesis of a complementary strand which remains hydrogen-bonded to the template RNA. This model is suggested by the fact that initiation of RNA synthesis on viral RNA templates is known to occur (17,19), and the product of synthesis on a single-stranded template is usually noted to be a hybrid molecule (4,5,24). 2. Initiation occurs on both strands of the DNA template at different frequencies, initiation on in vivo initiation sites being favored. Improper initiation on the non-coding strand may be favored with lambda DNA due to the presence of single-stranded ends (14,26) to which RNA polymerase can bind strongly (1). Subsequent to synthesis the complementary RNA molecules may anneal rapidly due to their close association in the synthetic complex (see introduction). The effect of Mn^{++} on synthesis

of complementary RNA may be due to the ability of Mn^{++} to increase the number of RNA polymerase molecules bound to lambda DNA (25).

The DNA saturation experiments reported here indicate that no more than 50% of the lambda genome, 25% of the total DNA, is transcribed in vitro by E. coli RNA polymerase. This is in agreement with the findings of Cohen & Hurwitz (6) and Cohen, Maitra & Hurwitz (7) that only the right half of the molecule is transcribed in vitro. The value of 50% (given here) for the fraction of the genome transcribed suggests that all of the right half of the molecule is asymmetrically transcribed in vitro.

In vivo studies indicate that 90% of the messenger synthesized early in infection is transcribed from the light strand (6,28). In view of the number of early genes known to be transcribed from the heavy strand (x,y, c_{II}, O, P, Q) it seems unlikely that 90% of the early genes are transcribed from the light strand. Thus a control mechanism that allows more copies of messenger to be transcribed from the light strand than from the heavy strand seems to be operating in vivo. This control is not inherently a part of the interaction of purified RNA polymerase with purified DNA. Gentle lysates of infected cells maintain the pattern of messenger synthesis observed in vivo but sonication of these lysates results in a pattern of synthesis observed in vitro with purified RNA polymerase, i.e. equal hybrid formation of synthetic RNA with both heavy and light strands (6).

In summary, purified RNA polymerase does not transcribe the entire genome of phage lambda. Those genes that it is capable of trans-

scribing, it transcribes in a manner not observed *in vivo* both with regard to the frequency of transcription of different genes and with regard to the asymmetry of transcription. The data presented here lend confidence to the belief that conditions can be found which will allow at least the asymmetry of transcription to be expressed *in vitro*.

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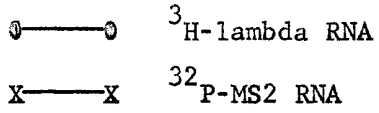
Figure 1. Ribonuclease-Sensitivity of Synthetic Lambda RNA and MS2 RNA.
(a) ^{3}H-Lambda RNA and ^{32}P-MS2 RNA are combined in 2 x SSC and treated with RNase (14 μg/ml) at 37°C for the indicated time followed by precipitation of an aliquot.
(b) ^{3}H-lambda RNA and ^{32}P-MS2 RNA are combined in 0.1 x SSC and treated with RNase (0.37 μg/ml) at 37°C as in (a). It is evident that the RNase is partially inhibited by the high salt concentration used in (a).
(c) ^{3}H-lambda RNA and ^{32}P-MS2 RNA are combined in 2 x SSC, 0.01M EDTA and treated with RNase (11 μg/ml) at 60°C as in (a).



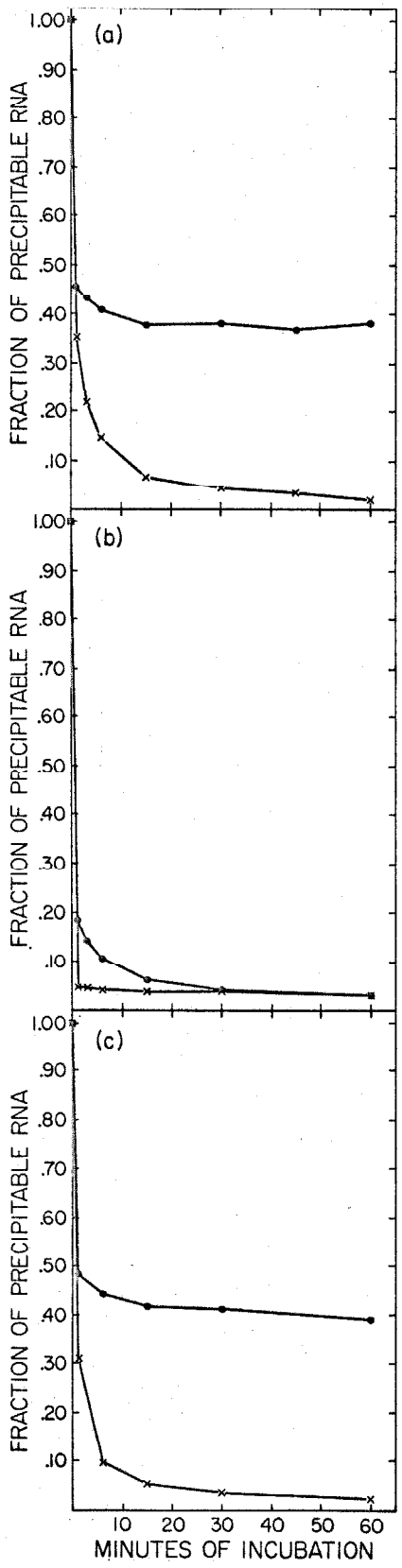
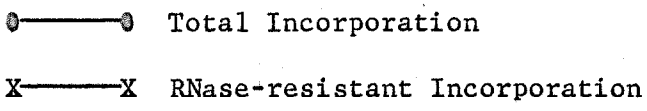

(a)
(b)
(c)
FRACTION OF PRECIPITABLE RNA
1.00
.90
.80
.70
.60
.50
.40
.30
.20
.10
10 20 30 40 50 60
MINUTES OF INCUBATION

Figure 1.

Figure 2. Kinetics of RNA Synthesis. RNA is synthesized as described in Methods and the incorporation of ^{3}H-UTP into acid precipitable form measured.



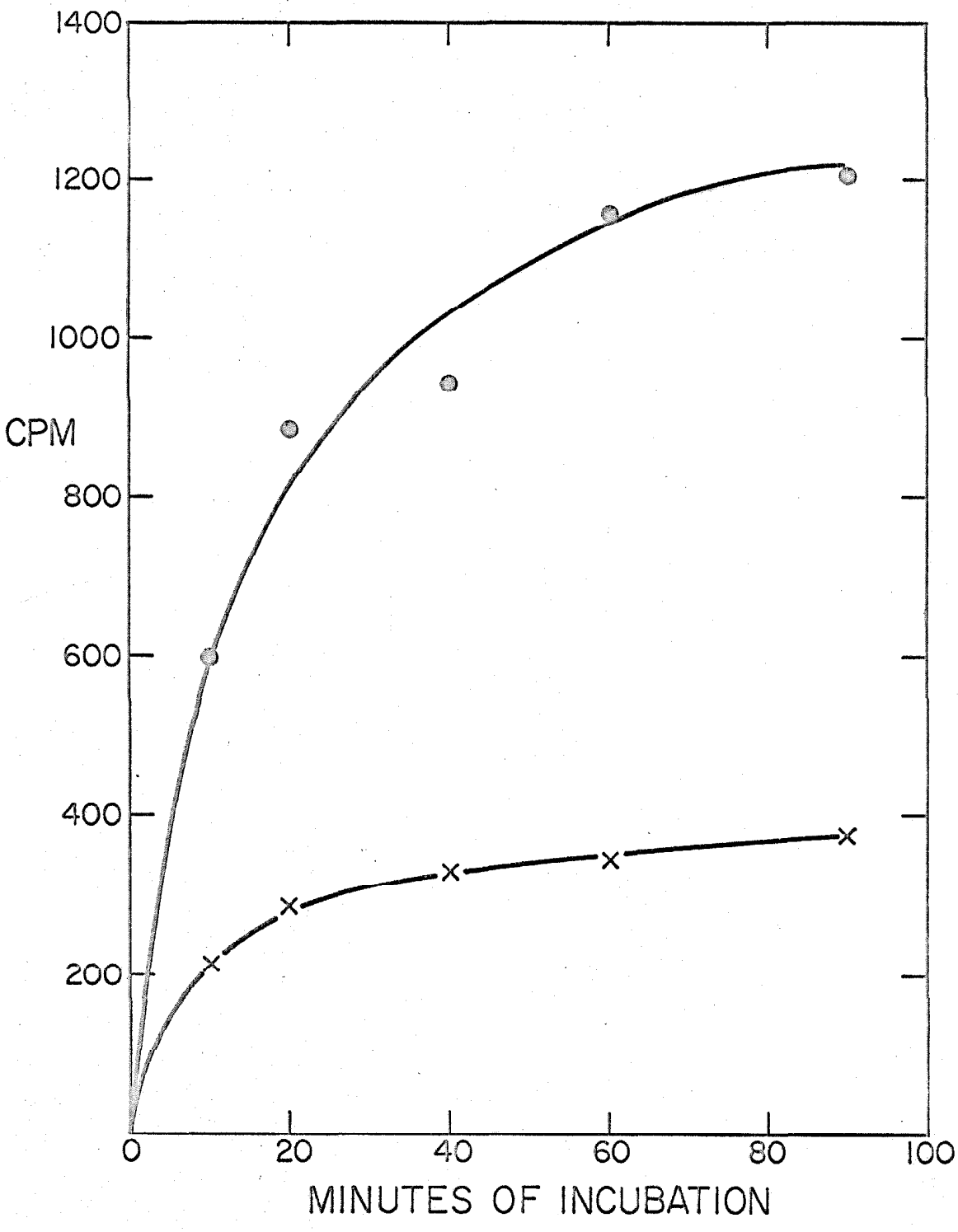

1400
1200
1000
CPM
800
600
400
200
0
20
40
60
80
100
MINUTES OF INCUBATION

Figure 2.

Figure 3. The Effect of Temperature on RNA Synthesis.

(a) RNA synthesized in equivalent reaction mixtures incubated for one hour at the indicated temperature. Composition of the reaction mixture is described in Methods.

(b) Fraction of RNase-resistant RNA synthesized in (a). RNase-resistance is determined as described in Figure 1a.

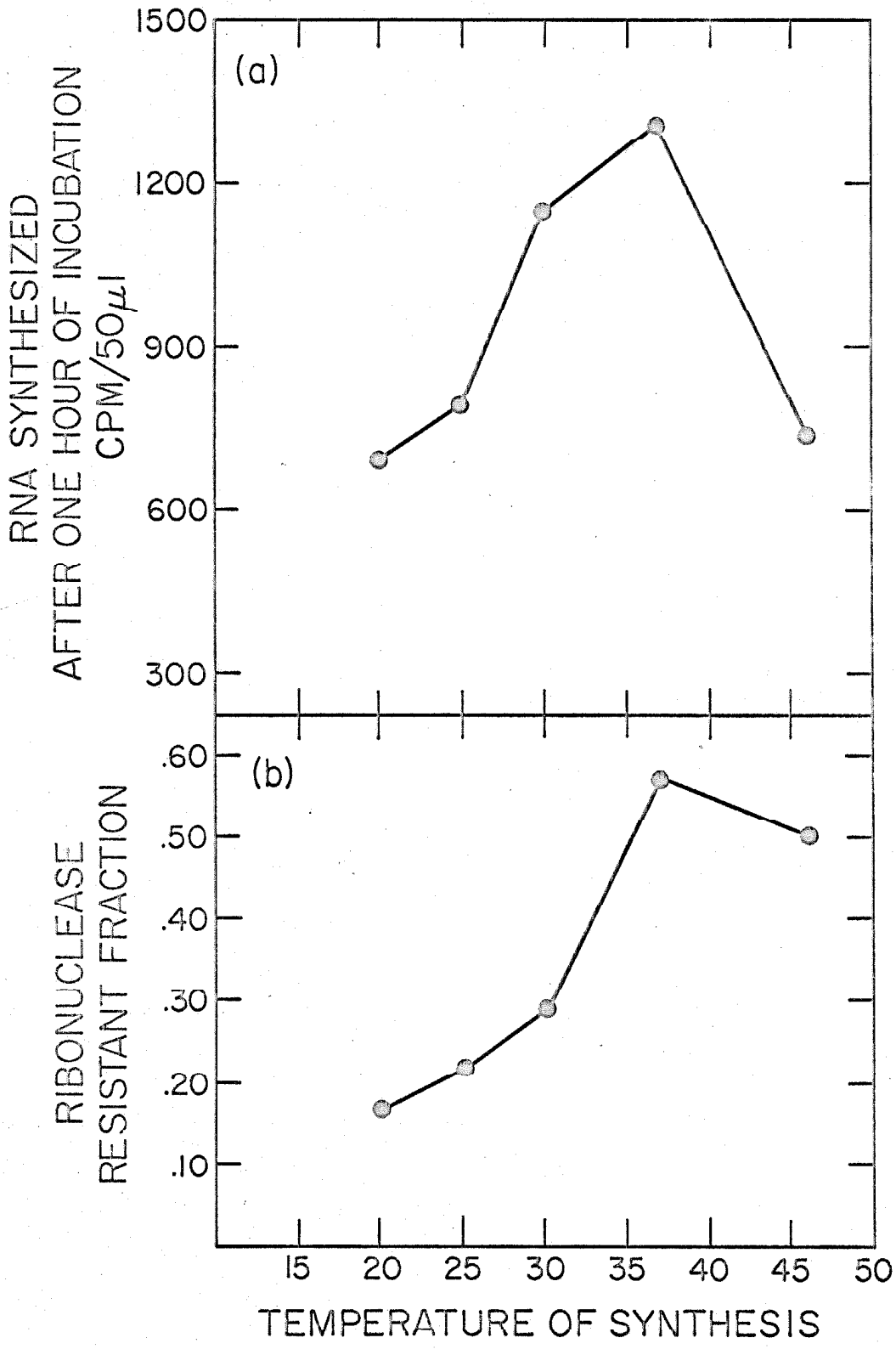
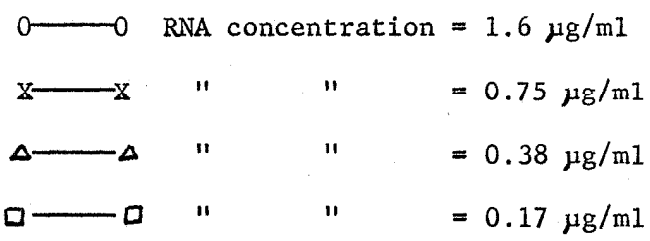
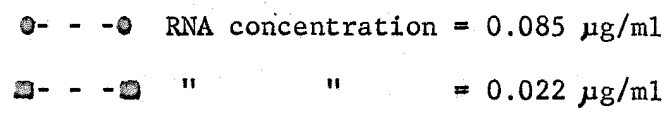

RNA SYNTHESIZED
AFTER ONE HOUR OF INCUBATION
CPM/50μl
1500
1200
900
600
300
(a)
RIBONUCLEASE
RESISTANT FRACTION
.60
.50
.40
.30
.20
.10
(b)
15
20
25
30
35
40
45
50
TEMPERATURE OF SYNTHESIS

Figure 3.

Figure 4. Reannealing of Complementary RNA. An RNA preparation containing 37% double-stranded RNA was melted as described in the text at a concentration of 1.7 μg/ml. The RNA was serially diluted, brought to 2 x SSC, incubated at 60°C for the indicated time and aliquots treated with RNase (50 μg/ml) at 37°C for 30 minutes followed by precipitation.



Another preparation of RNA containing 28% double-stranded RNA was melted at a concentration of 0.086 μg/ml, diluted, and treated as described above.



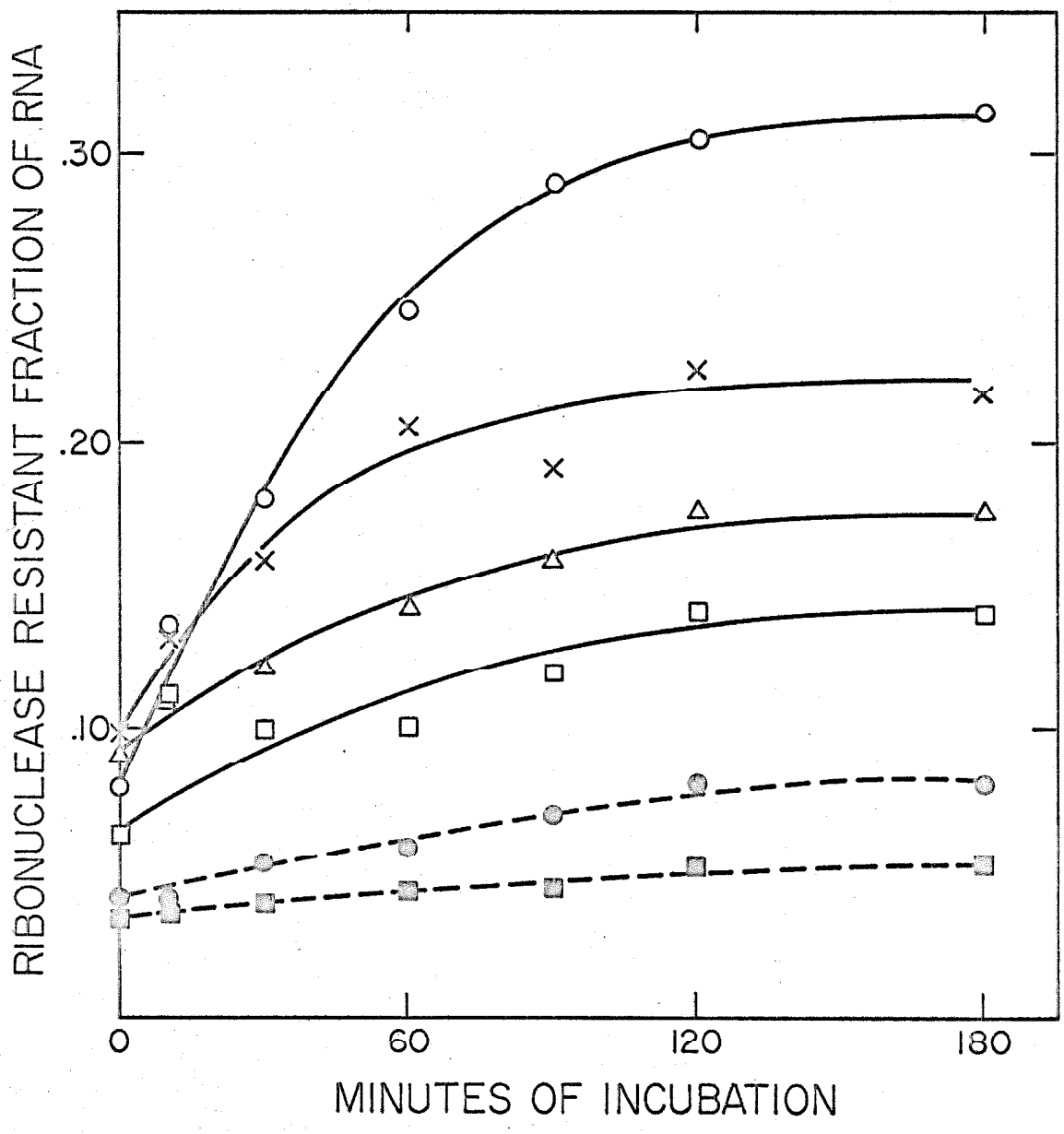
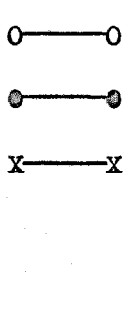

RIBONUCLEASE RESISTANT FRACTION OF RNA
.30
.20
.10
0
60
120
180
MINUTES OF INCUBATION

Figure 4.

Figure 5. Sedimentation of Synthetic Lambda RNA. Sedimentation is from right to left through a 5%-20% sucrose gradient in 2 x SSC in a Spinco SW 50 rotor at 50,000 rev./min. for 3 hours at 5°C.



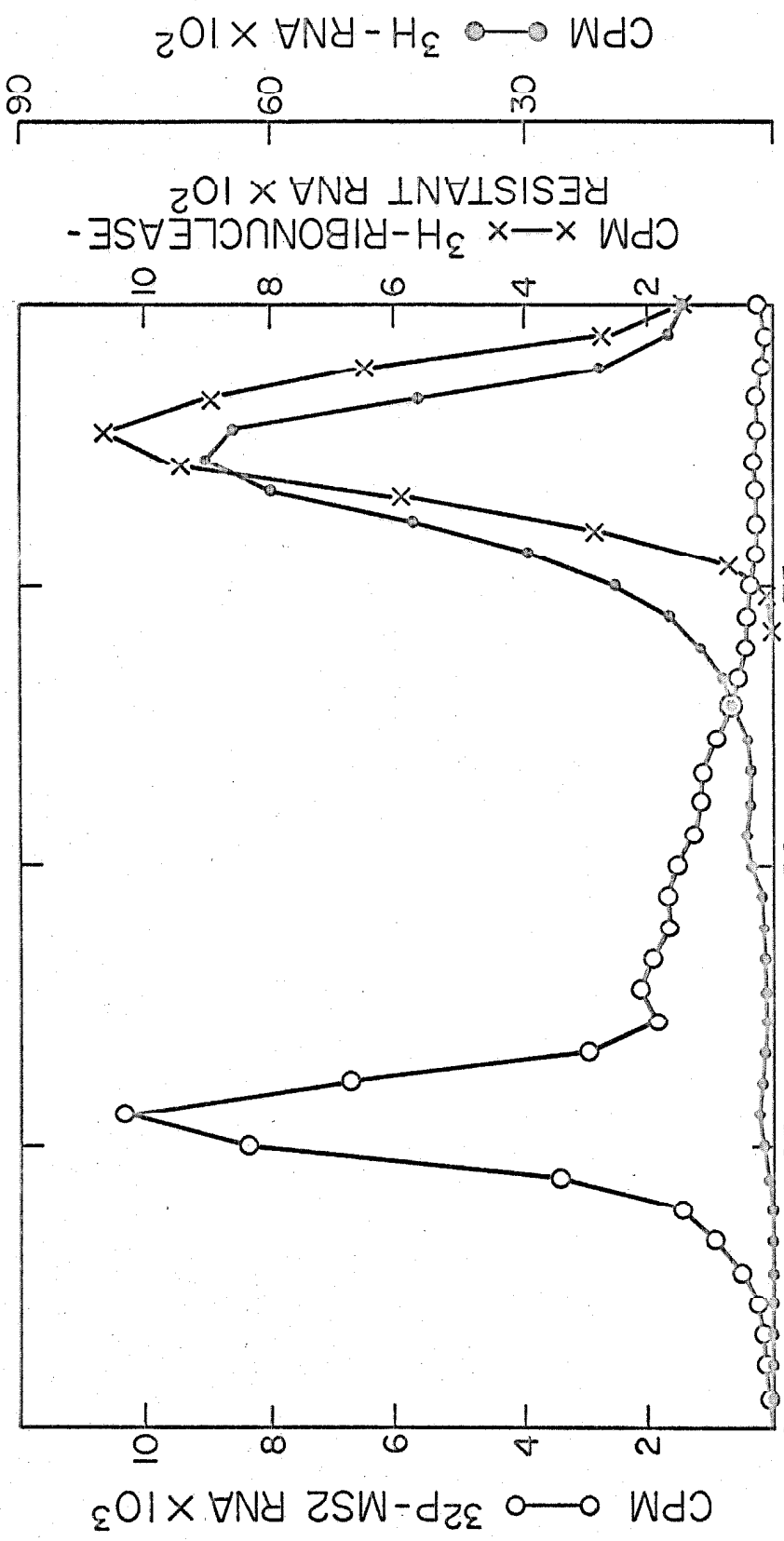

CPM ●—● ^{32}P-MS2 RNA × 10^3
CPM ×—× ^{3}H-RIBONUCLEASE-RESISTANT RNA × 10^2
CPM ●—● ^{3}H-RNA × 10^2
FRACTION OF GRADIENT
0
.25
.50
.75
2
4
6
8
10
30
60
90

Figure 5.

Figure 6. Hybrid Formation of ^{3}H-Lambda RNA with Denatured Lambda DNA. ^{3}H-RNA (1.8 µg/ml) is incubated in 3 x SSC at 60°C.

●——● Hybrid formation at a DNA concentration of 15 µg/ml

O——O Hybrid formation after the DNA concentration is raised to 30 µg/ml.

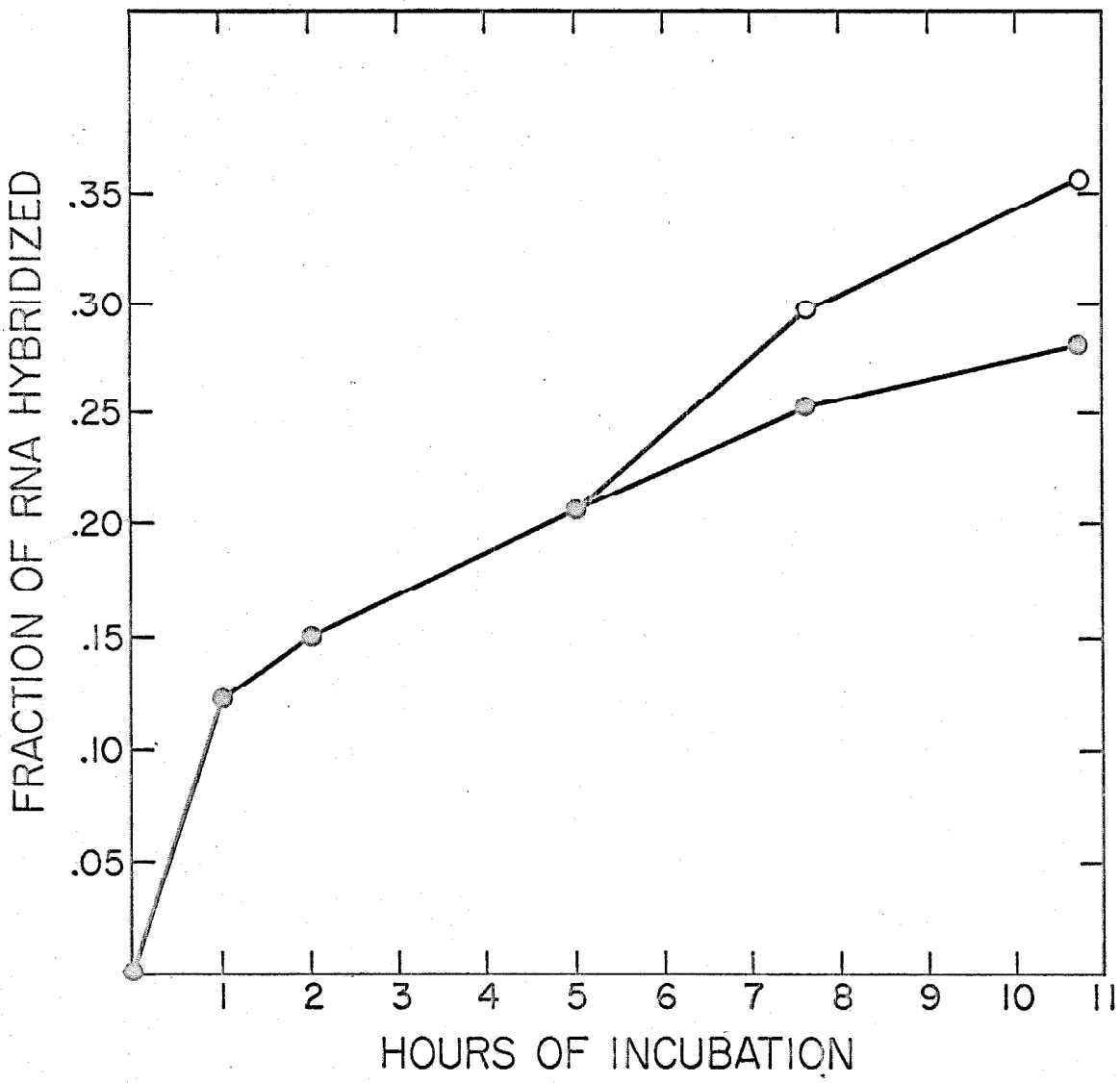
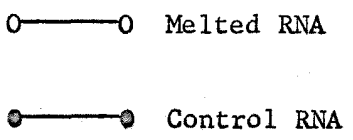

FRACTION OF RNA HYBRIDIZED
.35
.30
.25
.20
.15
.10
.05
1
2
3
4
5
6
7
8
9
10
11
HOURS OF INCUBATION

Figure 6.

Figure 7. The Effect of Prior Melting of RNA on the Formation of RNA-DNA Hybrid. Hybrid formation is in 2 x SSC at an RNA concentration of 0.019 μg/ml and a DNA concentration of 10 μg/ml.



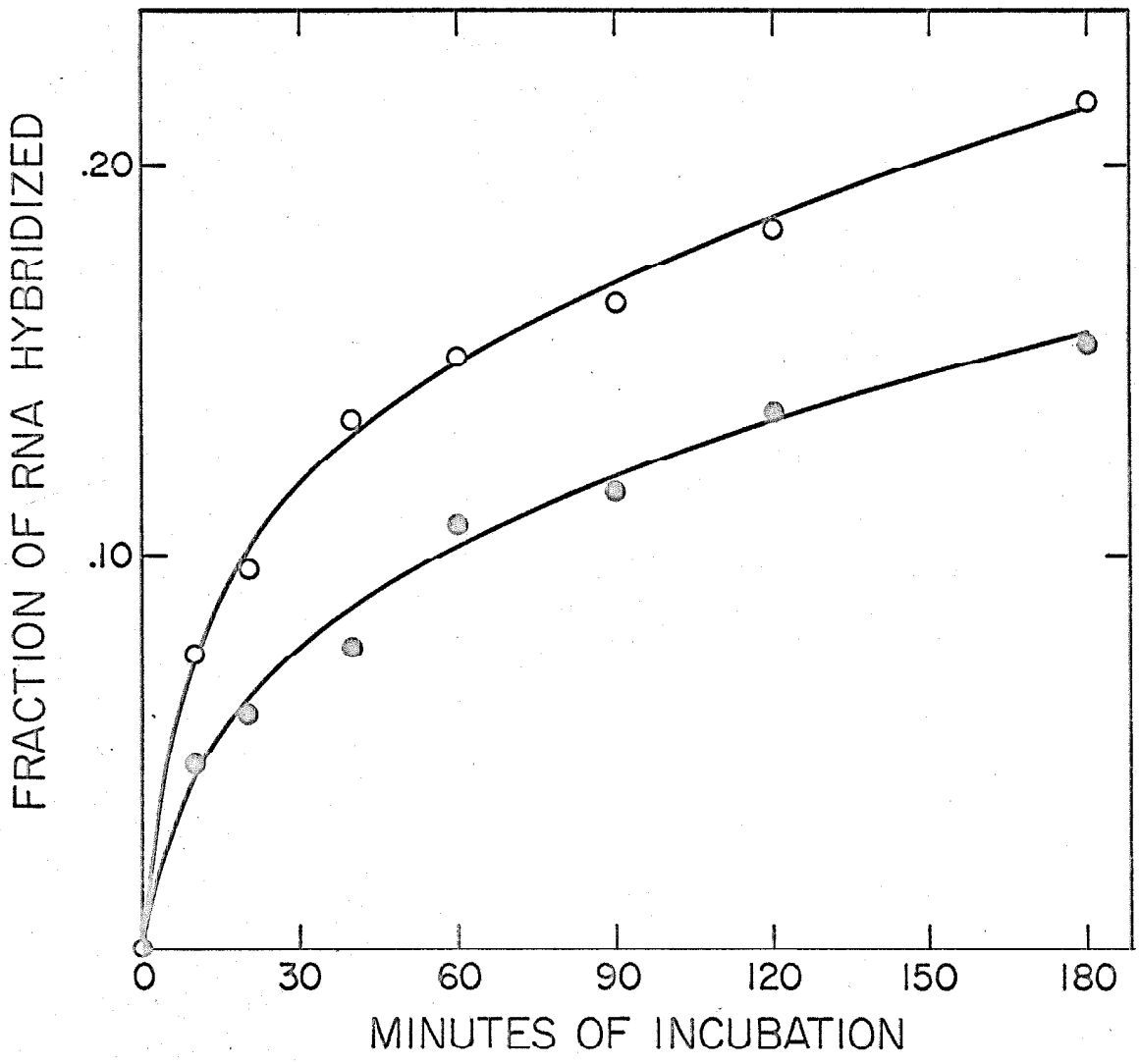
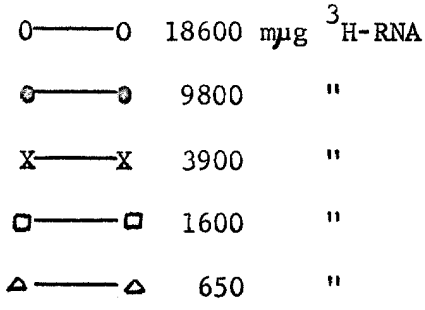

FRACTION OF RNA HYBRIDIZED
.20
.10
0
30
60
90
120
150
180
MINUTES OF INCUBATION

Figure 7.

Figure 8. Saturation of the Ability of Lambda DNA to Form Hybrid. Hybrid formation is at 66°C in 0.3 ml 6 x SSC containing a filter with 100mμg of bound denatured lambda DNA, a filter with no DNA and the indicated amount of ^{3}H-lambda RNA for the indicated time.



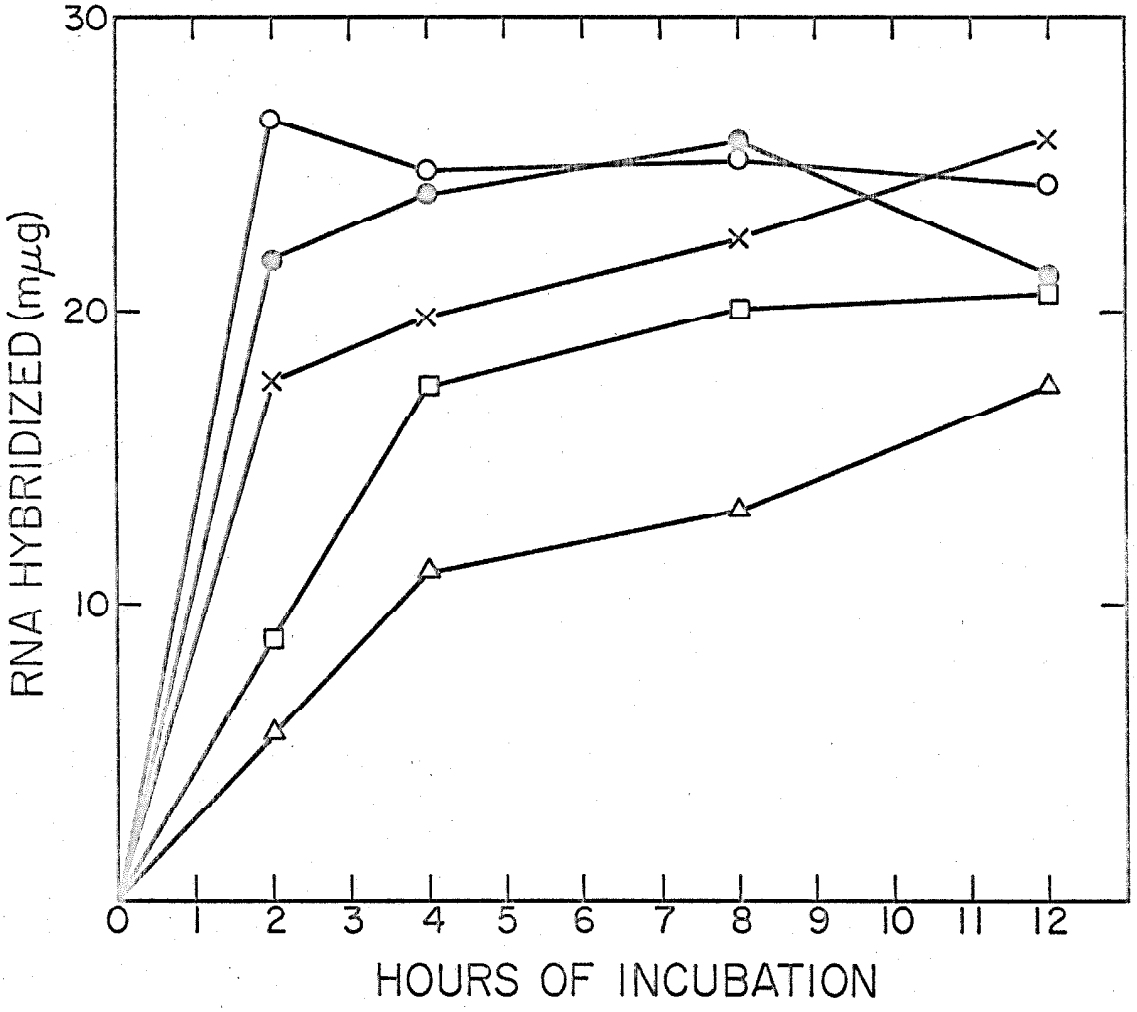
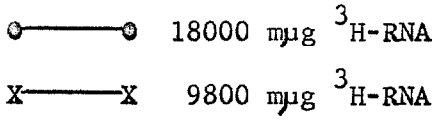

30
20
10
RNA HYBRIDIZED (mμg)
0
1
2
3
4
5
6
7
8
9
10
11
12
HOURS OF INCUBATION

Figure 8.

Figure 9. Saturation of ^{32}P-Lambda DNA. Hybrid formation is as described in Figure 8 except that 105 mμg of ^{32}P-lambda DNA is bound to each filter.



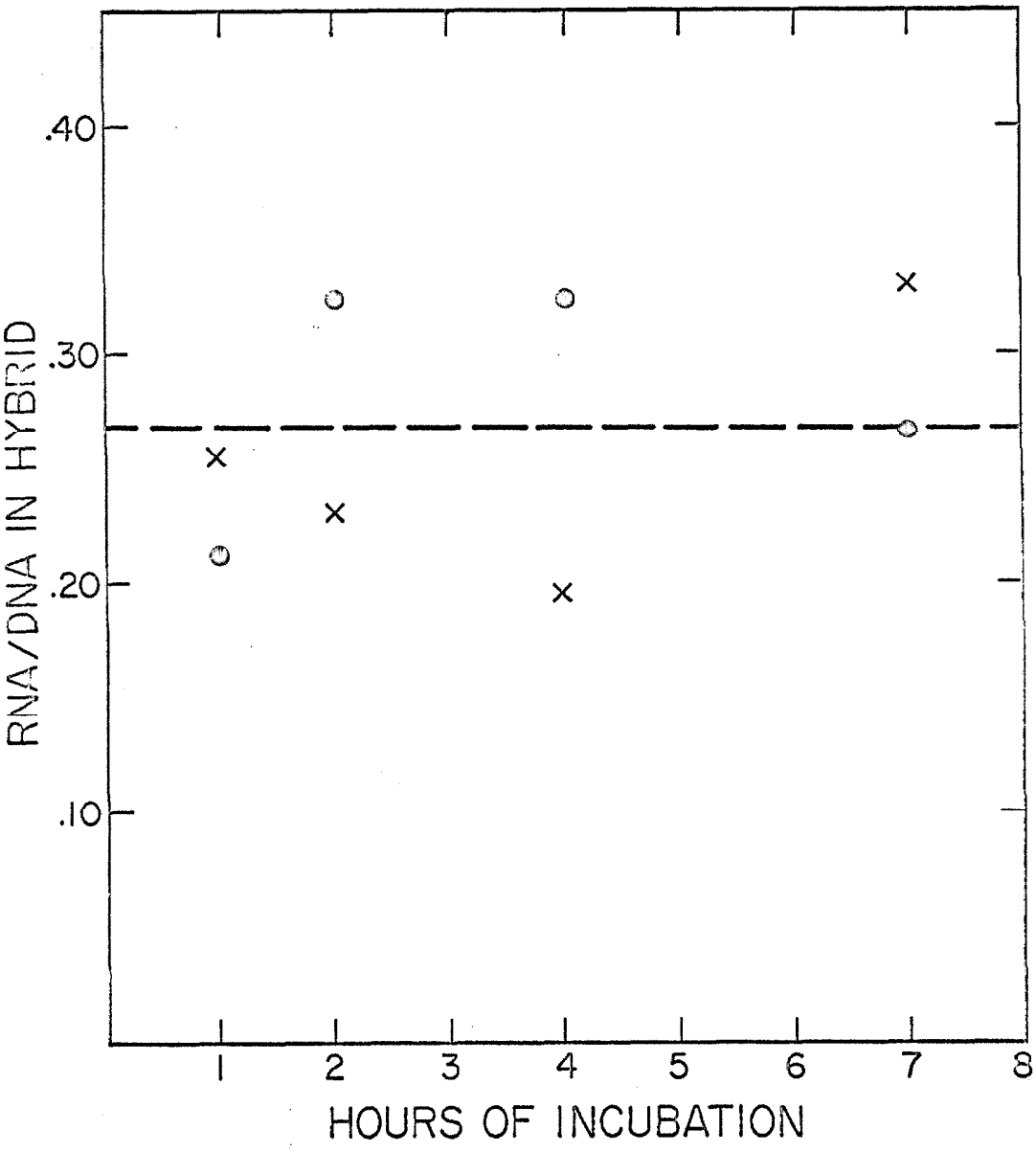


Figure 9.

Chapter 2

Transcription of Eucaryote DNA

1. MATERIALS AND METHODS

(a) Introduction

All solutions, chemicals and enzymes are described in Chapter 1 as are the techniques of RNA-DNA hybrid formation and the preparation of RNA polymerase. For some experiments the fraction 3 enzyme of Chamberlin & Berg (4) is used. The DNA saturation experiments performed here are done in solution and the hybrid assayed according to Nygaard & Hall (11) as described in Chapter 1.

(b) DNA Preparations

Most of the results to be reported are obtained with the DNA of Ischnochiton conspicuus (chiton). A few experiments are performed with DNA from Lottia gigantea (limpet) or Pisaster brevispinus (starfish). Animals are collected in the Corona del Mar - Newport Bay area of Southern California.

Sperm is collected from ripe males by excising the testes and allowing the sperm to drip into a beaker. Sperm is stored frozen until needed.

DNA is prepared by thawing the sperm in 2 x SSC, bringing the solution to 2% sodium dodecyl sulfate and shaking with one volume of re-distilled phenol or phenol-chloroform (1:1) (5) to form an emulsion. The emulsion is broken by centrifugation and the aqueous layer is re-

extracted until no protein remains at the interface. The DNA is precipitated at room temperature by layering two volumes of 95% ethanol over the DNA solution and collecting the DNA on a glass rod. The DNA is dissolved in 0.1 x SSC and re-precipitated and dissolved in 0.1 x SSC and stored in the refrigerator. DNA concentration is determined by the indole procedure of Keck (8) and by optical density at 2600 Å.

Thermal denaturation of DNA in 1 x SSC (9) yields a T_m of 86°C for chiton DNA and 84°C for limpet DNA indicating a base composition of 41% GC and 37% GC respectively.

(c) RNA Preparations

RNA is synthesized as described in Chapter 1 except when very high specific activity RNA is desired. High specific activity RNA is prepared by omitting unlabeled ATP or UTP and supplying only radioactively labeled precursor of high specific activity. This procedure reduces the mass of RNA synthesized to only a few percent of the mass of template DNA employed.

After incubation and DNase treatment as described in Chapter 1 the reaction mixture is extracted twice with re-distilled phenol saturated with 1 x SSC. The phenol layers are re-extracted with 1 x SSC and the aqueous layers combined and dialyzed against 1 x SSC. The RNA is stored frozen at -70°C. RNA concentrations are calculated from the specific activity of the reaction mixture.

2. RESULTS

(a) Properties of the Synthetic RNA

RNA synthesized with invertebrate DNA as template is found to be partially ribonuclease-resistant at high salt concentration (see Chapter 1). Incubation of the RNA under annealing conditions reveals very little if any change in the size of the RNase-resistant fraction. Figure 1 shows the effect of incubating chiton and limpet RNA at various RNA concentrations. Incubation at very low RNA concentrations actually seems to decrease the size of the RNase-resistant fraction slightly.

Under equivalent conditions less ribonuclease-resistant RNA is synthesized when invertebrate DNA is used as template than when lambda DNA is template. In Table 1 chiton and lambda DNA are compared as templates and some of the properties of their respective RNAs are recorded. It is evident that chiton DNA and lambda DNA have comparable template activity. The temperature of synthesis and the presence of Mn^{++} during synthesis affect the size of the ribonuclease-resistant fraction.

All the work presented below is with RNA synthesized at $37^{\circ}C$ in the presence of Mn^{++}.

(b) The Ability of the RNA to Form Hybrid with Complementary DNA

(i) Hybrid Formation in Aqueous Solution at High Temperature

The rate of hybrid formation of synthetic RNA with complementary DNA as a function of DNA concentration for chiton and starfish is shown in Figure 2. As the DNA concentration increases the amount of hybrid formed is seen to approach a maximum value that is considerably less than the amount of RNA present. The effect of temperature on the rate of hybrid formation by limpet RNA and DNA is shown in Figure 3. 60°C is seen to be the optimum temperature for hybrid formation and here too only a small fraction of the available RNA forms hybrid.

Two possible explanations for this limited hybrid formation have been considered. DNA renaturation may compete with RNA-DNA hybrid formation and this competition would be expected to increase with increasing DNA concentration. Or, RNA may become limiting after a certain fraction of the RNA has formed hybrid; there may be a heterogeneity in the ability of RNA molecules to form hybrid.

The former possibility is examined in an experiment shown in Figure 4. The kinetics of hybrid formation are followed and at the indicated times the concentration of denatured DNA is raised by addition of freshly denatured DNA. Increasing the DNA concentration is seen to increase the rate of hybrid formation (at least in the case of chiton) but the observed increase is not comparable to the initial rate of hybrid formation observed early in the incubation. We conclude that DNA renaturation is not limiting hybrid formation.

The latter explanation suggested above implies that it might be possible to fractionate the RNA into at least two fractions that differ in their ability to form hybrid. The kinetics of formation of RNA-DNA complexes (not treated with RNase but assayed identically) and ribonuclease-resistant hybrid are shown in Figure 5a. After 16 hours of incubation the mixture was passed through a nitrocellulose filter as described in Methods (Chapter 1). The RNA passing through the filter was collected while denatured DNA and RNA-DNA complexes were retained on the filter. This RNA which had not formed hybrid was tested for its ability to again form RNA-DNA complexes and ribonuclease-resistant hybrid in Figure 5b. It is evident that the rate of hybrid formation is markedly reduced by pre-hybridization of the RNA.

To demonstrate that this effect is due to pre-hybridization of the RNA and not simply to pre-incubation of the RNA the following experiments were performed. The effect of pre-incubation of the RNA under hybrid-forming conditions upon subsequent hybrid formation is shown in Figure 6. Incubation for up to 19 hours does not measurably affect the ability of the RNA to form hybrid with denatured DNA as might have been expected from the inability of the RNA to self-anneal (Figure 1).

The effect of pre-incubation on fractionation of the RNA as described in the experiment shown in Figure 5 is illustrated in Figure 7. Pre-hybridization and isolation of the unhybridized RNA by passage through a filter again is seen to result in an RNA that forms RNA-DNA complexes and ribonuclease-resistant hybrid at a decreased rate. RNA

exposed to an equal length of pre-incubation followed by passage through a filter is seen to form hybrid at a faster rate than the pre-hybridized RNA.

It may be noted that the ratio of RNA-DNA complex to ribonuclease-resistant hybrid is larger in the experiment described in Figure 7 than that in Figure 5. This variability is observed to depend on the RNA polymerase preparation used to transcribe the RNA. The RNA used in Figure 7 has a much larger average S value than the RNA used in Figure 5 - a reflection of the level of RNase contamination in the different preparations. This point has not been pursued.

(ii) Hybrid Formation in Formamide Solution at Low Temperature

The data presented in Table 1 suggest that the presence of secondary structure in the RNA may affect either the rate or extent of hybrid formation. It was believed desirable to find a method more gentle than boiling at low salt concentration to destroy the secondary structure of the RNA so that the extent of hybrid formation could be determined in the absence of RNA secondary structure. It has been demonstrated that formamide at sufficient concentration will disrupt the secondary structure of DNA and RNA (7,14,15). This method was employed, and a procedure for hybrid formation at low temperature simultaneously developed.

The melting of ribonuclease-resistant RNA in 49.5% formamide, 2 x SSC, is shown in Table 2. It is evident that even at 70°C all of the double-stranded RNA is not melted. Lowering the salt concentration to 0.1 x SSC, raising the formamide concentration to 89% and incubating

at 60°C for 15 minutes is, however, found to totally disrupt the secondary structure. RNA treated in this way is only 2% ribonuclease-resistant when assayed as described in the legend to Table 2.

RNA denatured in this way is diluted to 48% formamide and incubated with denatured DNA in 2 x SSC. The kinetics of formation of RNA-DNA complex (as assayed by the filter method which assays only RNA bound to DNA) and of ribonuclease-resistant RNA (which is an assay for both RNA-DNA hybrid and double-stranded RNA) at various temperatures of incubation is seen in Figure 8. Under these conditions also only a limited amount of the RNA forms complexes. A comparison of RNA-DNA complex formation and ribonuclease-resistant hybrid formation in formamide is made in Figure 9b. A similar comparison for the same RNA preparation incubated in the presence of denatured DNA in the absence of formamide at high temperature is shown in Figure 9a.

Comparison of the amount of ribonuclease-resistant hybrid with the amount of RNA-DNA complex (Figure 9) and with the total amount of ribonuclease-resistant RNA (Figure 8) formed during incubation in formamide indicates that RNA-RNA annealing takes place concomitantly with RNA-DNA hybrid formation.

The data presented in Figure 8 demonstrate that it is possible to form RNA-DNA hybrid in formamide solutions at reasonably low temperatures. Hybrid formation is optimal over a wide range of temperatures from 22°C (the lowest tested) to 41°C (data not shown). At 50°C hybrid formation is markedly reduced, however. A somewhat different method of hybrid formation in formamide solution has recently been described

by Bonner, Kung & Bekhor (1).

We conclude that only a small fraction of the RNA synthesized using chiton DNA as template is amenable to hybrid formation under our conditions. 10-15% of this RNA readily forms hybrid with denatured DNA under conditions that allow more than 50% of lambda RNA to form hybrid (Chapter 1). The maximum amount of ribonuclease-resistant RNA-DNA hybrid attained with chiton RNA is 20%. Even less of the RNA transcribed from limpet and starfish DNA readily forms hybrid under our conditions.

(c) The Ability of the DNA to Form Hybrid with Complementary RNA

The data presented in the previous section indicate that RNA transcribed in vitro from those eucaryote DNAs examined is heterogeneous with regard to the rate with which different RNA species form hybrid. In this section the ability of such RNA transcribed from chiton DNA to saturate complementary DNA or conversely the ability of denatured DNA to form RNA-DNA hybrid is examined.

The amount of denatured DNA forming hybrid when incubated with increasing amounts of RNA for 3 hours and 12 hours is seen in Figure 10. The data are presented in both a normal plot and a reciprocal plot. It is clear that only a small fraction of the DNA has formed hybrid. The data suggest that incubation for longer periods would not appreciably increase the amount of hybrid formed. The amount of hybrid formation is not linear with increasing RNA concentration which could be

interpreted to mean that the rate of hybrid formation decreases as a consequence of the second order kinetics known to govern hybrid formation (12,17).The reciprocal plot of the data suggests that such a simple interpretation is not in order as the values do not fit a straight line.

The experiment illustrated in Figure 10 employes RNA labeled in adenine. A similar experiment employing RNA labeled in cytosine and a lower concentration of denatured DNA is shown in Figure 11. Approximately the same level of hybrid formation is achieved and the reciprocal plot again does not produce a straight line. Increasing the temperature of incubation from 60°C to 68°C results in a marked increase in the amount of hybrid formed. The data in figure 3 indicate that 60°C is the optimum temperature of hybrid formation for RNA labeled in adenine. This observation suggests a further heterogeneity of the RNA species with regard to base composition (10). This latter heterogeneity clearly has only a second order effect on the amount of hybrid formed and appears to be qualitatively different from the heterogeneity suggested by the reciprocal plots.

It is of interest to know exactly how much of the DNA can form hybrid in order to determine if E. coli RNA polymerase is able to transcribe the entire chiton genome. The only conclusive way to answer this question is to convincingly show what fraction of the DNA finds complementary sequences in the transcribed RNA. As demonstrated above the resolution of this problem is complicated by the heterogeneity observed in the rate of hybrid formation by different regions of the DNA and RNA. We would like to be able to force the kinetics of the

reaction by exposing denatured DNA to as large an amount of RNA as is needed.

An attempt to accomplish this is illustrated in Figure 12. RNA: DNA ratios up to 280 fail to significantly increase the fraction of DNA forming hybrid. In none of the experiments reported here is more than 11% of the DNA involved in hybrid formation and that is in the experiment employing the highest DNA concentration (Figure 10).

3. Discussion

The results of hybrid formation with eucaryote RNA and DNA at high DNA/RNA ratios indicate that a fraction of the RNA anneals rapidly at a rate comparable to the rate at which lambda RNA anneals to lambda DNA. Another fraction anneals much more slowly but the kinetics do not allow us to state that this fraction is homogeneous. The isolation of a fraction of RNA by pre-hybridization which anneals very slowly is further evidence for at least two fractions of RNA which differ in their rate of hybrid formation. The presence of some secondary structure in the RNA as evidenced by ribonuclease-resistance does not affect this interpretation as RNA synthesized under conditions producing less secondary structure exhibits the same properties of hybrid formation. Nor does prior denaturation of RNA secondary structure markedly change the ability of the RNA to form hybrid (Table 1).

Since most of theRNA does not readily form hybrid with DNA, the fact that the RNA does not self-anneal (Figure 1) is not evidence for asymmetric transcription of the bulk of the genome. As in the case of

lambda, however, the rapidly annealing RNA does seem to be the product of asymmetric transcription since pre-incubation does not diminish its ability to form hybrid nor does incubation under annealing conditions lead to any increase in ribonuclease-resistant RNA.

The presence of heterogeneity in the RNA implies the existence of similar heterogeneity in the DNA. This is supported by the attempts made to saturate the DNA.

Assuming the simplest kinetics possible, let

R = RNA concentration

D = DNA concentration $\qquad R \gg D$

h = RNA-DNA hybrid

then

$$\frac{h}{(R-h)\ (D-h)} = K \quad \text{a constant}$$

$$\frac{h}{R\ (D-h)} = K \quad \text{since } (R-h) \cong R$$

$$h = \frac{K\ D\ R}{1 + K \cdot R}$$

taking the reciprocal

$$\frac{1}{h} = \frac{1}{K\ D\ R} + \frac{1}{D}$$

a straight line is obtained if 1/h is plotted against 1/R with 1/D as the intercept on the ordinate and 1/KD the slope.

Treating the data from DNA saturation experiments employing RNA transcribed <u>in vitro</u> in this way Paul & Gilmour (13) and Bekhor, Kung & Bonner (manuscript) have found their data to fit a straight line in a reciprocal plot which they extrapolate to infinite RNA concentration to obtain a saturation value for the DNA. These investigators find

that the extrapolated values obtained are very close to one half of the DNA forming hybrid at infinite RNA concentration. This value could be interpreted to mean that one half of the DNA is transcribed *in vitro*, possibly the entire genome being transcribed asymmetrically. This interpretation is questionable, however, for the work of Paul & Gilmour (13) as they employ *M. lysodeikticus* RNA polymerase which other investigators find to uniformly give symmetric transcription (6). Thus the data of Paul & Gilmour suggest that either only approximately 50% of their DNA (calf thymus) was transcribed *in vitro* or else only this portion of the DNA forms hybrid under their conditions, suggesting heterogeneity.

If heterogeneity of rate is present it seems inexplicable that a straight line should be obtained in a reciprocal plot. Indeed it is evident that the data presented here do not fit a straight line. We feel that this is strong evidence for the existance of heterogeneity in the rate with which RNA and DNA molecules form hybrid although this interpretation is subject to the uncertainties in our knowledge of the kinetics of hybrid formation. Extrapolation of such data to infinite RNA concentration with the intention of interpreting the intercept value can only be an *auto da fé*.

What can studies of hybrid formation contribute to our understanding of the transcription of eucaryote DNA by *E. coli* RNA polymerase? The saturation experiments indicate that at least 10-12% of the DNA is transcribed, and other experiments indicate that transcription of this fraction of the DNA is asymmetric. Hybrid formation at high DNA/RNA ratios has shown as much as 20% of the RNA to be capable

of hybrid formation. It seems reasonable that the 80% of the RNA that does not readily form hybrid is transcribed from the DNA that does not readily form hybrid and that a large fraction if not all of the eucaryote DNA is transcribed. The data do not compel acceptance of this hypothesis, however.

Renaturation studies of eucaryote DNA have shown that portions of the DNA renature at very different rates (2,3,16). These studies have indicated that a small fraction of most eucaryote DNAs renatures at rates comparable to those observed for phage or bacterial DNA renaturation. It is probable that the heterogeneity observed here in RNA-DNA hybrid formation is of the same nature as that detected in DNA renaturation studies.

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TABLE 1

Type	Conditions of Synthesis	RNA Synthesized	RNA Template	RNase Resistance	Fraction of RNA forming hybrid	
					untreated	melted
Chiton	37^{o} + Mn^{++}	7.1 µg	0.71	.134	.052	.077
	37^{o} - Mn^{++}	5.0 µg	0.50	.07	.094	.094
	30^{o} + Mn^{++}	14.4 µg	1.44	.07	.122	.150
	30^{o} - Mn^{++}	10.7 µg	1.07	.05	.168	.168
Lambda	30^{o} - Mn^{++}	5.0 µg	0.50	.14	--	.210
	37^{0} + Mn^{++}	6.8 µg	0.68	.29	.133	.192

RNA is synthesized as described in Methods (Chapter 1). Each reaction mixture contained 10 µg of DNA. Incubation is for 90 minutes under the indicated conditions.

Levels of hybridization are determined after 3 hours incubation in 2 x SSC at 60^{o} and represent ribonuclease-resistant hybrid. Hybridization is performed with a 300-500 fold excess of denatured DNA over RNA. The concentration of DNA in the hybridization mixture is 10 µg/ml.

TABLE 2

$T^{o}C$	Ribonuclease Resistance
0	.14
20	.12
25	.13
30	.12
35	.11
40	.10
50	.11
60	.08
70	.05

Chiton ^{3}H-RNA (0.41 µg/ml) in 49.5% Formamide, 2 x SSC, was held at the indicated temperature for 15 minutes, diluted 1:1 with 2 x SSC containing 10 µg/ml RNase, incubated at 37°C for 10 minutes and acid precipitated.

Figure 1. Incubation of Synthetic RNAs Under Annealing Conditions

RNA is incubated in 2 x SSC at 60°C for 4 hours followed by RNase (15 μg/ml) treatment in 2 x SSC at 37°C for 10 minutes and acid precipitation. The lines represent the level of ribonuclease-resistance determined before incubation: solid line - chiton RNA; dashed line - limpet RNA.

● - incubated chiton RNA X - incubated limpet RNA

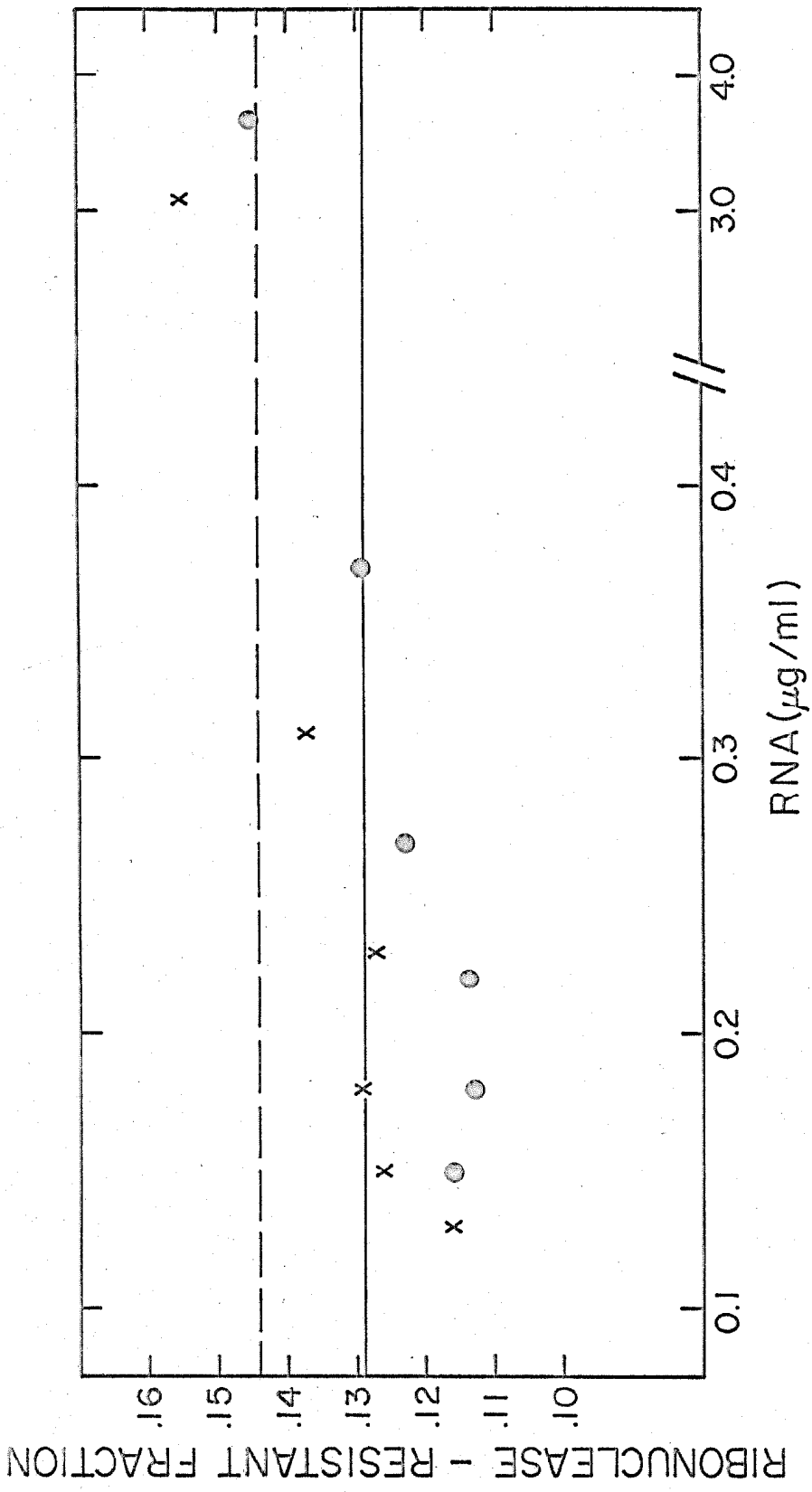
RIBONUCLEASE - RESISTANT FRACTION
.16
.15
.14
.13
.12
.11
.10
0.1
0.2
0.3
0.4
3.0
4.0
RNA (μg/ml)

Figure 1.

Figure 2. Limited Hybrid Formation between Synthetic RNA and DNA.
(a) ^{3}H-RNA transcribed from chiton DNA (0.08 μg/ml) is incubated with denatured chiton DNA at the concentration indicated for 4 hours at 60°C followed by RNase treatment and assay as described in Methods.
(b) ^{3}H-RNA transcribed from starfish DNA (0.023 μg/ml) is incubated with denatured starfish DNA as described in (a).

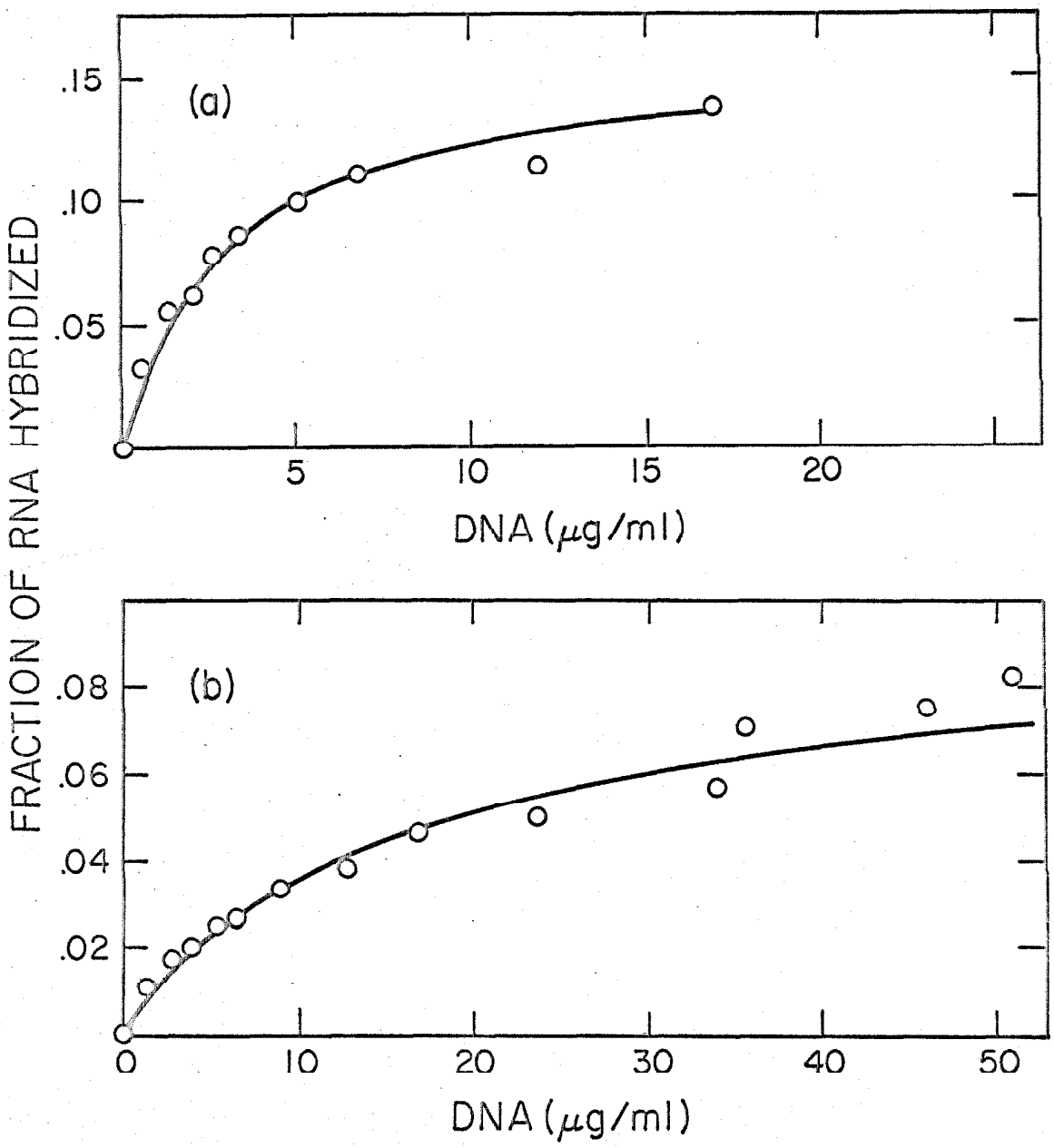
(a)
.15
.10
.05
5
10
15
20
DNA (μg/ml)
FRACTION OF RNA HYBRIDIZED
(b)
.08
.06
.04
.02
0
10
20
30
40
50
DNA (μg/ml)

Figure 2.

Figure 3. The Effect of Temperature on Hybrid Formation

^{3}H-RNA transcribed from limpet DNA (0.20 µg/ml) is incubated with denatured limpet DNA at the indicated concentration and temperature as described in Figure 2.

△——△ incubation at 45°C

□——□ " 52°C

0——0 " 60°C

X——X " 68°C

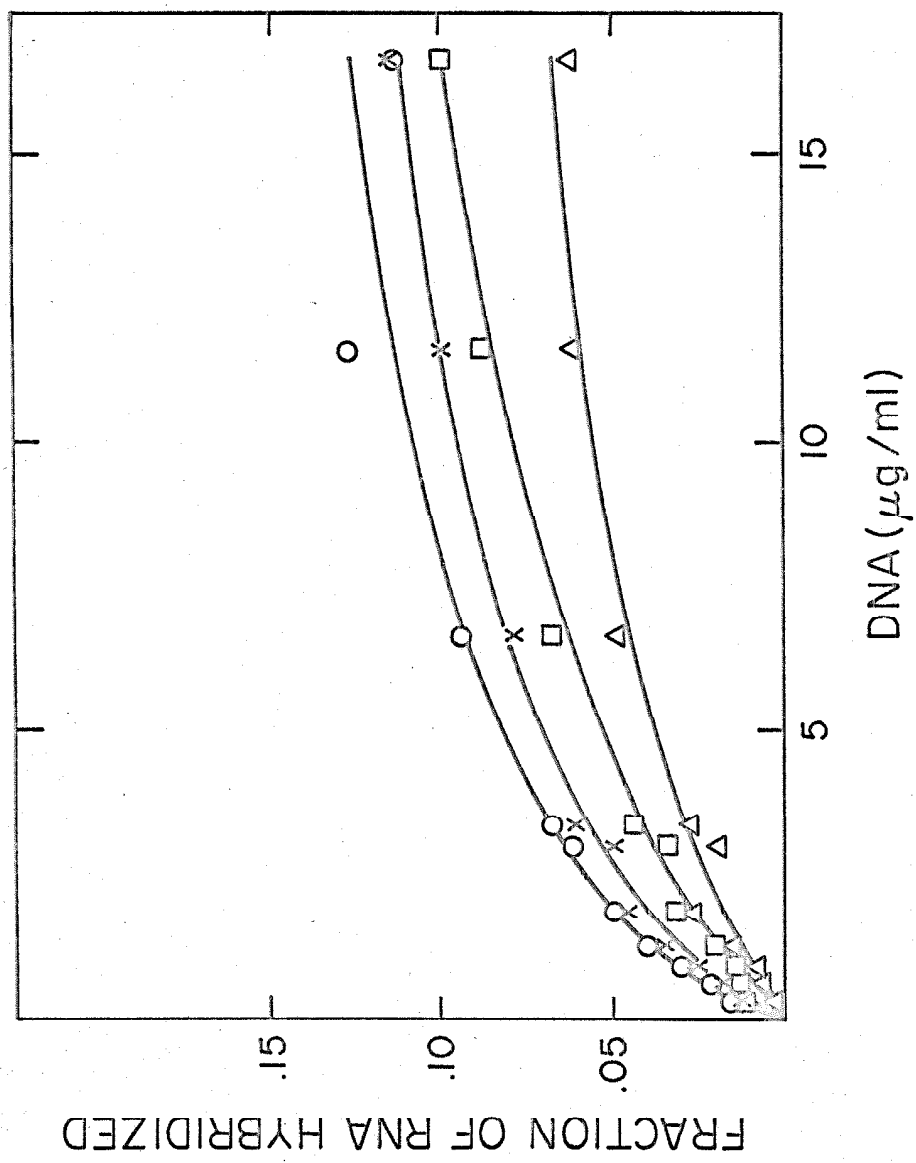

Figure 3.

Figure 4. The Effect of Denatured DNA Concentration of the Rate of Hybrid Formation.

(a) ^{3}H-RNA transcribed from chiton DNA (starting concentration = 0.19 µg/ml) is incubated with denatured chiton DNA for the indicated time. The concentration of denatured DNA is increased as indicated by the addition of freshly denatured DNA at times shown by the origin of new lines. Incubation and assay are as in Figure 2.

O———O DNA concentration - 17 µg/ml

X———X " " 27 µg/ml

□———□ " " 38 µg/ml

(b) ^{3}H-RNA transcribed from limpet DNA (starting concentration = 0.20 µg/ml) is incubated with denatured limpet DNA as described above.

O———O DNA concentration = 17 µg/ml

X———X " " 31 µg/ml

□———□ " " 48 µg/ml

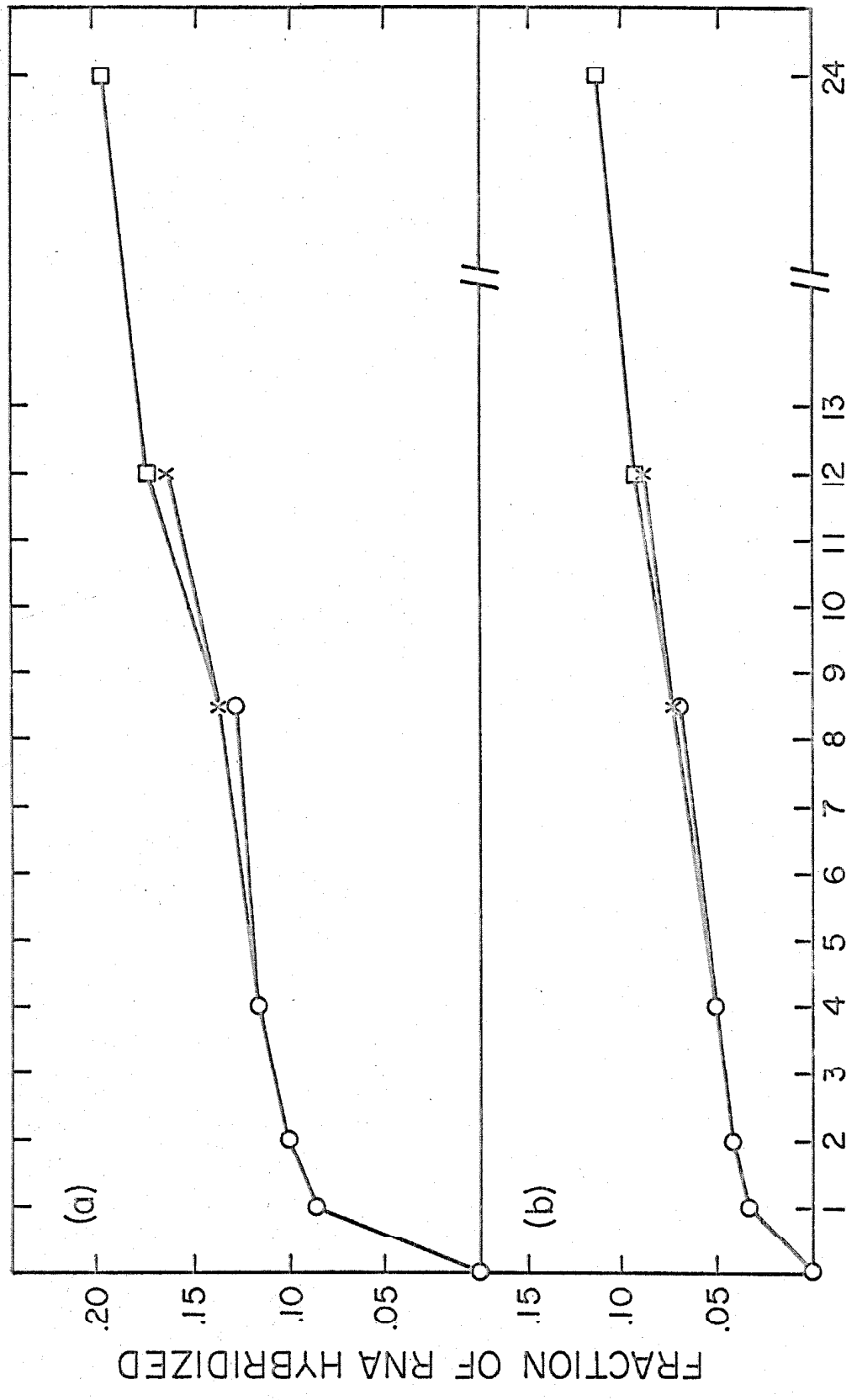
FRACTION OF RNA HYBRIDIZED
.20
.15
.10
.05
(a)
(b)
.15
.10
.05
1
2
3
4
5
6
7
8
9
10
11
12
13
24
HOURS OF INCUBATION

Figure 4.

Figure 5. Fractionation of Chiton RNA by Hybrid Formation.

(a) ^{3}H-RNA (0.19 μg/ml) is incubated with denatured DNA (24 μg/ml) and the kinetics of annealing followed by assaying ribonuclease-resistant hybrid (O———O) and RNA-DNA complexes (not treated with RNase but assayed in an identical manner) (●———●). After the indicated duration of incubation at 60°C the solution was passed through a nitrocellulose filter and the RNA in the filtrate collected.

(b) Denatured DNA is added to the above filtrate to give a concentration of 24 μg/ml. The RNA concentration is 0.03 μg/ml. Annealing is followed as described above.

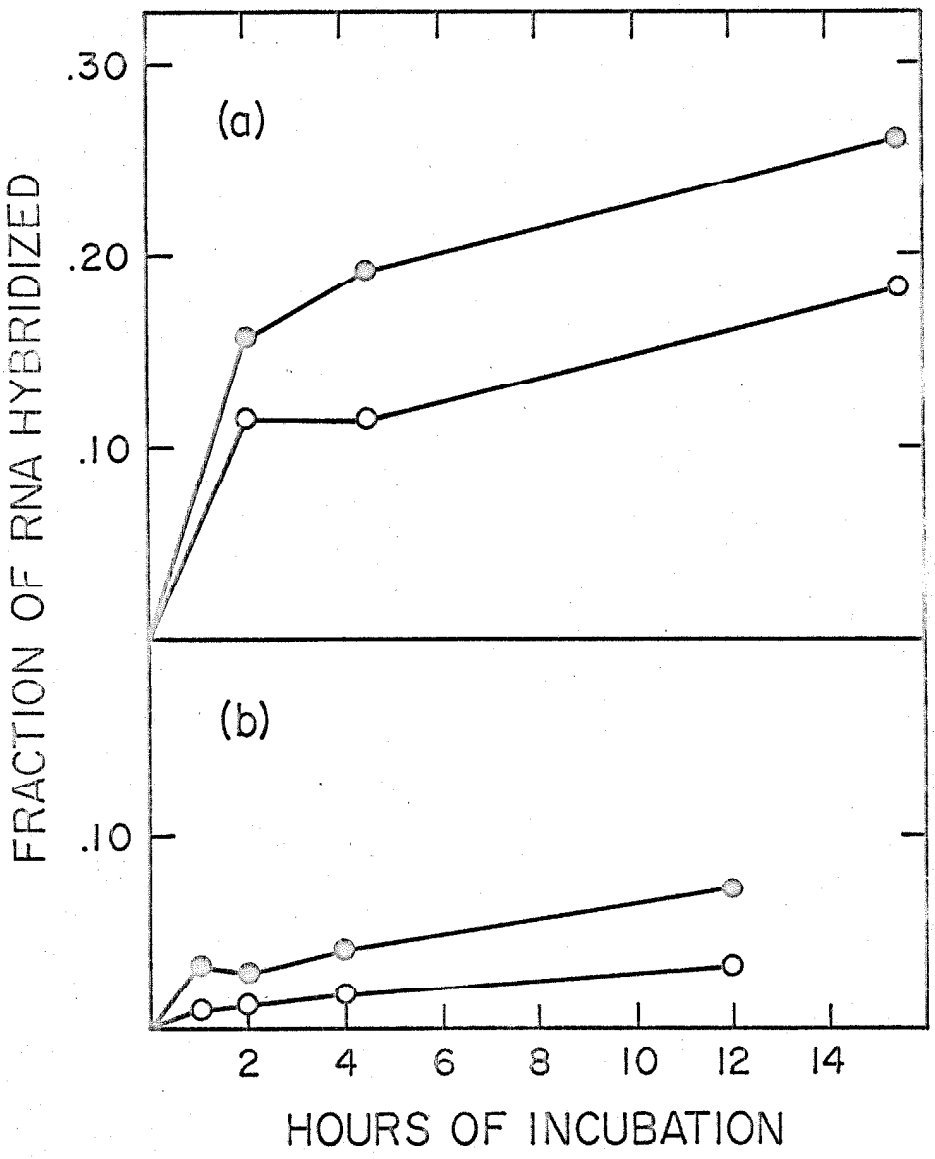
.30
.20
.10
(a)
(b)
.10
FRACTION OF RNA HYBRIDIZED
2
4
6
8
10
12
14
HOURS OF INCUBATION

Figure 5.

Figure 6. The Effect of Pre-incubation of the RNA on Hybrid Formation. ^{3}H-RNA transcribed from chiton DNA (0.34 µg/ml) in incubated at 60°C in 2 x SSC for the indicated time followed by addition of denatured DNA to give 24 µg/ml and continued incubation at 60°C in 2 x SSC for 4.5 hours. Ribonuclease-resistant hybrid is assayed as previously described.

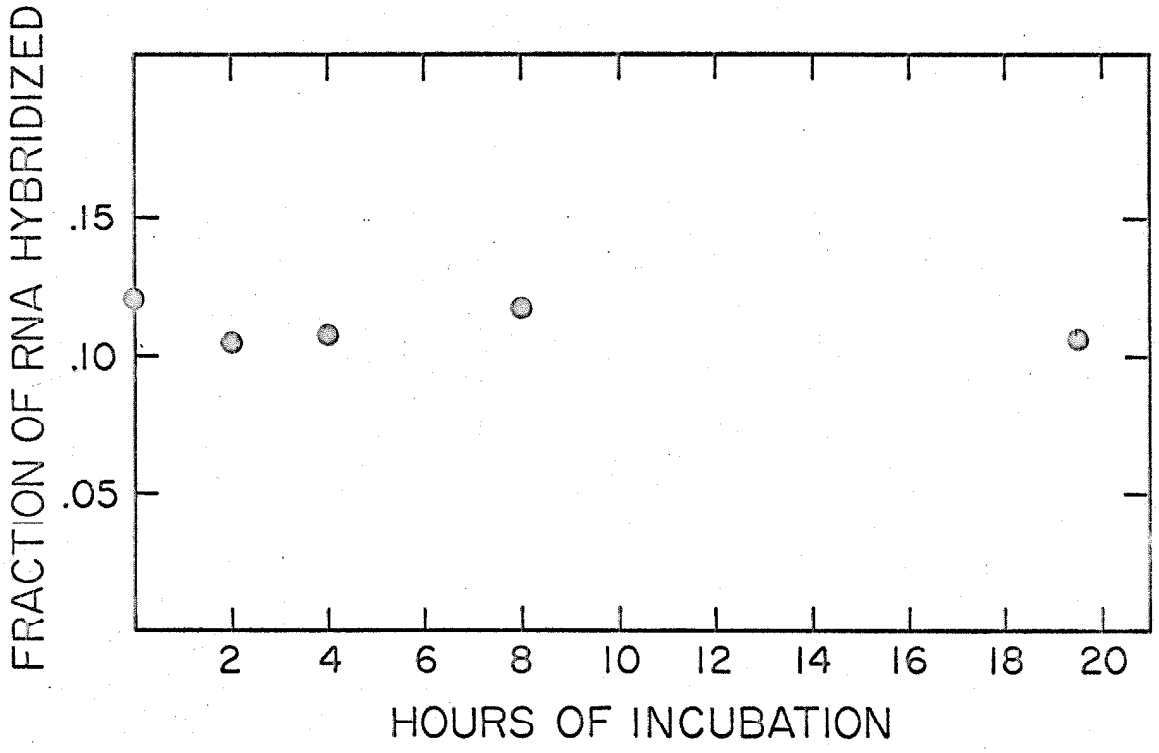
FRACTION OF RNA HYBRIDIZED
.15
.10
.05
2
4
6
8
10
12
14
16
18
20
HOURS OF INCUBATION

Figure 6.

Figure 7. Fractionation of Chiton RNA by Hybrid Formation.

(a) ^{3}H-RNA (0.17 µg/ml) is incubated with denatured DNA (17.4 µg/ml) as described in Figure 5a. ^{3}H-RNA at the same concentration is incubated for the same length of time and also passed through a nitrocellulose filter.

(b) Denatured DNA is added to each of the above filtrates to give a concentration of 18.2 µg/ml. and incubation continued at 60°C.

0- - - 0 RNA-DNA complexes formed by pre-hybridized RNA

0- - - 0 Ribonuclease-resistant hybrid formed by pre-hybridized RNA

0———0 RNA-DNA complexes formed by pre-incubated RNA

0———0 Ribonuclease-resistant hybrid formed by pre-incubated RNA

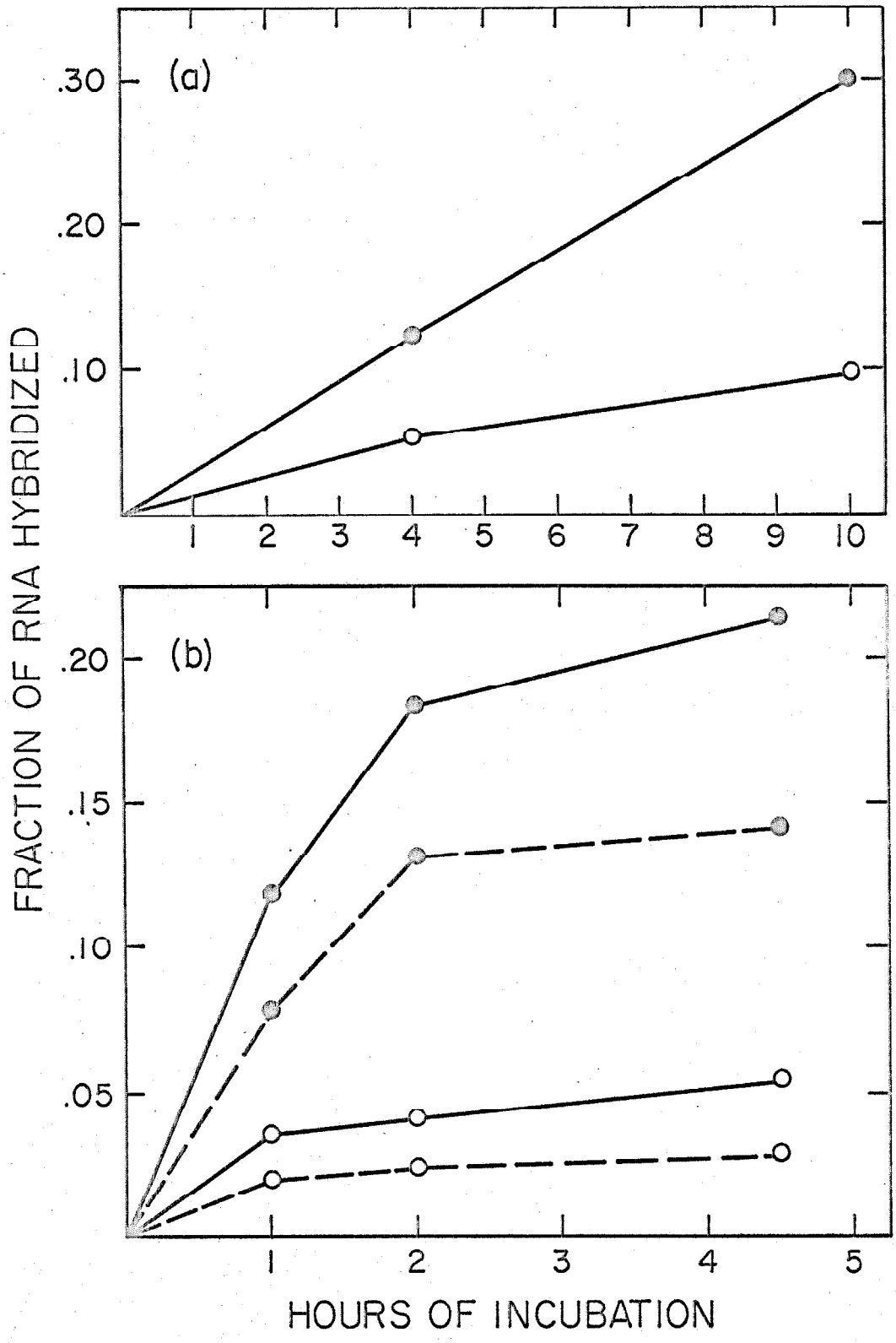
(a)
.30
.20
.10
1
2
3
4
5
6
7
8
9
10
(b)
.20
.15
.10
.05
1
2
3
4
5
FRACTION OF RNA HYBRIDIZED
HOURS OF INCUBATION

Figure 7.

Figure 8. Annealing of RNA and DNA in Formamide.

Formamide denatured ^{3}H-RNA transcribed from chiton (0.22 μg/ml) is incubated with denatured DNA (19 μg/ml) in 48% formamide, 2 x SSC, and RNA-DNA complexes (●————●) and total ribonuclease-resistant RNA (X————X) determined at various times. Temperature of incubation is (a) 22°C (b) 35°C (c) 50°C.

RNA-DNA complexes are assayed by filtration through nitrocellulose filters after 1/100 dilution into 2 x SSC. Total ribonuclease-resistant RNA is determined by RNase treatment (5 μg/ml) at 37°C for 10 minutes followed by acid precipitation.

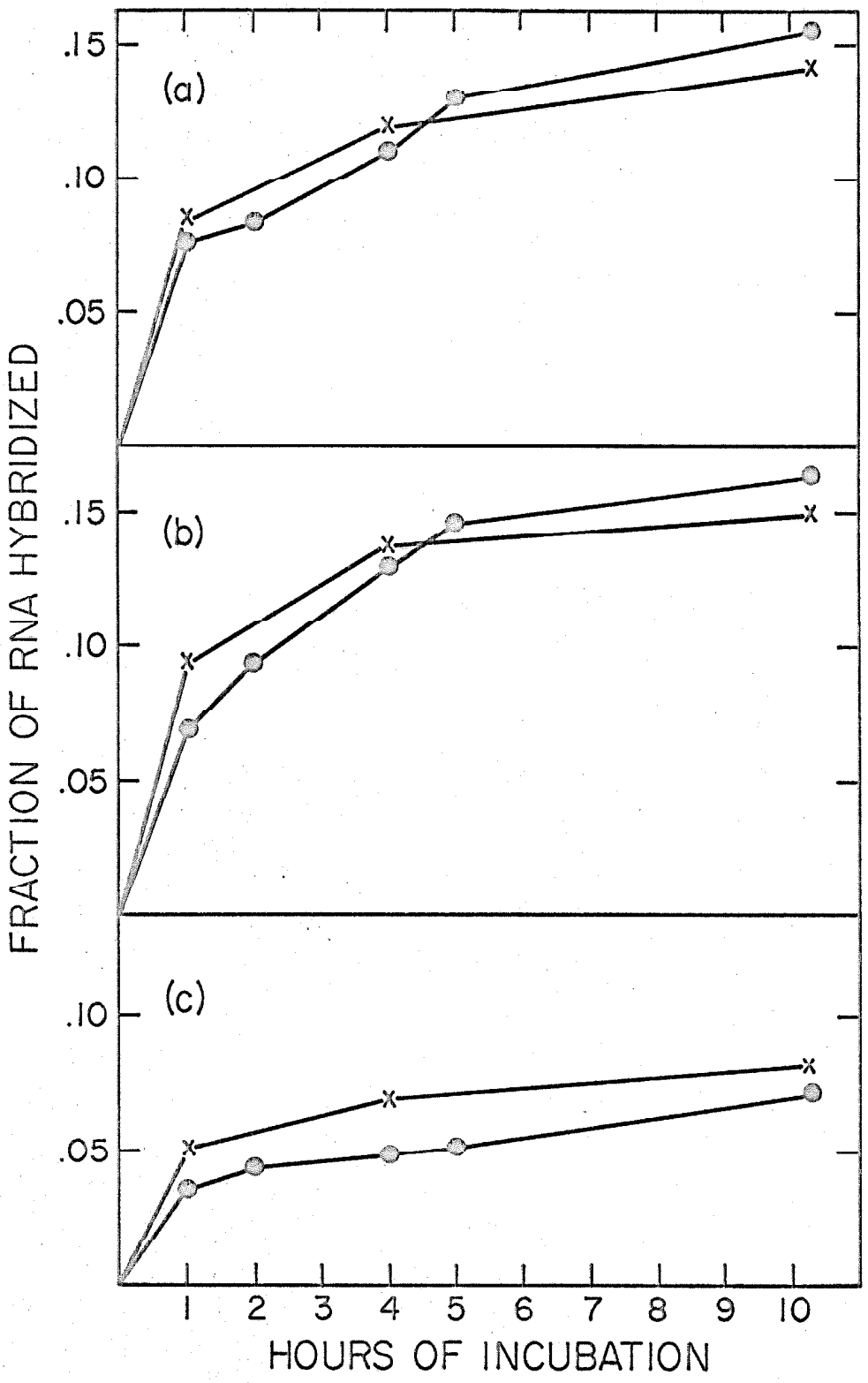
.15
(a)
.10
.05
FRACTION OF RNA HYBRIDIZED
.15
(b)
.10
.05
.10
(c)
.05
1
2
3
4
5
6
7
8
9
10
HOURS OF INCUBATION

Figure 8.

Figure 9. Kinetics of Annealing of Chiton RNA and DNA.

(a) ^{3}H-RNA (0.19 µg/ml) is incubated with denatured DNA (17.4 µg/ml) at 60°C in 2 x SSC and RNA-DNA complexes (●———●) and ribonuclease-resistant hybrid (○———○) assayed.

(b) Formamide denatured ^{3}H-RNA is incubated with denatured DNA (14.3 µg/ml) in 48% formamide, 2 x SSC, at 30°C and RNA-DNA complexes (●———●) assayed as in Figure 8. Ribonuclease-resistant hybrid (○———○) is assayed as previously described.

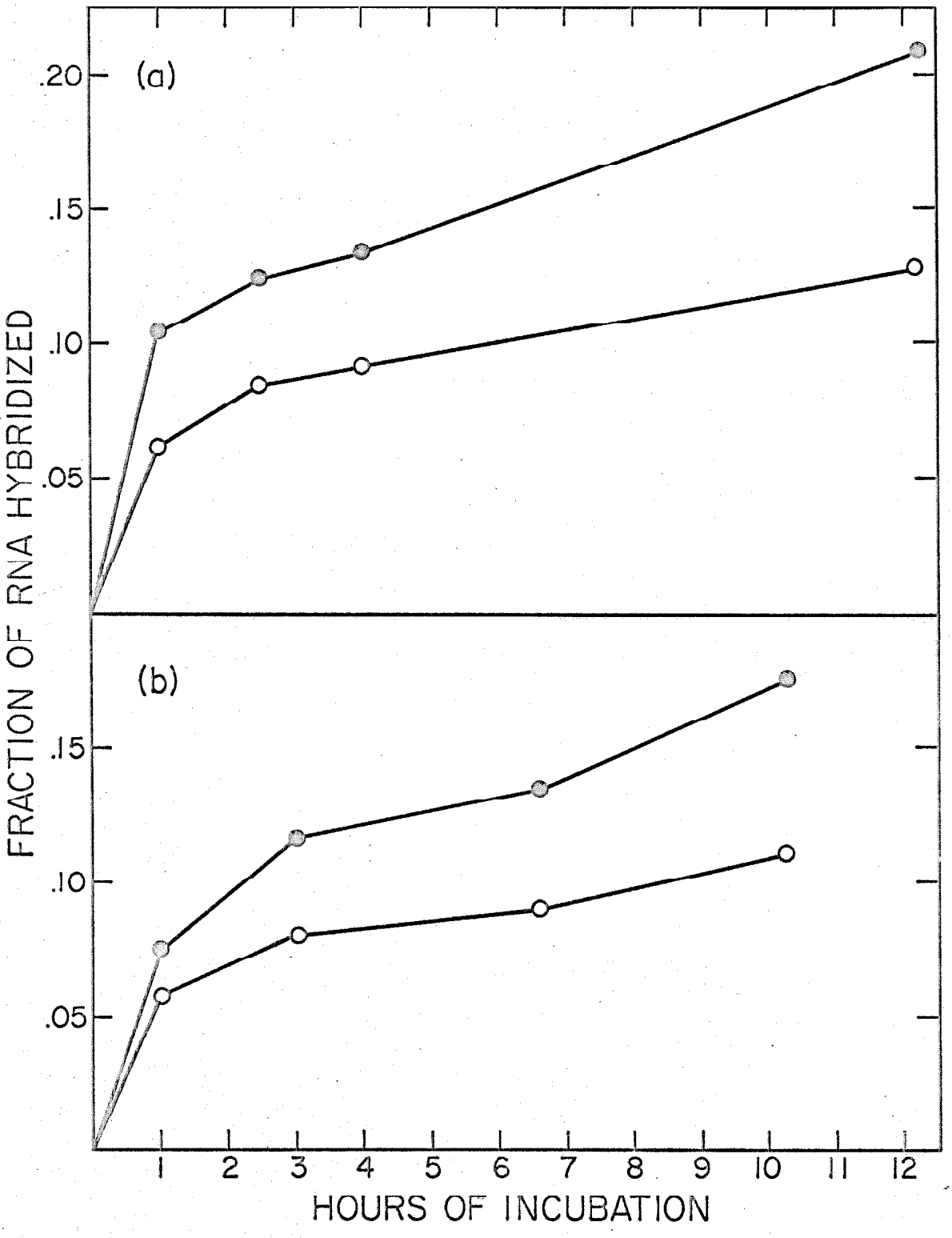
(a)
(b)
.20
.15
.10
.05
.15
.10
.05
FRACTION OF RNA HYBRIDIZED
1
2
3
4
5
6
7
8
9
10
11
12
HOURS OF INCUBATION

Figure 9.

Figure 10. Kinetics of Hybrid Formation at High RNA/DNA Ratios. Denatured chiton DNA (0.38 μg) and ^{3}H-Adenine chiton RNA are incubated in 0.12 ml 2 x SSC at 60°C for 3 hours (X———X) and 12 hours (●———●). Hybrid assayed as described in Methods.

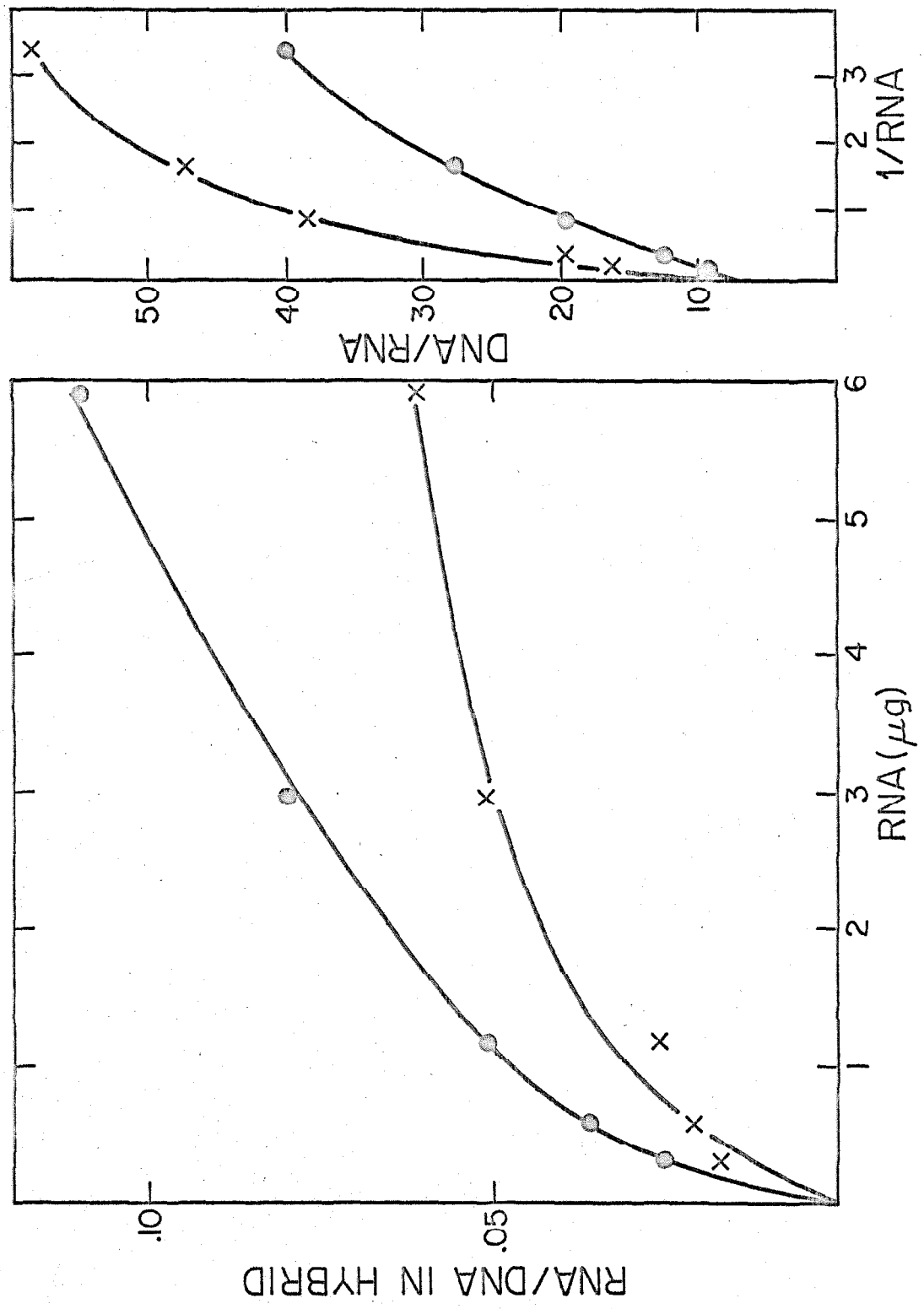
RNA/DNA IN HYBRID
.10
.05
1
2
3
4
5
6
RNA(μg)
DNA/RNA
50
40
30
20
10
1
2
3
1/RNA

Figure 10.

Figure 11. Hybrid Formation at High RNA/DNA Ratios.

Denatured chiton DNA (0.072 μg) and ^{3}H-Cytosine chiton RNA are incubated in 0.12 ml 2 x SSC for 12 hours at 60°C (■———■) and 68°C (●———●). Hybrid is assayed as described in Methods.

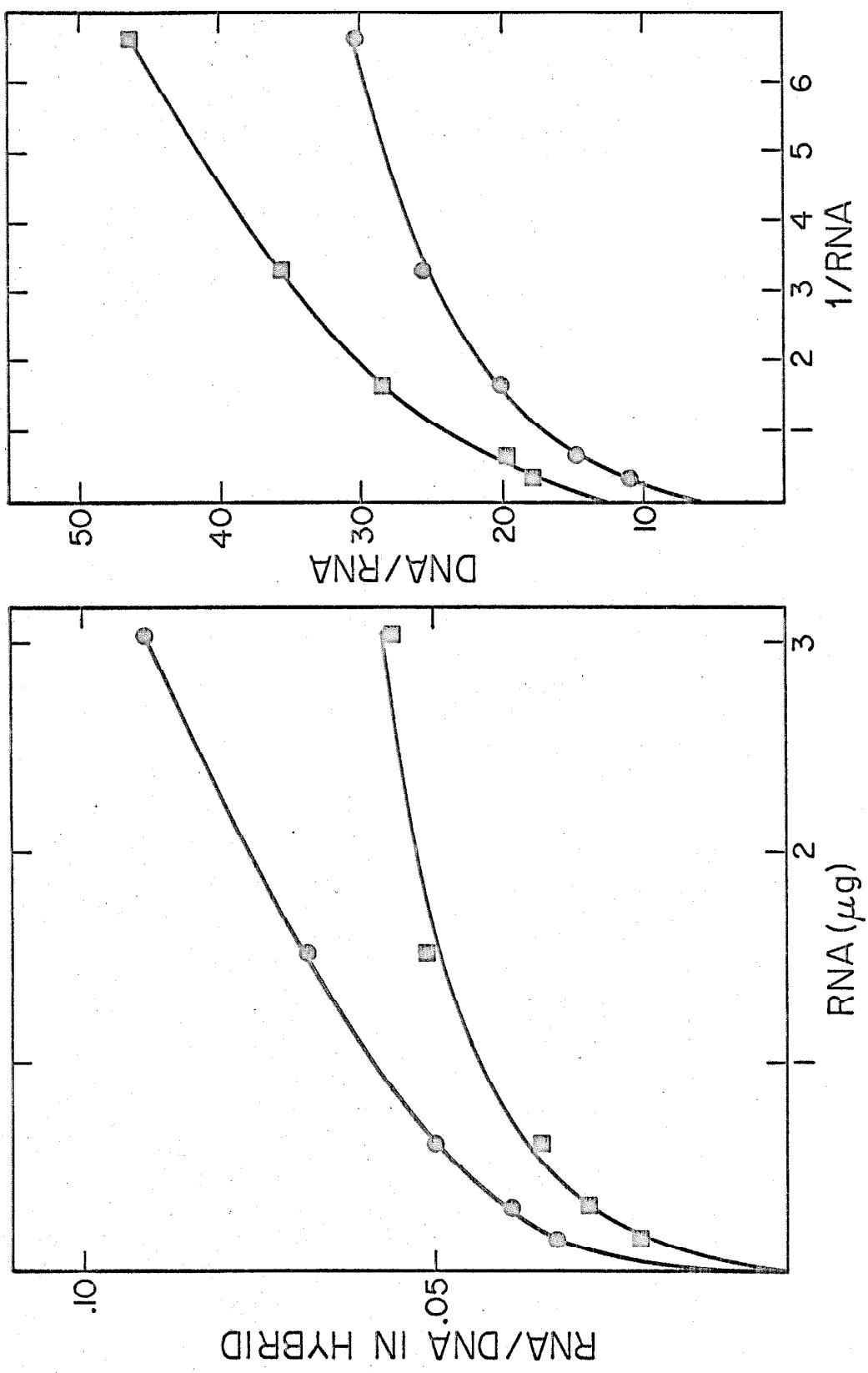
RNA/DNA IN HYBRID
.10
.05
RNA (μg)
1
2
3
DNA/RNA
50
40
30
20
10
1/RNA
1
2
3
4
5
6

Figure 11.

Figure 12. Hybrid Formation at High RNA/DNA Ratios.

Denatured chiton DNA (0.036 μg) and ^{3}H-Adenine chiton RNA are incubated in 0.12 ml 2 x SSC for 18 hours at 61°C. Hybrid is assayed as described in Methods.

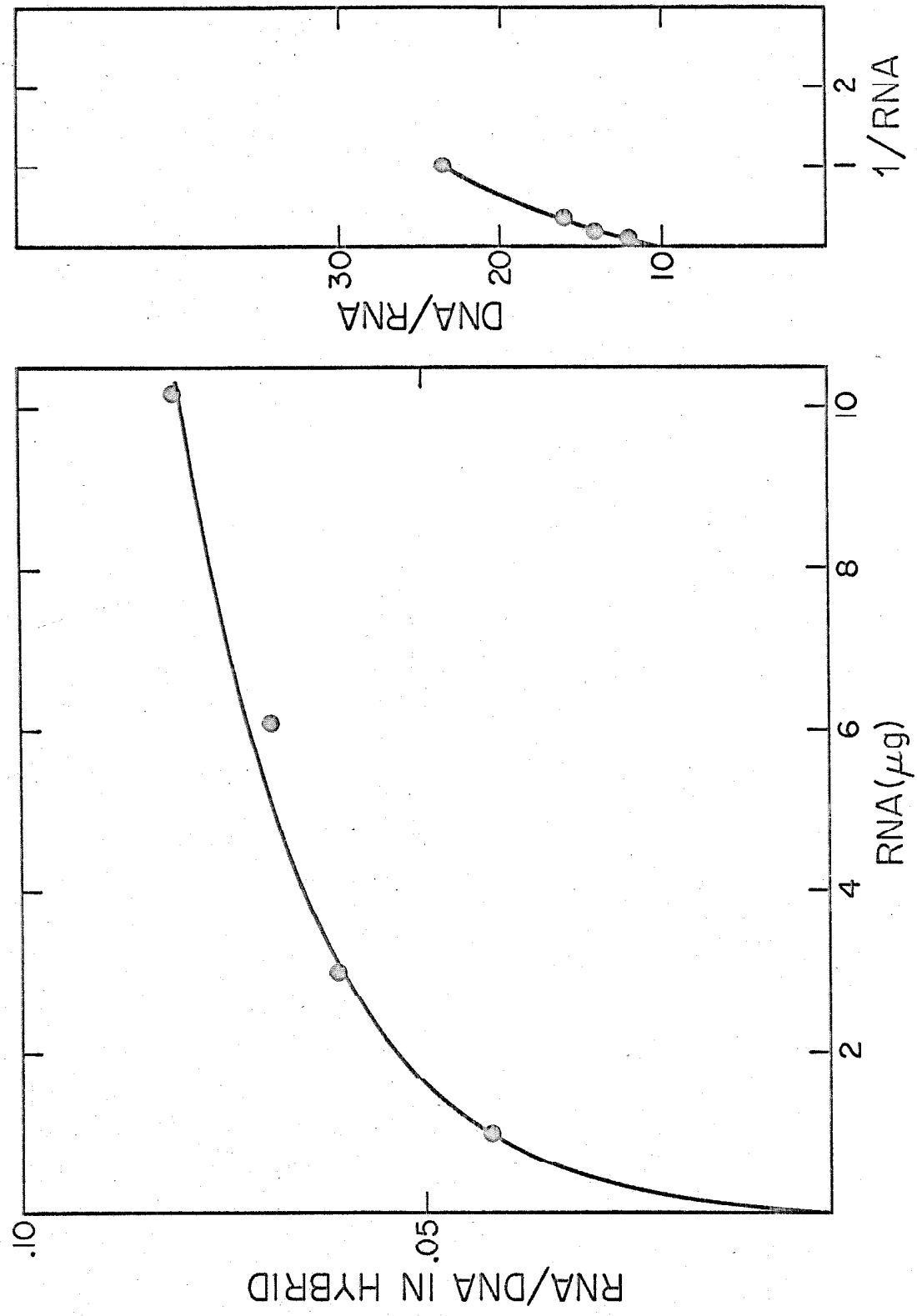

Figure 12.

PART II

The Structure and Replication of Intracellular Bacteriophage Lambda DNA

Chapter 1

The Structure and Properties of Non-Replicating Intracellular Lambda DNA

Summary

Intracellular lambda DNA has been purified from immune bacteria infected with lambda phage.

The prediction from sedimentation properties that one intracellular form of lambda DNA is a twisted, closed-circular molecule has been confirmed by observation in the electron microscope. Two unique forms of lambda DNA are present in infected cells: twisted, closed-circular molecules (component I) and open-circular molecules (component II). One single-strand scission is sufficient to convert component I to component II.

Both components I and II, either native or denatured, are able to infect spheroplasts. The infective entity present after denaturation of component I is a molecule in which strand separation has not occurred. That present after denaturation of component II is a single-strand ring; single-stranded linear molecules are not infective.

The ionic strength dependence of the sedimentation rate of components I and II and phage lambda DNA has been studied. For comparative purposes, similar studies have been made with ØX 174 RF. A new consequence of DNA circularity is evident from these studies.

The work presented in this chapter was done in collaboration with Dr. E.T. Young,II and is presented in the form in which it was published or submitted for publication.

1. INTRODUCTION

The DNA of mature particles of phage lambda exists as a linear, double-stranded molecule. Each 5' end of this linear molecule possesses a short single-stranded region of about 16-20 nucleotides (Kaiser, personal communication). The base sequence of the single-stranded region at one end of the molecule is complementary to that at the opposite end of the molecule. As a consequence of this complementarity the ends of the molecules are able to form hydrogen-bonded complexes in vitro resulting in intramolecular circles and intermolecular dimers, trimers, etc. of the whole molecule (12,13,20,23,26).

Upon infection by phage lambda of either sensitive or immune bacteria, some of the infecting DNA is found to possess a circular form (1, 21, 34). It has been suggested that this circular form is the replicative form of lambda DNA (36,37). Several forms of intracellular lambda DNA are known, including: component I, a twisted-circular DNA in which both strands are covalently closed; component II, an open-circular DNA, possessing one or more single-stranded breaks per molecule; and component III, which is identical to linear phage DNA. All three components are biologically active in a spheroplast assay (35).

This paper presents a preparative procedure for obtaining intracellular lambda DNA free of bacterial DNA and RNA and a study of the biological and physical properties of the purified DNA. In addition to the biological interest of components I and II, the supercoiled form of lambda DNA (component I) is potentially useful for a variety of physical studies concerned with the origin and consequences of DNA circularity as

it is the highest molecular weight closed-circular DNA presently available. Further, the high degree of both regional and temporal control of RNA transcription from lambda DNA (6,7,29) makes intracellular lambda DNA a natural choice for studying RNA transcription and control of transcription in vitro.

2. MATERIALS AND METHODS

(a) Media and Solutions

All solutions are made with distilled deionized H_2O and are sterilized before use.

2 x M9 medium contains 14 g Na_2HPO_4, 6 g KH_2PO_4, 2 g NH_4Cl, 10 ml. 1 M $MgSO_4$, 4 ml. 25% NaCl, and 40 ml. 10% glucose per liter. The glucose and $MgSO_4$ are added sterily after autoclaving.

K medium contains 500 ml. of a 3% solution of vitamin-free casamino acids (Difco) and 500 ml. of 2 x M9. The two solutions are autoclaved separately and mixed after cooling to room temperature.

Modified K medium contains 548 ml. H_2O, 91 ml. concentrated salts, 36 ml. 10% glucose, 320 ml. 3% casamino acids (Difco), 4.5 ml. 1.0 M $MgSO_4$ and 0.18 ml. 0.5 M $CaCl_2$ per liter. Concentrated salts contains 70 g Na_2HPO_4, 30 g KH_2PO_4, 5 g NaCl, 10 g NH_4Cl and 1 liter H_2O. All solutions are autoclaved separately and mixed after cooling to room temperature.

Tris buffers, either pH 8.1 or 7.2 are made with Trizma base (Sigma Chemical Co.) and HCl.

TM is 0.01 M tris, pH 7.2, 0.01 M $MgSO_4$.

TE is 0.01 M tris, 0.001 M EDTA pH 8.1.

SSC is 0.15 M NaCl, 0.015 M Na_3 citrate. 1x, 2x, etc. indicates the degree of concentration of this solution.

(b) Chemicals and Enzymes

Brij 58 (polyoxyethylene (20) cetyl ether) a non-ionic detergent, Atlas Chemical Industries, Inc.; Ribonuclease-A, Type II-A, 90 Kunitz units/mg., Sigma Chemical Co., is dissolved at 5 mg/ml. in 0.01 M Na_3 citrate-HCl, pH 5.0, boiled 10 minutes, quickly cooled and stored frozen; ethidium bromide, a gift from N. Davidson, is available from Boots Pure Drug Co., Ltd., Nottingham, England; Lysozyme, Grade 1, 20,000 units/mg., Sigma Chemical Co., used for the lysis of infected bacteria is dissolved just before use in 0.25 M tris, pH 8.1; Cytochrome-C (equine heart), A grade, Cal Biochemical Co.; E. coli exonuclease I (4,000 units/ml.) was a gift from I.R. Lehman; Yeast s-RNA, Type III, Sigma Chemical Co.

(c) Growth of Bacteriophage

Bacteriophage and bacterial strains or lysogenic derivatives of them employed here are described by Young and Sinsheimer (35) as are the conditions for the growth and purification of radioactively labelled phage stocks. Non-radioactively labelled phage stocks are produced by growing W3110 to 3-4 x 10^8 cells/ml. in modified K medium and infecting with 0.01 pfu[1]/cell. Lysis is usually complete by 3 hours after infection. Titers in excess of 1 x 10^{11} phage/ml. are routinely obtained using modified K medium.

(d) Assay for biologically active DNA

The spheroplast assay described by Young and Sinsheimer (35) is used with one alteration of the procedure. Bacteria are grown in 60 ml. of 3xD to a concentration of 1 x 10^8 cells/ml. and concentrated to provide 20 ml. of bacteria at 3 x 10^8 cells/ml. This change makes the assay more reproducible.

(e) pH Measurement

A Radiometer Titrator with a G222B glass electrode and a K 130 saturated KCl, liquid junction electrode, is used. No corrections have been made in the pH values reported. The accuracy, determined with a saturated solution of a $Ca(OH)_2$, is within 0 to +0.04 pH units, and the precision is about $\pm$ 0.05 pH units, in the range pH 12-13.

(f) DNA denaturation

Buffers for alkaline titration of DNA are prepared by mixing 5.0 ml. of 0.20 M K_2HPO_4, x ml. of 1.0 M KOH, and H_2O to produce the desired pH in a final volume of 20 ml. Usually 0.30 ml. of DNA solution in low ionic strength buffer is mixed with 1.7 ml. of alkaline buffer and incubated 15 minutes at 37°C and then neutralized with HCl to a pH of about 8.1. Other procedures are occasionally used and these will be described as they occur.

(g) Ultraviolet irradiation of DNA

A low pressure mercury germicidal lamp which emits most of its radiation at 2537 Å is used as a source. DNA is irradiated in 0.05 M

phosphate buffer pH 7.4. The solution is contained in a watch glass and stirred by a magnetic stirrer during irradiation.

(h) Exonuclease I treatment

To 0.60 ml. of DNA in 0.1 x SSC, 0.025 ml. of 5 N NaOH is added and the solution is incubated at 37°C for 10 min. 0.030 ml. of 2.0 M glycine is then added along with sufficient 6 N HCl to bring the solution to pH 9.3, 0.005 ml. of Yeast s-RNA (1.68 mg/ml. dissolved in 0.01 x SSC) and 0.005 ml. of 1.0 M $MgCl_2$. The solution is divided into portions and each portion is treated with a given amount of exonuclease I at 45°C for 15 minutes. The reaction is stopped by adding 0.5 M EDTA pH 8.1 to give a concentration of 0.03 M EDTA.

(i) Electron Microscopy

DNA is observed in the electron microscope after a preparation essentially according to the procedure of Kleinschmidt and Zahn (17). DNA is spread on a water-air interface in the presence of cytochrome-C, picked up on 3% parlodion-coated copper grids and shadowed with Pt-Pd. The electron microscope facilities were graciously made available by Dr. A. Hodge.

(j) Analytical Ultracentrifugation

Sedimentation analysis is carried out in a Beckman model E ultracentrifuge equipped with a photoelectric scanner and multiplex accessory operating at 2650 Å.

Band sedimentation is performed using 12 mm. charcoal filled epon centerpieces of Type III (32). Sedimentation is usually at 25,980 rev./min. In order to minimize convective instability the heater unit is not used during centrifugation. The temperature change during a run is between one and two degrees. In calculating $S_{20,w}$, the average temperature is used. In general 0.02 ml. of a DNA solution of about 0.2 A_{260} units/ml. or less is placed in the sample well. When the bulk solution is less than 1.0 M NaCl it is made up in 50% D_2O. When the bulk solution is alkaline, the sample is denatured prior to centrifugation by addition of concentrated NaOH to the sample well to give a final concentration of 0.2 N NaOH. The solvent density and viscosity factors given by Studier (27) are used to calculate S_w. In comparative studies at least two DNA species are always present and are sedimented simultaneously in the same cell.

Sedimentation into alkaline buoyant CsCl is performed at 39,460 rev./min. with the temperature controlled at 25°C.

For sedimentation studies ØX174RF II is produced from RFI by making the DNA sample in the well 1.0 mM in hydroquinone (31) which converts about half of the RFI to RFII.

(k) Preparation of intracellular lambda DNA

(i) E. coli W3110 lysogenic for the strain of phage to be used in the infection is grown to about 1 x 10^9 cells/ml. in modified K medium (unless stated otherwise) at 37°C, infected with 20-25 pfu/cell, and vigorously aerated at 37°C. After 45-60 minutes the culture is cooled

and the cells are collected by centrifugation. The cells are resuspended in cold TM and uninjected phage are removed from the bacteria by blending at full speed in a Waring Blender for 2 min at 4°C, followed by centrifugation to collect the cells. When radioactively labelled phage infect sensitive bacteria at low multiplicity, this blending procedure removes a significant fraction of the adsorbed radioactivity without changing the number of infective centers.

(ii) The cell paste from a one-liter culture is then resuspended in 10 ml. of cold 25% sucrose in 0.04 M tris, pH 8.1, blended in a Waring Blender to disperse the cells, and treated according to the procedure of Godson (1966). The cells are converted to spheroplasts by the addition of 1.0 ml. of lysozyme (6.4 mg/ml.) and 1.0 ml. of 2% (w/v) EDTA (disodium salt). After three minutes on ice, during which time spheroplasts are formed, a solution containing 1.1 ml. of 0.1 M $MgSO_4$ and 1.5 ml. of 5% (w/v) Brij (in 0.01 M tris, pH 7.2) is added with mixing. The viscous solution is transferred to a polycarbonate screw cap centrifuge tube and centrifuged for 15 minutes at 30,000 rev./min in a Spinco 30 rotor. The non-viscous supernatant is decanted from the gelatinous pellet which contains cell membranes and DNA.

(iii) The supernatant solution is brought to 1 x SSC by the addition of 20 x SSC and extracted once with redistilled phenol previously equilibrated with 1 x SSC. The aqueous phase is collected after centrifugation at 5,000 rev./min in a Sorvall centrifuge GSA rotor. Phenol is removed from the aqueous solution by ether extraction, and the ether is removed by bubbling nitrogen gas through the solution.

(iv) From this stage several diverse procedures have been used to further purify the intracellular lambda DNA.

(1) NaCl is added to 1.0 M and the solution is stored in the refrigerator overnight. The precipitated RNA is removed by centrifugation and discarded. The solution is diluted to 0.4 M NaCl with H_2O and passed through a methylated albumin-Kieselguhr column, previously washed with 0.4 M NaCl, as described by Sueoka and Cheng (28). The column is washed further with 0.4 M NaCl and then the material on the column is eluted with a linear 0.4 to 1.0 M NaCl gradient (buffered with 0.05 M sodium phosphate, pH 6.7). Fractions containing the DNA, which elutes at 0.75 M NaCl, are combined and dialysed against TE.

(2) The preparation is treated with 10 µg/ml. of RNase at 37°C for one hour and then brought to pH 5.4 with HCl and passed thru a 6 x 25 cm Sephadex G-100 column equilibrated with 0.01 M Na_3 citrate, 0.02 M NaCl, pH 5.4. The DNA comes out in the void column while the RNase digestion products are retarded in their passage through the column. The fractions containing DNA are pooled and passed through a column containing IRC-50 (which has been heated to 60°C for 10 minutes and washed thoroughly) to remove RNase. The pH of the DNA solution is raised to 8 with KOH. Exposure of the DNA to pH 5.4 during this procedure does not introduce single-strand breaks.

(3) A step gradient of CsCl in 15% sucrose containing 0.04 M tris, pH 8.1 is prepared in a centrifuge tube and consists of 2 ml. 65%, 3 ml. 55% and 2 ml. 45% CsCl (w/v), respectively. The phenol-extracted supernatant is layered gently over the CsCl gradient to fill the tube

which is then centrifuged in the Spinco SW25.1 rotor at 25,000 rev./min for 20-24 hours at 6°C. A hole is pierced in the bottom of the tube and one-ml. fractions are collected. This procedure concentrates the DNA in the middle of the CsCl gradient. Fractions containing DNA are pooled and dialysed against TE.

(v) The procedures described above give preparations containing three species of lambda DNA; closed-circular DNA (component I); open-circular DNA (component II); and linear DNA (component III). Closed-circular DNA can be purified free of components II and III by CsCl equilibrium density gradient centrifugation in the presence of ethidium bromide (22). The preparation is brought to 100 µg/ml. ethidium bromide, the density adjusted to 1.56 g/cm^3 by addition of solid CsCl, and is then centrifuged for 2-3 days at 37,000 rev./min and 10°C in the Spinco SW 50 rotor. RNA, if present, forms a red pellet on the bottom of the tube. The DNA is present at two density positions in the tube, the denser of which is the closed-circular DNA. Fractions are collected as usual. The free and intercalated dye can be removed from the DNA solution by passage through a cation exchange resin as described by Radloff, Bauer and Vinograd (22).

(vi) Concentration of the preparation has usually been necessary at various stages. This has been accomplished either by evaporation using a Buchler Flash Evaporator or by dialysis against dry sucrose.

3. RESULTS AND DISCUSSION

I. Purification and Biological Properties of Intracellular Lambda DNA.

(a) Separation of ^{32}P-intracellular lambda DNA and ^{3}H-E. coli DNA.

Each E. coli cell contains an amount of DNA equivalent to about 100-300 lambda genomes, depending on the number of bacterial chromosomes in a cell. Lambda DNA and E. coli DNA have the same base-composition and thus cannot be separated by conventional methods of DNA fractionation. Advantage has been taken of the fact that with gentle lysis of the cells with a non-ionic detergent the bacterial DNA remains attached to, or contained within the cell membranes and can be removed by a brief centrifugation, while much of the cytoplasmic content of the cells is released and does not sediment with the cell DNA. Godson and Sinsheimer (11) used this technique, which we have adapted, to prepare E. coli polyribosomes. In order to test the efficiency of this method for separating intracellular lambda DNA from the bulk of the E. coli DNA, the experiment described below was performed.

E. coli W3110 (λ) was grown to a cell concentration of 1×10^8 cells/ml. in 20 ml. of K medium at 37°C. Deoxyadenosine at a final concentration of 250 μg/ml. and 0.1 mc. of ^{3}H-thymidine were added and growth allowed to continue for over an hour, after which time the cells were collected by filtration, washed to remove unincorporated tritium and resuspended in 20 ml. of nonradioactive medium. (Deoxyadenosine enhances the incorporation of exogenous thymidine in a non-thymine-requiring host (2).) When the cell density had reached 2.7×10^9 cells/ml.,

^{32}P-lambda C_{26} was added at a multiplicity of about 7 pfu/cell. After further incubation for 30 minutes the cells were collected by centrifugation, blended and treated as described in Methods with the exception that the concentration of cells in the lysis medium was 0.2 x that described. An aliquot of the blended resuspended cells was removed before lysis to determine the amount of TCA-insoluble ^{3}H and ^{32}P present.

The pellet (containing membranes and DNA) obtained from the high speed centrifugation of the detergent-treated spheroplasts was resuspended in a volume of TE equivalent to that of the supernatant by vigorous vortexing. The NaCl concentration was increased to 0.2 M and both fractions (resuspended pellet and supernatant) were extracted with phenol, the phenol removed from the aqueous phase by ether extraction, and the ether removed by bubbling N_2 gas through the solution. Aliquots of the pellet and supernatant fractions were precipitated with TCA before and after phenol extraction; the precipitates were collected on filters and counted in a scintillation counter to determine the ^{3}H and ^{32}P activities of each fraction. The results are shown in Table 1. The recovery of ^{32}P-lambda in the supernatant was 31%. About 99.6% of the ^{3}H-*E. coli* DNA was in the pellet fraction.

The specific activity of the ^{3}H-*E. coli* DNA in the pellet, determined from the amount of ^{3}H in that fraction and the amount of DNA assayed chemically by the indole procedure of Keck (15), was 1.7×10^{4} cts/min/μg DNA. The specific activity of the ^{32}P-lambda DNA was calculated to be 6.4×10^{4} cts/min/μg DNA from the ^{32}P content and the absorbance at 2600 Å of the phage preparation. Using these values and the total amount of ^{3}H and ^{32}P in the pellet plus supernatant fractions,

the ratio of bacterial DNA to lambda DNA before centrifugation of the lysate was 10.4 μg:1.0 μg. The ratio in the phenol-extracted supernatant after centrifugation was 0.065 μg *E. coli* DNA: 1.0 μg lambda DNA, a purification relative to *E. coli* DNA of 160-fold.[2] The phenol-extracted supernatant contained 8.8 μg of lambda DNA and 0.57 μg of bacterial DNA.

Further purification of this preparation was performed by procedure 1 of Methods. No further separation of lambda and *E. coli* DNA was obtained, but the bulk of the RNA remaining after the salt precipitation was separated from the DNA by passage through the MAK column as shown in Fig. 1. DNA which eluted from the column was sedimented through a neutral sucrose gradient. Approximately 60% of the lambda DNA sedimented as component I; the remainder of the ^{32}P sedimented as component II or degraded material.

The DNA obtained from the MAK column was examined in the electron microscope after preparation according to the method of Kleinschmidt and Zahn (17) (Plate 1). Three types of DNA molecules, distinguishable by their unique configurations, were observed: tightly twisted circular molecules; open-circular molecules; and linear molecules of various lengths. Approximately 50% of the molecules seen on the grids are of the tightly twisted type, in agreement with the sedimentation analysis which revealed about 60% of the preparation to be component I. To exclude the possibility that the tightly twisted molecules observed were an artifact introduced by the preparation of the grids, hydrogen-bonded circular DNA was prepared from phage DNA by the method of Hershey,

Burgi & Ingraham (13) and examined in the electron microscope as described above. As can be seen in Plate 2, tightly twisted circular molecules were not observed. Thus the prediction from sedimentation properties (1, 35) that component I is a tightly twisted, circular form of lambda DNA, analogous to ØX174 RF (4,24) or polyoma viral DNA (31) is substantiated by direct observation.

(b) Separation of closed-circular lambda DNA from linear and open-circular DNA

Preparations of intracellular lambda DNA almost (93%) free of E.coli DNA can be obtained by the methods just described. However, these preparations still contain three species of lambda DNA (linear, open circular, and closed circular molecules) and, occasionally (when the gentle lysis procedure does not confine all of the bacterial DNA to the pellet fraction) significant amounts of E. coli DNA. Closed-circular lambda DNA can be separated from these other DNA species by CsCl density gradient centrifugation in ethidium bromide as described by Radloff, Bauer & Vinograd (22). Figure 3 of that publication shows the separation of component I from components II and III achieved by this technique; the material is lambda b_2b_5c intracellular DNA prepared by us using method 2.

The sedimentation of lambda b_2b_5c component I, prepared by CsCl equilibrium centrifugation in ethidium bromide and passage through a cation exchange resin (Methods (v)), in the analytical ultracentrifuge is shown in Figure 2. The material seen at the meniscus is diffusing

down the cell and not sedimenting. It is a low molecular weight contaminant which does not form a band during CsCl equilibrium density gradient centrifugation. It can be separated from the DNA by passage through a G-100 Sephadex column. The small amount of component II observed is due to subsequent conversion of component I by strand scission (see below) and does not reflect any inadequacy of the ethidium bromide technique.

(c) A note on the recovery of closed-circular DNA

Starting with 10^{14} phage particles (100 A_{260} units or 5 mg. of DNA) which would be used to infect a 5-liter culture, the best recovery obtained has been only 3-5%. Usually only 50% of the phage inject. Of this 50%, only 30% is recovered in the supernatant after gentle lysis and centrifugation. In the final equilibrium centrifugation in ethidium bromide to separate closed circular DNA from other forms of DNA, typically only 15-30% of the DNA is supercoiled as compared to 60% or more at the time of lysis. The step or steps at which component I is preferentially lost have not been identified. The relative loss of component I observed may be due to conversion of component I to component II. Such a conversion is partially inherent in the large size of the lambda molecule relative to other closed circular DNA species such as ØX RF or polyoma DNA that have been studied. Thus, one single-strand scission per 30 x 10^6 daltons of DNA would degrade 63% of lambda component I but less than 10% of ØX RF I.

(d) Infectivity of native and denatured purified intracellular lambda DNA

The circular intracellular forms of lambda DNA are infective in a spheroplast assay (35). After alkaline denaturation of preparations of intracellular lambda DNA a reproducible but variable increase in the infectivity is observed. This increase was originally attributed to a loss of the ordered secondary structure of component I without concomitant separation of the strands, resulting in anincreased ability to infect spheroplasts. Double-strandedness was assumed to be a prerequisite to infection with lambda DNA since denatured phage DNA was not infective (or was much less so than native DNA) in either the helper assay (14) or the spheroplast assay described by Brody, Coleman, Mackal, Weringhaus & Evans(3). Since the closed-circular form of polyoma DNA denatures at a higher pH than the open-circular form (30), an alkaline titration of the infectivity present in intracellular lambda DNA and in lambda phage DNA was performed to determine the pH at which intracellular DNA would increase in infectivity and phage DNA would decrease in infectivity, (see below). Unexpectedly, the transition in both cases occurred at the same pH. This result led us to other experiments described below and elsewhere (16) which indicate that the increase in infectivity observed for intracellular lambda DNA upon denaturation is due to single-stranded DNA arising from the denaturation of component II and that it is the single-stranded rings produced from component II that are infective. The infectivity of component I remains virtually unchanged after incubation at pH which denatures component II.

(i) Alkaline titration

Intracellular lambda DNA prepared by Method 2 was further purified by sedimentation through a neutral sucrose gradient. Fractions containing components I and II were pooled, dialysed, the volume reduced to 3 ml., and then dialysed against 0.01 x SSC. Portions of this solution were treated with alkali as described in Methods. After denaturation the samples were assayed in spheroplasts. The same experiment was performed with b_2b_5c phage DNA. Figure 3 shows the biological activity of the intracellular (i^{λ}) and phage DNA (i^{b5}) as a function of pH. The midpoint of the transition is pH 11.8 $\pm$ 0.1, both for the inactivation of phage DNA and the activation of intracellular DNA. The biological activity of the phage DNA decreased 100-fold between pH 11.5 and pH 12.0 while the activity of the intracellular DNA increased 25-fold in the same pH range. The pH-induced activation curve is quite sharp, unlike the denaturation of polyoma DNA component I observed by hypochromicity (30).

(ii) Buoyant density and infectivity

In order to determine whether the infectivity present after treatment of the intracellular DNA with alkali is due to material with the buoyant density of native or denatured DNA, a sample of a preparation containing ^{32}P-b_2b_5c intracellular DNA prepared by Method 2 was brought to pH 12.2 with NaOH for 15 minutes at 25°C and then neutralized with HCl. A buoyant density marker, native ^{3}H-lambda C_{26} DNA, was added and the density raised to 1.71 g/cm^3 with CsCl. The sample was then centrifuged for 37 hours at 30,000 rev./min and 20°C. After deceleration,

fractions were collected from a hole punched in the bottom of the tube and assayed for radioactivity and biological activity. The results are shown in Figure 4.

The radioactivity (^{32}P) and infectivity (i^{b5}) on the gradient containing denatured intracellular DNA are present in two peaks. The density difference between the marker, native ^{3}H-lambda DNA and the heavy ^{32}P peak is about 0.015 g/cm^3, the density difference expected if the heavy peak represents denatured DNA.

Comparable amounts of infectivity are present in the heavy and the light peaks, indicating that some denatured form of intracellular lambda DNA is much more infective than denatured, linear phage DNA (which has been shown to have very little infectivity) (16). It does not seem likely that the infectivity in the heavy (denatured) peak could be that of denatured RFI as a sample of the heavy peak sediments through an alkaline sucrose gradient at very nearly the same rate as denatured phage DNA without any clear indication of the presence of a more rapidly sedimenting component (Figure 5 (b)).

Actually, most or all of the component I appears to have renatured. The light peak of ^{32}P (Fig. 4) represents 30% of the material on the gradient and is composed almost equally of renatured (or undenatured) component I and renatured components II or III, as indicated by sedimentation of material from the peak through an alkaline sucrose gradient (Figure 5 (c)). Component I is known to comprise 16% of the ^{32}P-intracellular lambda DNA used for this experiment as determined by sedimentation through a neutral sucrose gradient (Figure 5 (a)) and also by

CsCl equilibrium density sedimentation in ethidium bromide. Thus, most or all of the component I initially present is in the light peak. This suggests that the infectivity present in the heavy peak is in a single-stranded DNA, arising from denatured component II.

(iii) Ultraviolet sensitivity of the infectivity of native and denatured intracellular DNA

The experiment described above (ii) does not rule out the possibility that the heavy infective entity in denatured intracellular DNA is renatured in the process of assay. To clarify this point, the ultraviolet sensitivity of the infectivity of native and denatured intracellular lambda DNA was tested.

Native intracellular lambda DNA prepared by Method 2 was irradiated as described in Methods. Aliquots which had been irradiated were divided into two portions and one portion treated with alkali at pH 12.3 for 15 minutes at 25°C, and reneutralized with HCl. The biological activity of each portion (native and denatured) was then assayed in spheroplasts. The result is shown in Figure 6.

The aliquot which received no irradiation shows a 12-fold increase in infectivity upon denaturation. The native DNA exhibits a linear inactivation with dose. The inactivation curve for the alkali treated DNA has a break in it, indicating that two components with different ultraviolet sensitivities contribute to the infectivity of denatured intra-cellular DNA. More than 90% of the infectivity present after denaturation is 5x more sensitive to ultraviolet radiation than the infectivity of native DNA, as would be expected if this infectivity were

due to single-stranded DNA. The less sensitive component present in denatured DNA has about the same sensitivity as native DNA suggesting that it is due either to a component which was not denatured (or renatured rapidly) or which did not undergo strand separation upon denaturation. This is probably component I. If the curve for denatured DNA at high dose is extrapolated to zero dose a value is obtained which is 65% of the infectivity present at zero dose for native DNA. The preparation of intracellular DNA used in this experiment consisted of only 20-30% component I. This may indicate that alkali treated component I can increase in infectivity by as much as a factor of 2 or 3 (while retaining its UV resistance).

More than 90% of the infectivity present after denaturation has the enhanced ultraviolet sensitivity of single-stranded DNA (33) in an HCR^+ host. Thus, it may be concluded that the most infective species present after denaturation is a single-stranded molecule arising from the denaturation of component II. This infectivity present upon denaturation of component II sediments in alkali at a rate expected for single-stranded rings (16; see below). That single-stranded rings are responsible for the increased infectivity of denatured intracellular DNA was confirmed by the following experiment.

(iv) Exonuclease I sensitivity of infective single-stranded intracellular lambda DNA

The same preparation of intracellular lambda DNA used for (iii) was denatured and treated with exonuclease I as described in Methods.

S-RNA was added to inhibit endonuclease I, which is known to contaminate some exonuclease I preparations (19). Endonuclease action was monitored during the treatment with exonuclease I by noting the conversion of component I to component II. Samples treated with exonuclease I were analyzed by band sedimentation in the analytical ultracentrifuge and circular and linear lambda single strands identified by their sedimentation rate. Representative traces are shown in Figure 7.

Upon denaturation an increase in infectivity of 5-fold was observed. Treatment with 70 units of exonuclease I, sufficient to render more than 85% of the linear DNA present (most of the linear single-stranded DNA in this preparation was E.coli DNA) non-sedimenting, resulted in virtually no loss of single-stranded rings or of infectivity. Treatment with 350 units, however, resulted in a large loss of infectivity. This loss can be attributed to the action of endonuclease since at this enzyme level all of the component I present before treatment had disappeared when the sample was analysed by band sedimentation.

II. Physical Characterization of Intracellular Lambda DNA and a Comparison with ØX174 RF[3]

(a) Alkaline band buoyancy sedimentation and conversion rates of lambda component I and ØX174 RFI to single strands

Covalently closed double-stranded circular DNA exhibits a greater density in buoyant alkaline CsCl than do single strands. This is true for component I of polyoma viral DNA, SV40 viral DNA and ØX RF (see 30,

for a review of the properties of circular DNA) and for unpurified preparations of intracellular lambda DNA. Demonstration of this fact for lambda intracellular DNA was complicated by the fact that alkaline CsCl promotes single-strand chain scissions (36). This is demonstrated by band sedimentation into alkaline buoyant CsCl in the analytical ultracentrifuge (Figure 8).

Both component I and the single-strands arising from component II can be seen to sediment to their buoyant equilibrium positions in the density gradient. In the course of sedimentation, single-stranded rings are partially separated from single-stranded linear molecules for a time. Before the single strands reach their equilibrium position, single strands produced by conversion of component I can be seen to appear at their characteristic position in the gradient. The rate of single strand scissions under these conditions can thus be determined. At equilibrium the density difference observed between component I and single strands is 0.019 g/cm^3. No resolution of the single strands into heavy and light fractions is observed under our conditions as has been reported by Doerfler & Hogness (8).

The conversion rates for both component I of lambda b_2h_5c and of ØX RF have been determined in this way, under identical conditions (CsCl dissolved in 0.05 M K_3PO_4 to give ρ = 1.767 g/cm^3; pH 12.4 before addition of CsCl, pH 12.8 after addition of CsCl, at 25°C, centrifuged at 39,460 rev./min). In both cases the amount of component I decreases exponentially with time. This can be interpreted to mean that one single-strand scission is sufficient to convert a twisted-circular molecule to

an open-circular molecule. The half life for lambda component I is 219 minutes and for ØX RFI is 1015 minutes. The molecular weight of lambda b_2b_5c DNA is 25.4 x 10^6 daltons (5, 20) and that of ØX RF is 3.2 x 10^6 daltons (18); these molecular weights correspond to 43,500 base pairs and 5,500 base pairs respectively. The rate of single-strand scissions is thus 1.1 x 10^{-7} scissions/base pair/min for lambda b_2b_5c and 1.8 x 10^{-7} scissions/base pair/min for ØX. An explanation of the difference in the rate of scission in the two molecules will probably have to await elucidation of the mechanism of scission in alkali.

(b) Sedimentation properties of circular DNA

The sedimentation properties of lambda component I have been studied preparatively on unpurified material by sedimentation through sucrose gradients at both neutral and alkaline pH (1,36). This work revealed an unusual dependence of sedimentation rate at neutral pH upon ionic strength. These studies have been extended using purified material (Methods iv, 3 & v) in the analytical ultracentrifuge (see Methods). Similar experiments were performed with ØX RF for comparative purposes.

(i) Alkaline sedimentation

Band sedimentation of a mixture of lambda b_2b_5c components I and II into 0.9 M NaCl, 0.1 M NaOH reveals 3 components with $S_{20,w}$ values of 156, 45.5 and 39.5. The fastest sedimenting component is I. It sediments 3.0 times as fast in alkali as it does at neutral pH at the same ionic strength. The two slower sedimenting components arise from denaturation of component II. The species with $S_{20,w}$ of 45.5 is a single-stranded ring

and the other a linear molecule (see above). Studier (27) has found lambda dg DNA to have an $S_{20,w}$ value of 40.1 in alkali.

(ii) Ionic strength dependence of $S_{20,w}$ for lambda and ØX DNA at neutral pH.

The ionic strength dependence of $S_{20,w}$ for lambda b_2b_5c components I and II and phage DNA is shown in Figure 9. It is evident that the sedimentation rates of both I and II increase below 1.0 M NaCl while the sedimentation rate of phage DNA shows either no change or decreases. Below 0.01 M NaCl, however, all three components show a decrease in sedimentation rate. These changes are better illustrated in Figures 10 and 12. Similar data for ØX 174 RF I and II are presented in Figures 11 and 12. In general the magnitude of these changes is greater for lambda DNA than for ØX DNA.

The slight decrease in sedimentation rate of linear DNA with decreasing ionic strength has been explained as an increase in the frictional coefficient of the molecule due to an extension of the molecule resulting from intramolecular electrostatic repulsion. There are two possible explanations for the opposite effect observed for component I: (1) the frictional coefficient decreases because the molecule becomes more compact at lower ionic strength; (2) the frictional coefficient decreases due to a change in hydration of the molecule with salt concentration. The latter explanation seems unlikely since no radical change in hydration seems to occur over the same range of salt concentration for linear molecules, as evidenced by their sedimentation rate. The former explanation requires that the molecule become more compact as

the intramolecular electrostatic repulsion increases. This seems possible only through a change in the pitch of the Watson-Crick helix. If the pitch were to decrease at decreasing salt concentration (down to 0.01 M), this would result in an increase in the number of super-helical twists in the molecule (30) which would cause it to compact.[4] The conformation of the molecule at any salt concentration would be the result of the opposing forces of electrostatic repulsion and the twisting imposed by the pitch of the helix. The decrease in sedimentation rate of component I below 0.01 M NaCl must indicate an extension of the molecule which could be due to either electrostatic repulsion or to incipient denaturation of enough base pairs to remove some of the super-helical twists (30), or to both.

The slight increase in sedimentation rate of component II between 1.0 M and 0.1 M NaCl as compared to linear DNA is noteworthy but an explanation cannot be offered.

Gellert (9) has observed a similar effect of salt concentration on the sedimentation rate of covalently closed circular lambda DNA formed *in vitro* by an extract of *E. coli* acting on hydrogen-bonded circles, and Upholt & Vinograd (personal communication) have demonstrated this effect on components I and II of polyoma viral DNA.

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FOOTNOTES

1. Abbreviations used: pfu, plaque forming unit; TCA, trichloroacetic acid; MAK, methylated-albumin Kieselguhr.

2. This includes a preferential loss of *E. coli* DNA during phenol extraction.

3. The ØX174 RF I used in these experiments was the generous gift of Dr. T. Komano and was prepared by the method of Komano and Sinsheimer (manuscript in preparation).

4. While a change in pitch of the helix is also envisioned for open circular and linear molecules, a change of pitch in these molecules would only have the effect of changing the length of the molecule by a small fraction.

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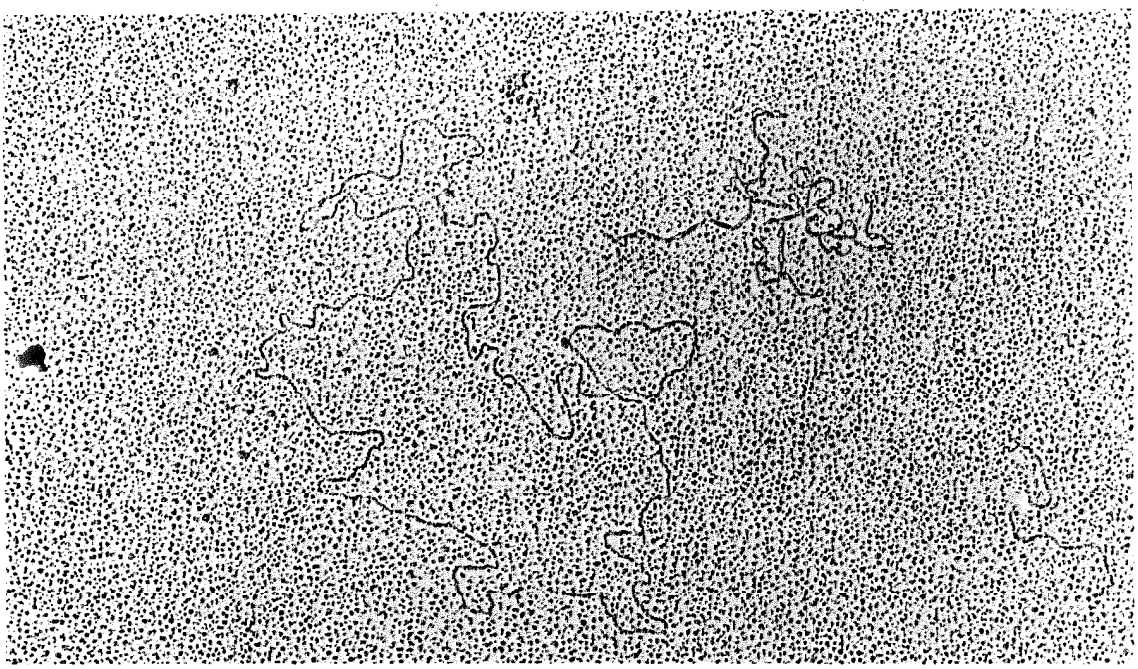
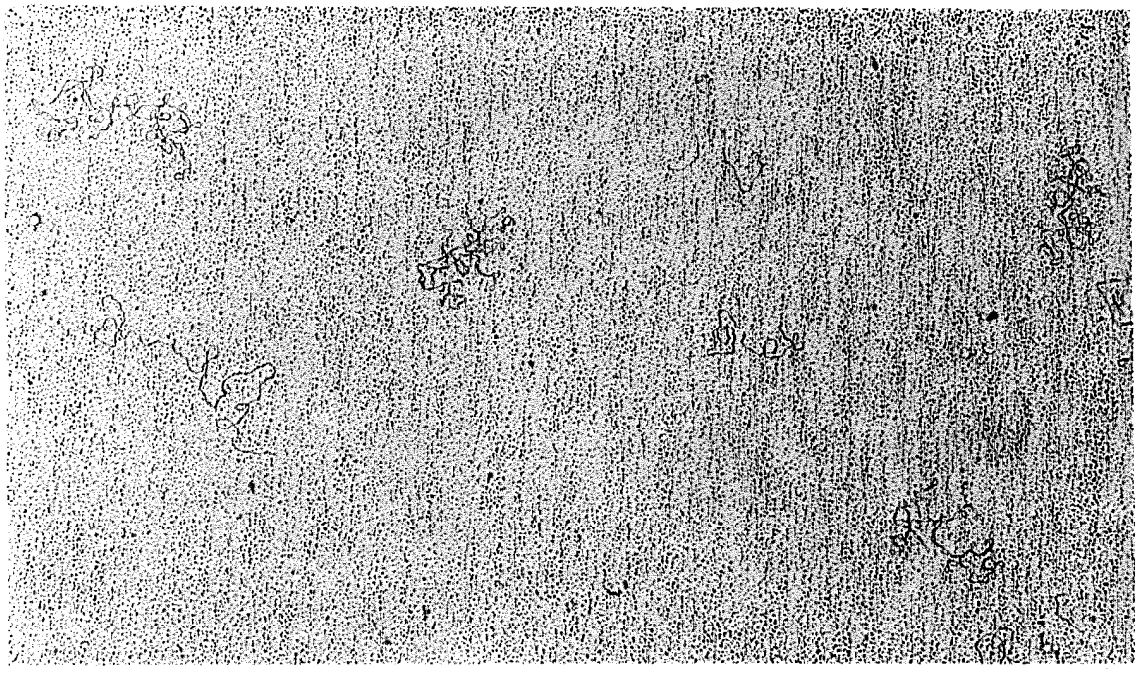
TABLE I

	cts/min/50λ ^{3}H-coli DNA	cts/min/50λ ^{32}P-lambda DNA
lysate supernatant	294	7,700
lysate pellet	67,980	17,000
phenol-extracted supernatant	97	5,660
phenol-extracted pellet	26,202	11,500

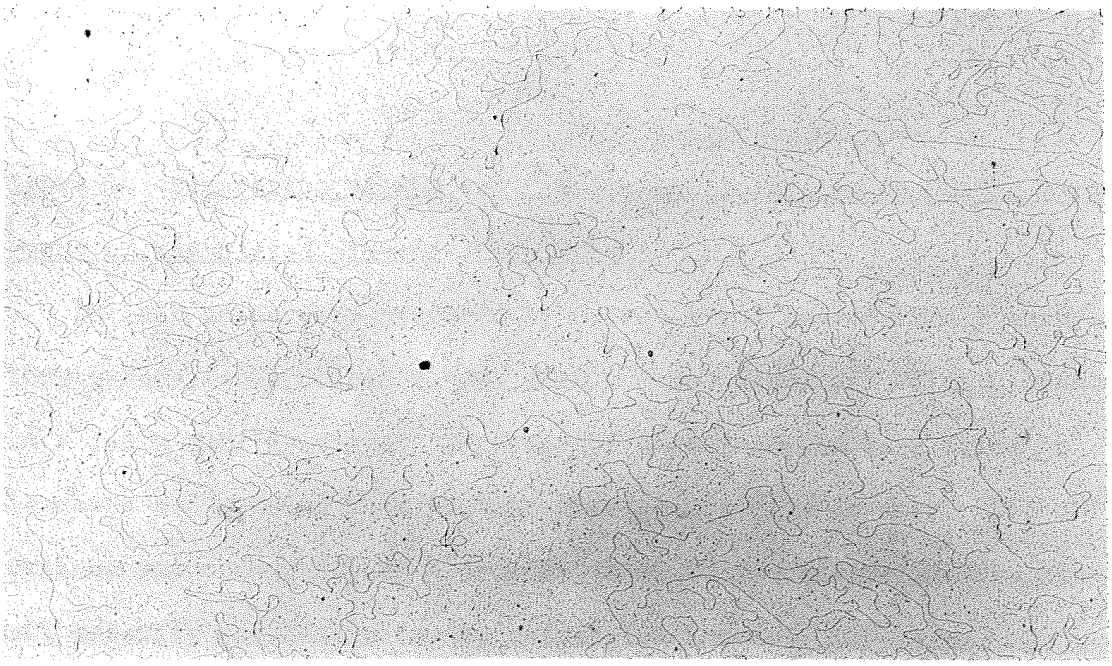


(a) x 25,000

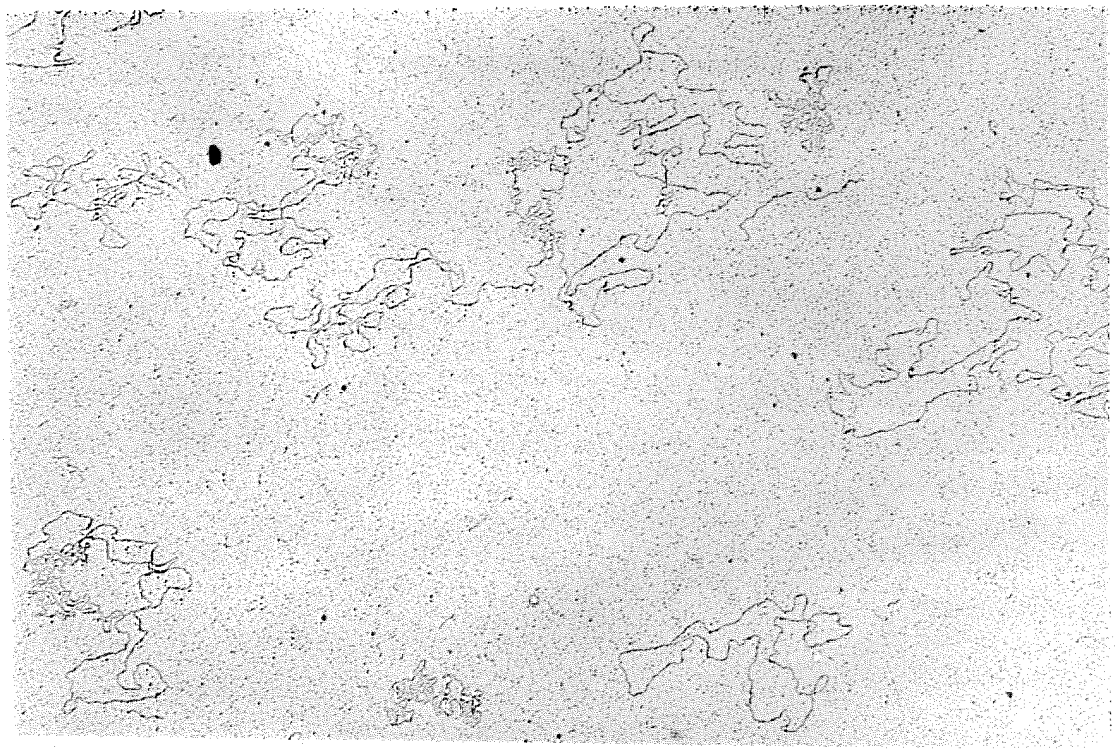


(b) x 16,000

Plate 1. Purified intracellular lambda DNA



(a) x 12,900



(b) x 12,900

Plate 2. Annealed lambda phage DNA

Figure 1. Separation of RNA and ^{32}P-intracellular lambda DNA by chromatography on methylated albumin-Kieselguhr.

Nucleic acids were prepared by Method 1 and passed thru a methylated albumin-Kieselguhr column (1 cm. high x 4 cm. diameter) using a linear 0.4 - 1.0 M NaCl gradient. Portions of each fraction were dried on planchets and counted in a Nuclear Chicago Gas Flow counter. The absorbance was measured with a Beckman model DU spectrophotometer. The NaCl molarity was determined by refractive index.

O------O NaCl molarity

——— ^{32}P cpm, intracellular lambda DNA

- - - - - A_{260}

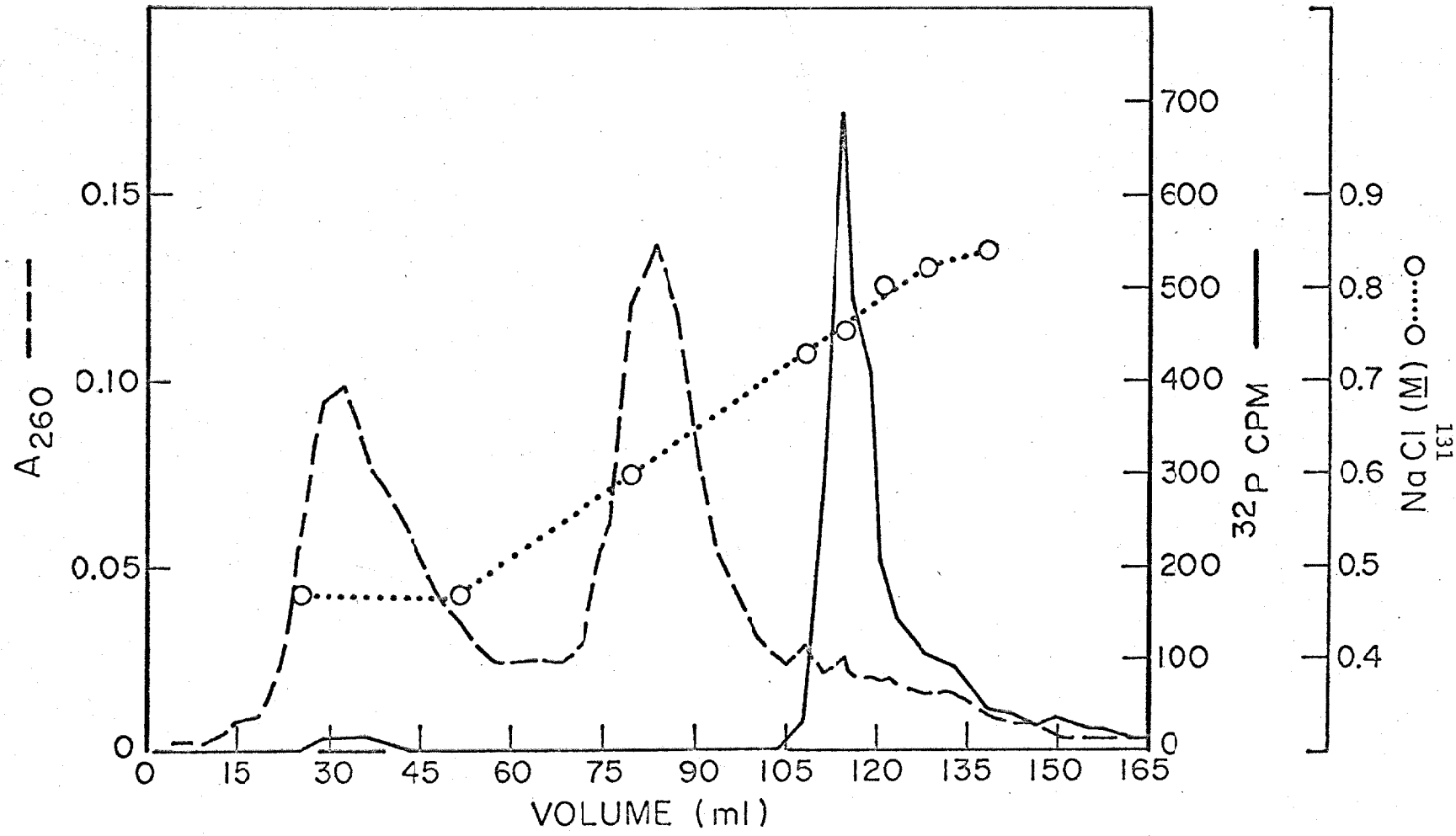

A260
0.15
0.10
0.05
0
0
15
30
45
60
75
90
105
120
135
150
165
VOLUME (ml)
700
600
500
400
300
200
100
0
32P CPM
0.9
0.8
0.7
0.6
0.5
0.4
NaCl (M) O.....O
131I

Figure 1

Figure 2. Analytical band sedimentation of component I prepared by equilibrium sedimentation in CsCl and ethidium bromide.

Sedimentation is into 3M CsCl, 0.01 M tris, pH 7.0, 20°C, 25,980 rev./min from left to right. Scan was taken 48 minutes after coming to speed.

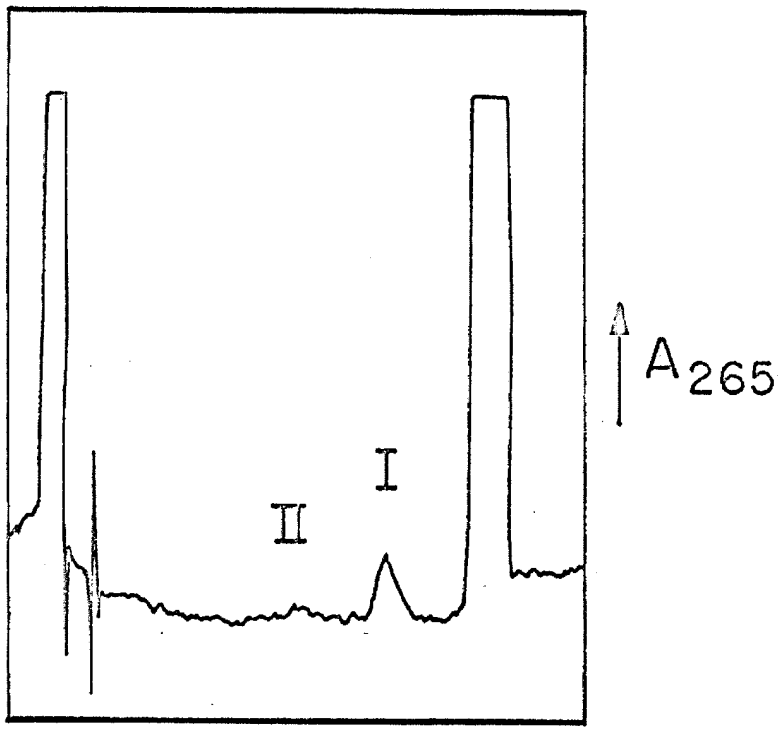
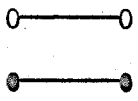


Figure 2

Figure 3. Alkaline titration of the biological activity of intracellular lambda DNA and phage DNA.

Intracellular lambda c_{26} DNA purified by procedure 2 of Methods was further purified by sedimentation through a neutral sucrose gradient. Fractions containing components I and II were pooled, dialyzed, the volume reduced to 3 ml. by flash evaporation, and the solution finally dialyzed against 0.01 x SSC. Different 0.3 ml. portions of this DNA solution were mixed with 1.7 ml. of an alkaline buffer as described in Methods. The pH was measured and then the sample was incubated for 15 minutes at 37°C. The pH was then adjusted to about 8 with HCl and portions of each sample were assayed in spheroplasts after dilution with 0.05 M tris, pH 8.1. Lambda b_2b_5c phage DNA was denatured in the same buffer and assayed identically.



i^{λ} intracellular DNA

i^{b5} phage DNA

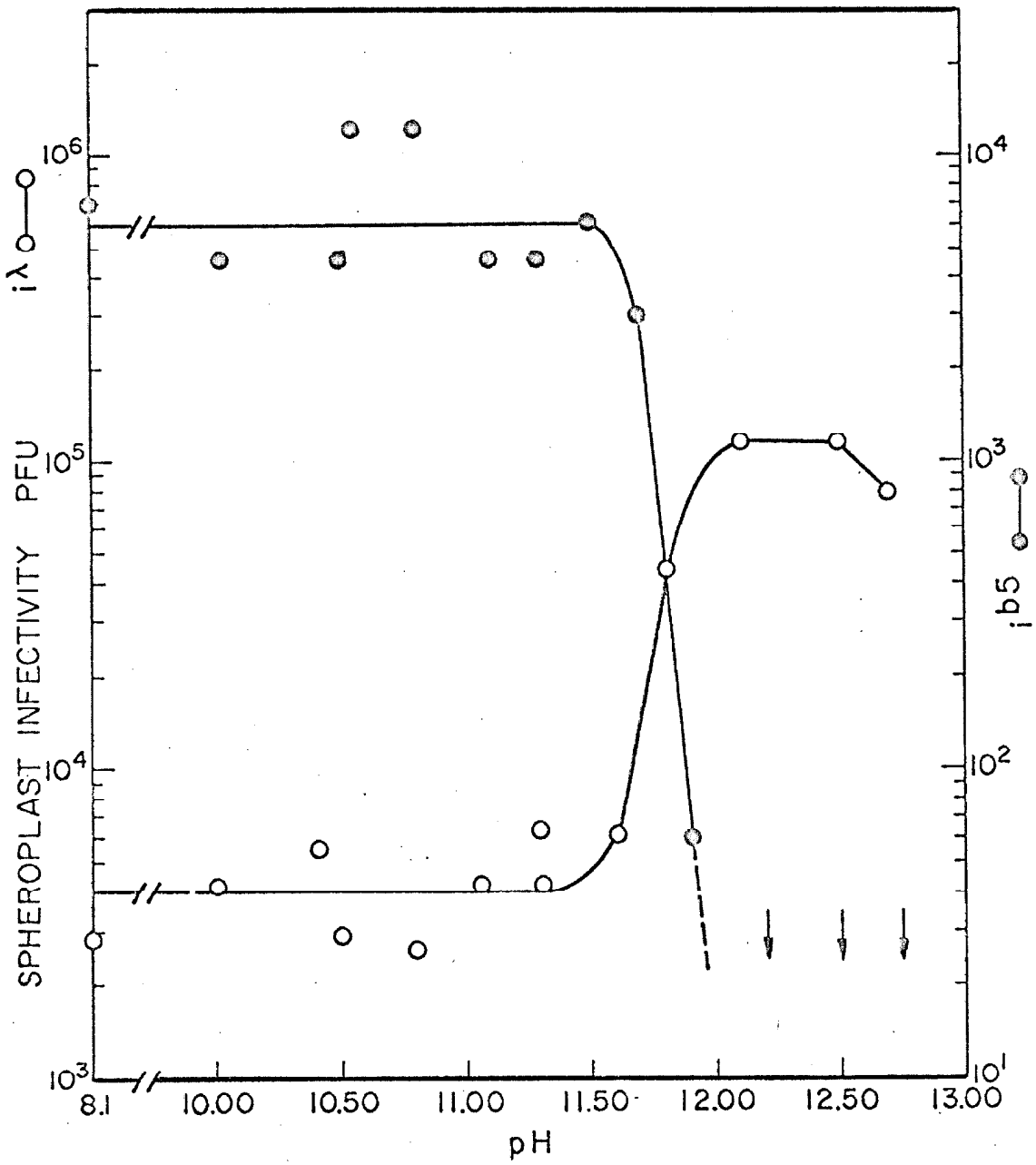

SPHEROPLAST INFECTIVITY PFU
iλ
ib5
pH
8.1
10.00
10.50
11.00
11.50
12.00
12.50
13.00
10^6
10^5
10^4
10^3
10^4
10^3
10^2
10^1

Figure 3

Figure 4. Distribution of infectivity of denatured intracellular lambda DNA after centrifugation in a CsCl equilibrium density gradient.

Lambda b_2b_5c intracellular DNA was denatured with NaOH, pH 12.2, for 15 minutes at 25°C, and then neutralized with HCl. Native ^3H-i^λ phage DNA was added and the density raised to 1.71 g/cm^3 with CsCl. Centrifugation was for 37 hours at 30,000 rev./min in a Spinco SW-50 rotor. Fractions were collected into buffer and assayed for radioactivity by liquid scintillation counting. Those fractions containing the peaks of radioactivity were dialyzed (25) against 0.01 M tris, pH 8.1, and subsequently assayed for infectivity in spheroplasts. The density gradient was measured by collecting fractions into paraffin oil and weighing 50 µl of the solution. Regions of the gradient not shown did not contain significant amounts of radioactivity.

X———X ^3H cpm, i^λ phage DNA

X------X infectivity, i^λ phage DNA

0———0 ^{32}P cpm, intracellular DNA

●———● infectivity, i^{b5} intracellular DNA

□———□ density

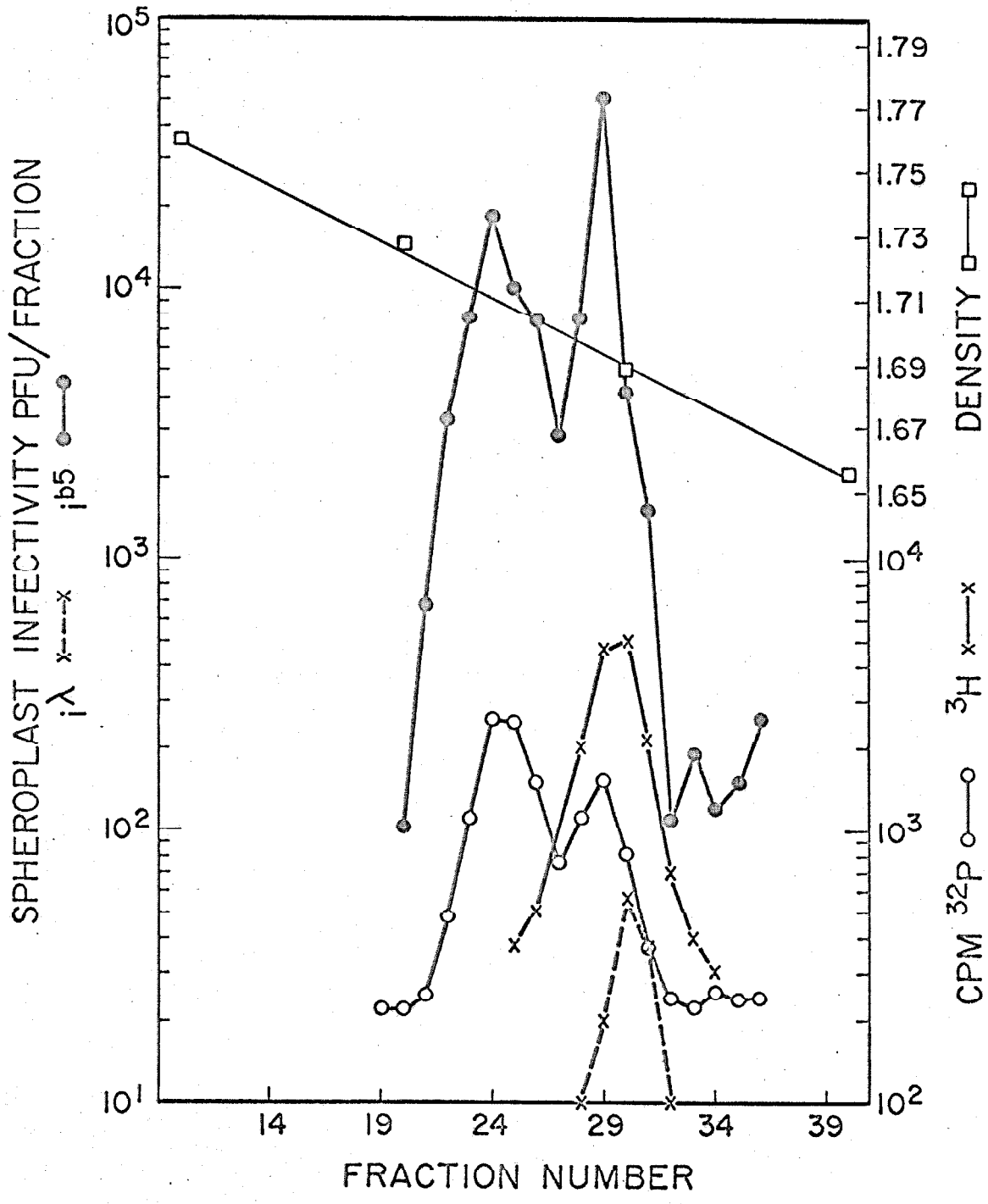

SPHEROPLAST INFECTIVITY PFU/FRACTION
iλ x---x
ib5
DENSITY
CPM 32P
3H x—x
FRACTION NUMBER
10^5
10^4
10^3
10^2
10^1
1.79
1.77
1.75
1.73
1.71
1.69
1.67
1.65
14
19
24
29
34
39

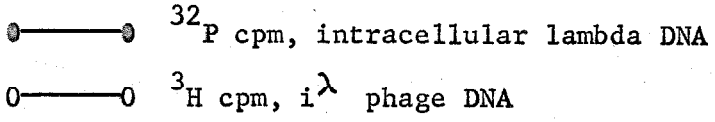
Figure 4

FIGURE 5. Sedimentation of intracellular lambda DNA before and after fractionation by denaturation and CsCl equilibrium density centrifugation.

(a) Distribution of ^{32}P intracellular lambda DNA after sedimentation through a neutral 5-20% sucrose gradient in TE for 5 hours at 25,000 rev/min at 6°C in a Spinco SW-25.1 rotor. Fractions were collected by pumping the gradient from the centrifuge tube. Background has not been subtracted. Sedimentation is from right to left.

(b) Distribution of ^{32}P intracellular lambda DNA, from the heavy peak shown in Figure 4, after sedimentation through an alkaline 5-20% sucrose gradient containing 0.3 M NaOH for 3 hours at 25,000 rev/min at 6°C in a Spinco SW-25.1 rotor. ^{3}H-i^{λ} phage DNA, used as a marker, was added to the intracellular lambda DNA before centrifugation and the mixture denatured in 0.2 M NaOH at 37°C for 10 min. Fractions were collected by pumping the gradient from the centrifuge tube. Background has not been subtracted.

(c) Distribution of ^{32}P intracellular lambda DNA, from the light peak shown in Figure 4, after sedimentation as described in (b) above. Background has not been subtracted.



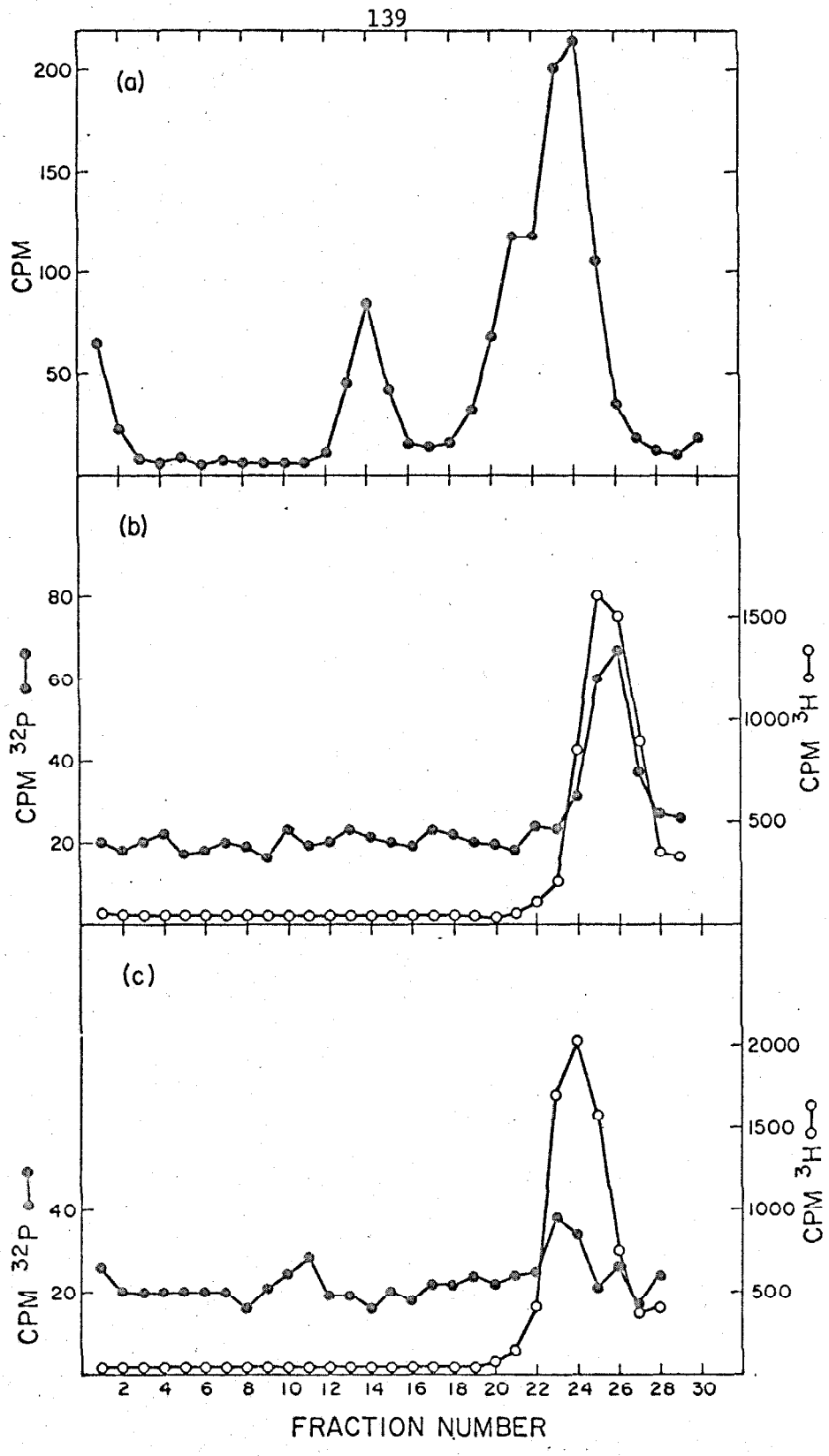

(a)
CPM
200
150
100
50
(b)
CPM 32P
80
60
40
20
CPM 3H
1500
1000
500
(c)
CPM 32P
40
20
CPM 3H
2000
1500
1000
500
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
FRACTION NUMBER

Figure 5

Figure 6. Ultraviolet sensitivity of native and denatured intracellular lambda DNA.

Lambda c_{26} intracellular DNA prepared by Method (2) was irradiated and treated as described in the text. Samples were assayed for infectivity in spheroplasts prepared from a uvr^+ strain.

●——● infectivity of native intracellular DNA

X——X infectivity of denatured intracellular DNA

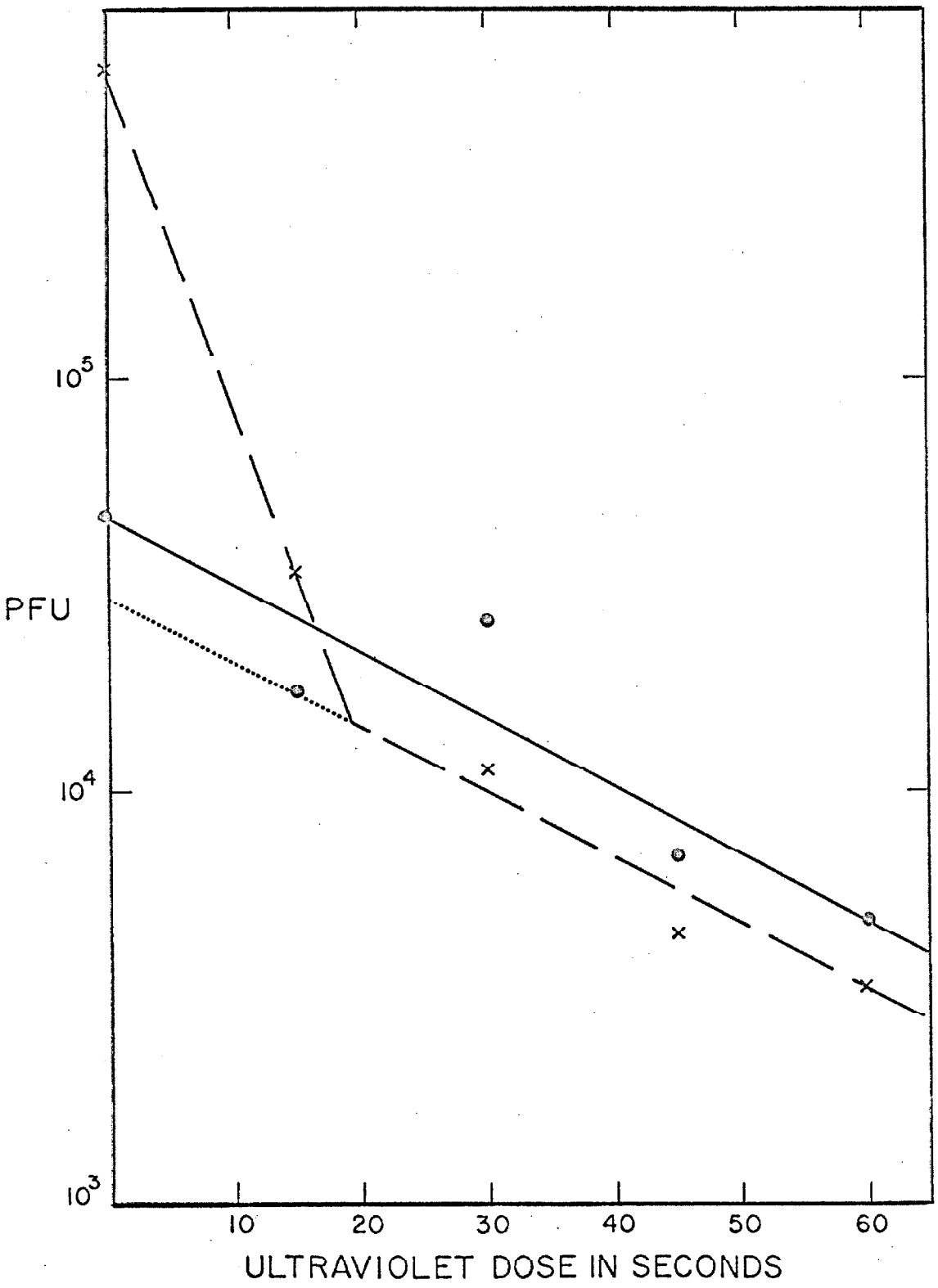


Figure 6

Figure 7. Exonuclease I treatment of denatured lambda intracellular DNA.

Lambda c_{26} intracellular DNA prepared by Method 2 was alkali-denatured, neutralized, and treated with exonuclease I as described in Methods. Each reaction mixture was analyzed by band sedimentation of a 20 µl. portion into 3 M CsCl, 0.05 M phosphate, pH 12.5, in the analytical ultracentrifuge. The remainder of each reaction mixture was assayed for infectivity in spheroplasts.

(a) No exonuclease I. Sedimentation is from left to right.

A - single-strand lambda rings

B - single-strand lambda linears

C - E. coli single-strands

The reaction mixture contained 2.6×10^6 pfu in the spheroplast assay.

(b) 7 units of exonuclease I. Sedimentation as in (a). The reaction mixture contained 3.0×10^6 pfu.

(c) 70 units of exonuclease I. Sedimentation as in (a). The reaction mixture contained 1.8×10^6 pfu.

(d) 350 units of exonuclease I. Sedimentation as in (a). The reaction mixture contained 0.07×10^6 pfu. The small peak ahead of the marked positions of single-stranded rings is an artifact which does not sediment.

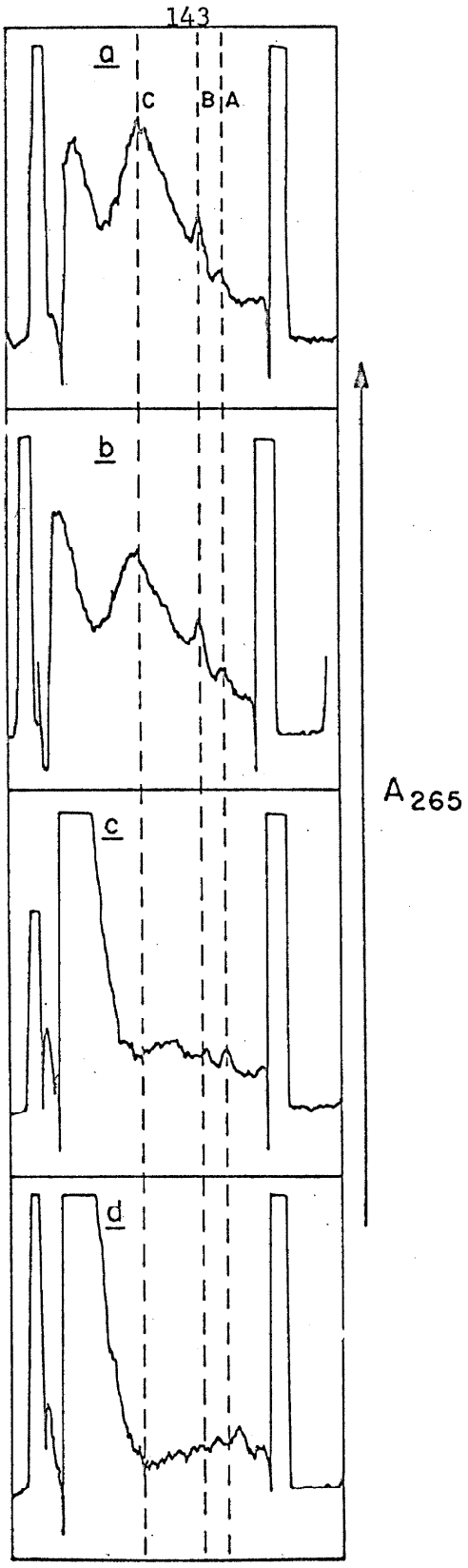


Figure 7

Figure 8. Alkaline band buoyancy sedimentation of intracellular lambda DNA.

Lambda b_2b_5c component I was prepared by Method v and a portion of it allowed to convert spontaneously to component II before sedimentation into alkaline CsCl, 0.05 M phosphate, pH 12.4, ρ = 1.748 g/cm^3. Each component is identified by its initial sedimentation rate and its equilibrium buoyant density.

●———● component I

0———0 single strands initially present

X———X single strands arising from component I during centrifugation (some of these arrive at their equilibrium density before any single strands arising from component II arrive in this region of the cell)

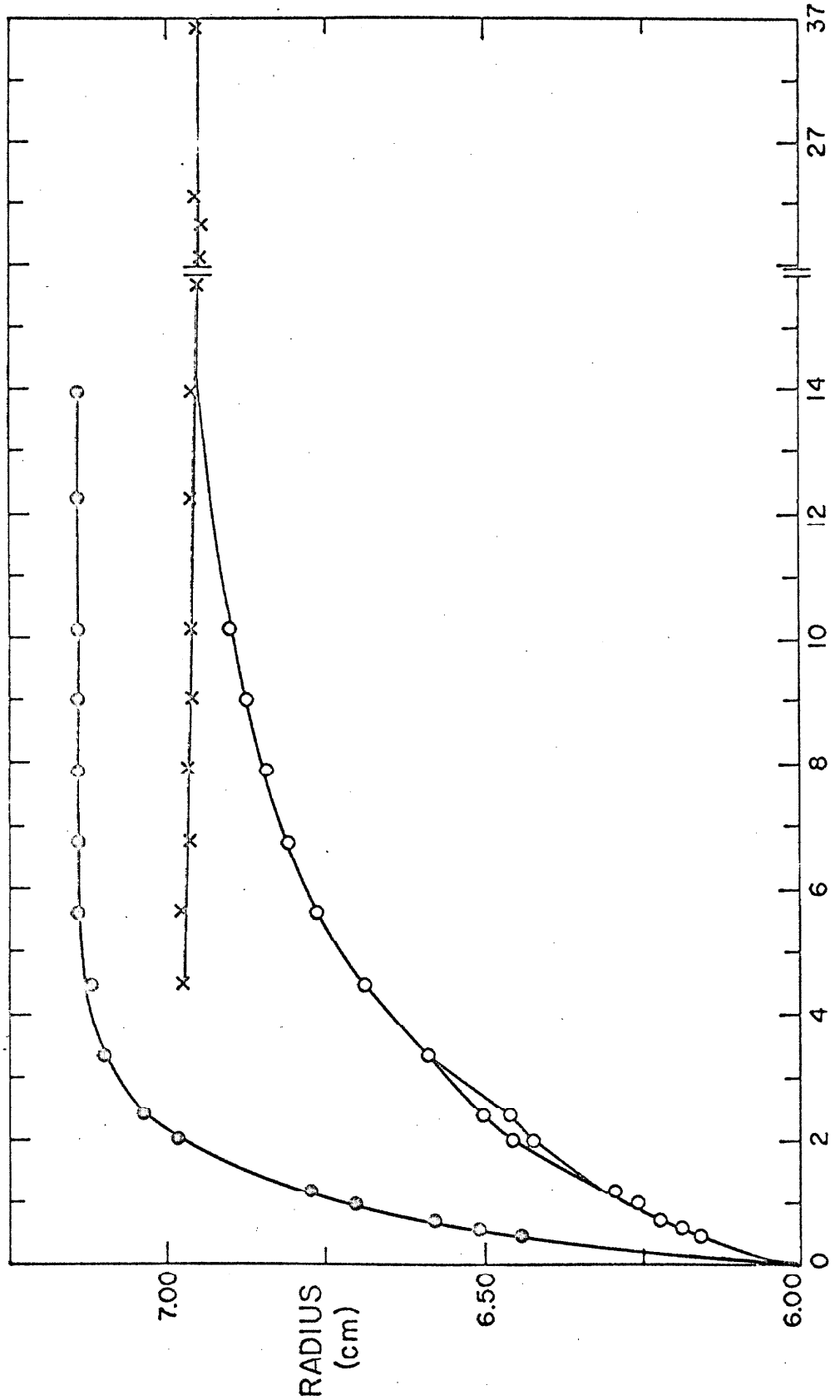
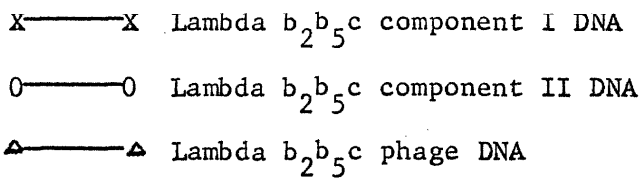


Figure 8

Figure 9. Na^+ concentration dependence of $S_{20,w}$ of lambda DNA.



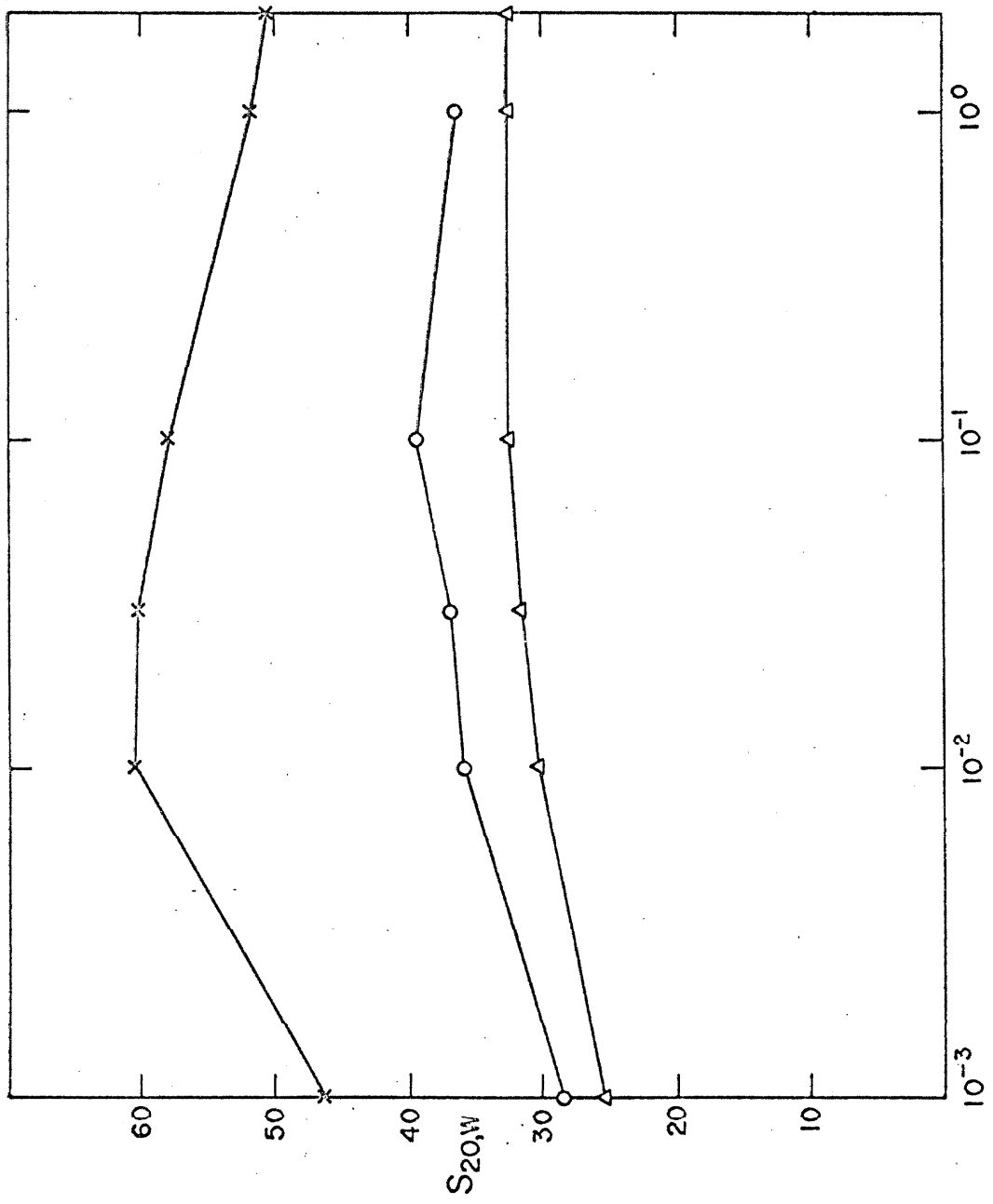


Figure 9

Figure 10. Relative sedimentation rates of lambda b_2b_5c DNA species as a function of Na^+ concentration.

Ratio of $S_{20,w}$, component I: $S_{20,w}$ phage DNA. 0———0

Ratio of $S_{20,w}$, component I: $S_{20,w}$ component II X———X

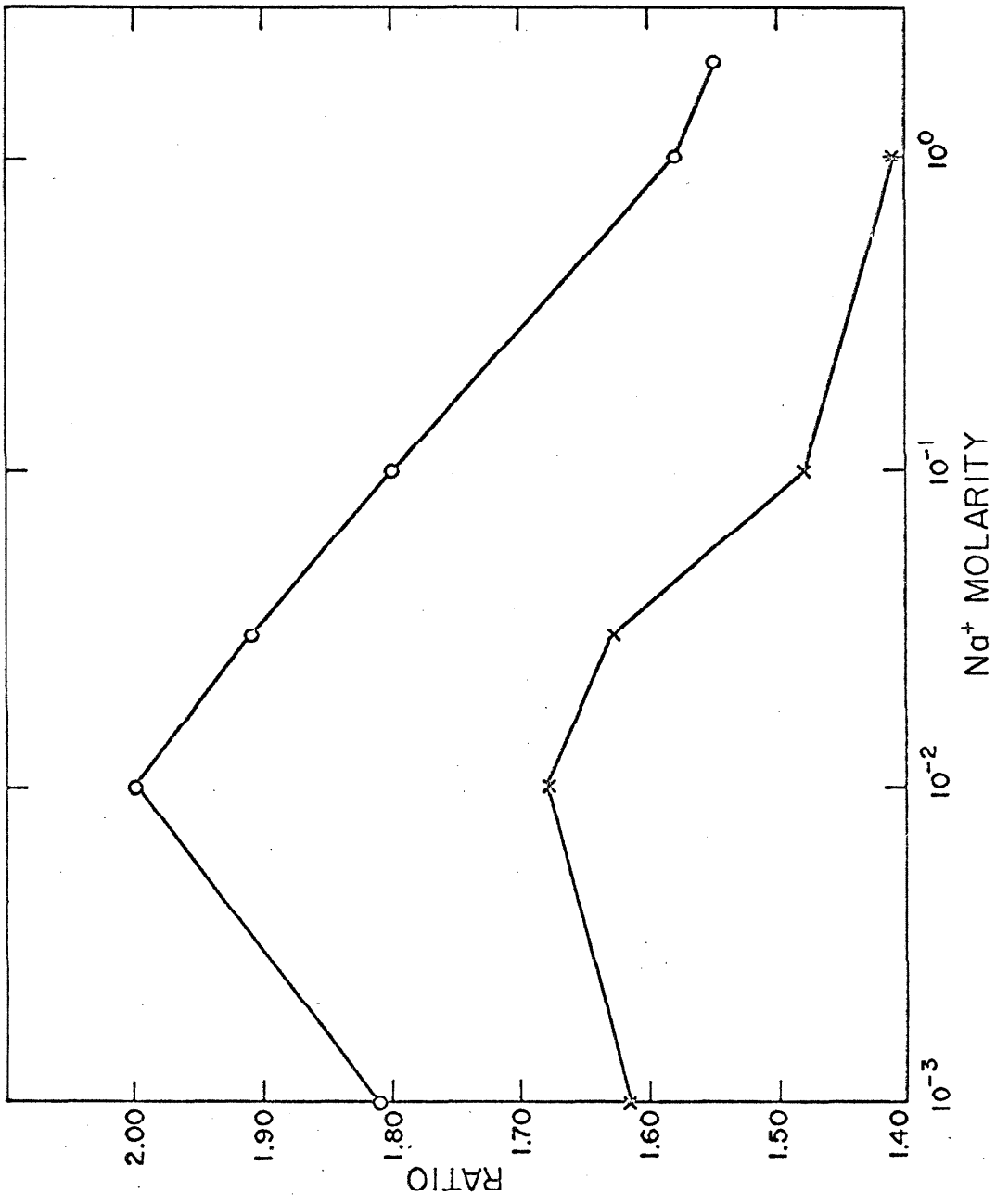


Figure 10

Figure 11. Sedimentation properties of ØX174 RF components I and II as a function of Na^+ concentration.

(a) $S_{20,w}$ of component I (X———X) and of component II (0———0).

(b) Ratio of $S_{20,w}$ component I: $S_{20,w}$ component II.

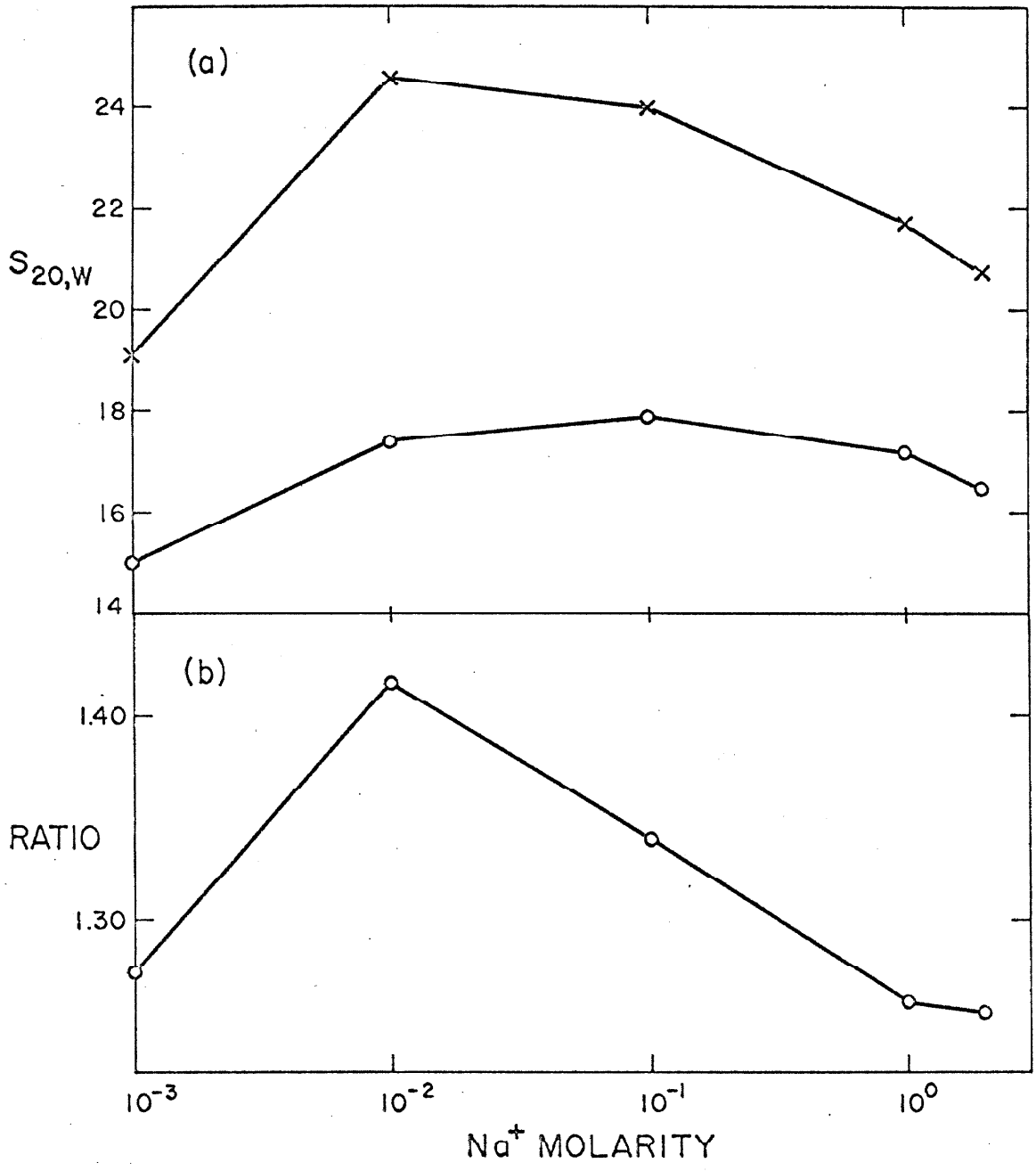

(a)
24
22
$S_{20,w}$
20
18
16
14
(b)
1.40
RATIO
1.30
10^{-3}
10^{-2}
10^{-1}
10^{0}
Na^{+} MOLARITY

Figure 11

Figure 12. Relative change of $S_{20,w}$ as a function of Na^+ concentration for lambda b_2b_5c DNA species and ØX174 RF species. (Normalized to $S_{20,w}$ in 1M NaCl.)

X——X Lambda b_2b_5c component I

O——O Lambda b_2b_5c component II

△——△ Lambda b_2b_5c phage DNA

X- - - X ØX174 RFI

O- - - O ØX174 RFII

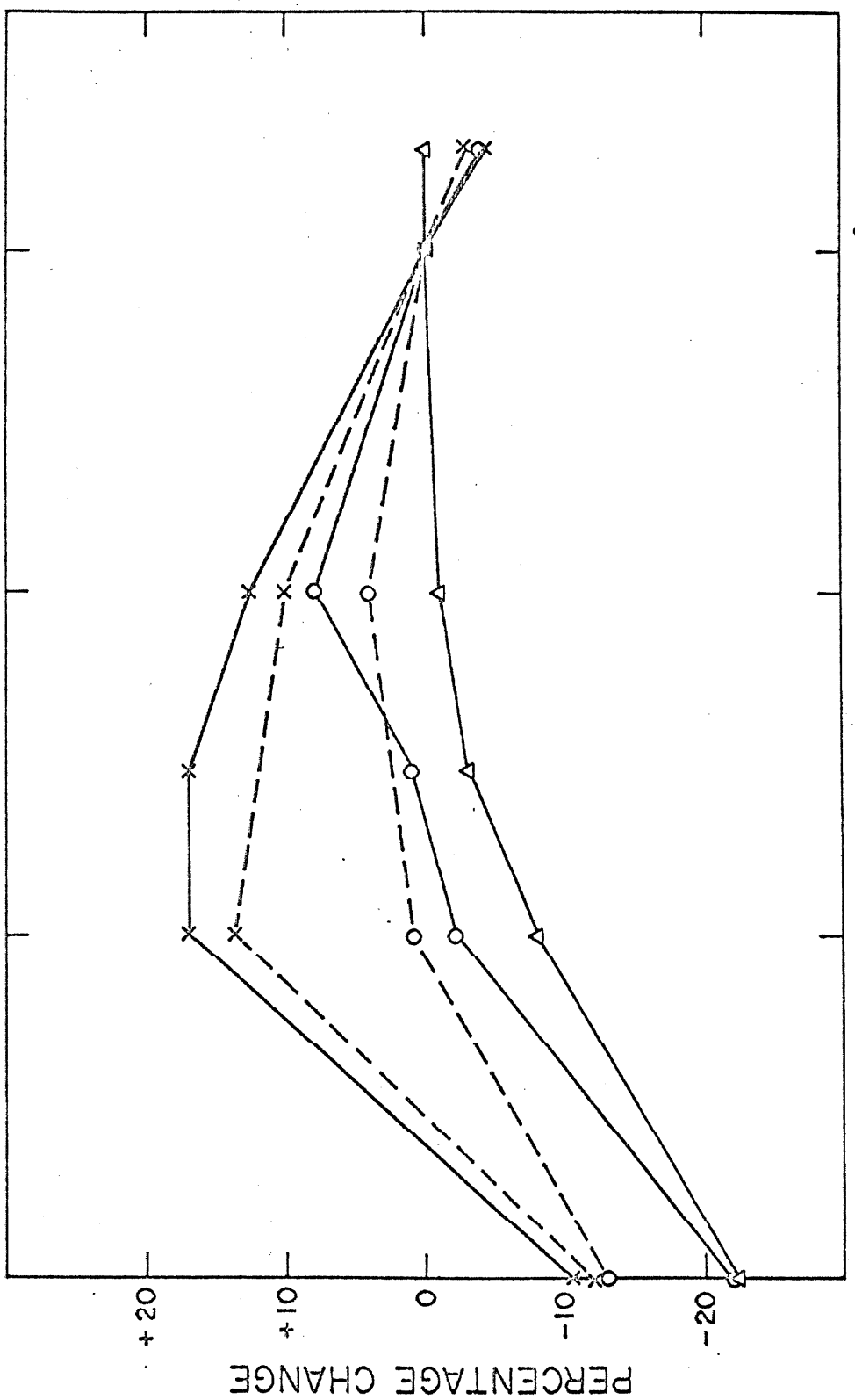


Figure 12

J. Mol. Biol. (1967) 28, 157–160

LETTERS TO THE EDITOR

Infectivity of Single-stranded Rings of Bacteriophage Lambda DNA

Upon injection of bacteriophage lambda DNA into either a sensitive or immune bacterium some of the DNA becomes circular (Young & Sinsheimer, 1964; Bode & Kaiser, 1965; Ogawa & Tomizawa, 1967). Two forms of circular lambda DNA are known to exist: I, a twisted circular DNA in which both strands are covalently closed, and II, an open circular DNA in which one or more single-strand breaks are present. A third component, III, identical to linear phage DNA, is also present in the infected bacterium. All three of these forms of lambda DNA are able to infect spheroplasts (Young & Sinsheimer, manuscript in preparation). Evidence will be presented to show that single-stranded rings of lambda DNA are also able to infect spheroplasts and that they can do so more efficiently than either double-stranded circular or double-stranded phage DNA.

W3110(b5), a prototroph lysogenic for lambda b5, was grown to a cell concentration of 1×10^9 /ml. and infected at a multiplicity of 25 with ^{32}P-labeled lambda b2b5c. After aeration at 37°C for one hour, the cells were collected by centrifugation. Intracellular ^{32}P-labeled lambda DNA was prepared free of bacterial DNA (Kiger & Young, manuscript in preparation).

Sedimentation of the intracellular ^{32}P-labeled lambda DNA on a neutral sucrose gradient separates component I from the more slowly sedimenting components II and III (Fig. 1). Material hereafter referred to as component I was prepared by pooling the fractions containing the fast sedimenting peak of radioactivity as shown in Fig. 1. Similarly, component II was prepared by pooling the fractions from the leading edge

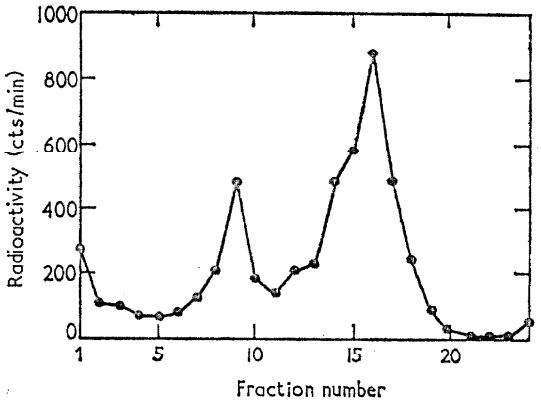


FIG. 1. Distribution of ^{32}P-labeled lambda intracellular DNA after sedimentation through a 5 to 20% w/v sucrose gradient containing 0·01 M-Tris, pH 8·1, 0·001 M-EDTA. One-ml. fractions were collected and 0·02 ml. of each fraction assayed for radioactivity. Centrifugation was for 5 hr at 25,000 rev./min at 6°C in a Spinco SW25·1 rotor. Sedimentation is from right to left.

of the slowly sedimenting peak. Linear phage DNA was prepared by phenol extraction of some of the ^{32}P-labeled lambda b2b5c phage used in the infection above. All DNA preparations were dialyzed against 0·001 M-Tris, pH 7·4, 0·05 M-NaCl before use.

Samples of components I and II and phage DNA were denatured in 0·2 N-NaOH at 37°C for ten minutes and then neutralized with HCl. The infectivity of the native and denatured DNA was assayed in spheroplasts. The specific infectivities (plaque-forming units/cts/min) calculated from the linear portion of the assay and normalized to native phage DNA are given in Table 1. It will be noted that denaturation of

TABLE 1

Specific infectivities of lambda DNA

		Plaque-forming units/cts/min	Normalized specific infectivity
Component I	native	129	3·3
	denatured	62	1·6
Component II	native	19	0·49
	denatured	571	14·7
Phage DNA	native	39	1·00
	denatured	1·6	0·041

component II but not of component I produces molecules that are approximately 15 times more infective than native linear molecules.† The infectivity present in denatured component II is several-fold more sensitive to ultraviolet radiation than is the infectivity present before denaturation. Denaturation of linear molecules results in a large decrease in infectivity. The small amount of infectivity associated with the denatured phage DNA is probably due to renaturation of a portion of the molecules prior to infection of the spheroplasts (Brody, Coleman, Mackal, Werninghaus & Evans, 1964).

Alkaline denaturation of component II gives rise to two species of DNA molecules: single-stranded rings and single-stranded linear molecules. Denaturation of phage DNA produces only single-stranded linear molecules. Single-stranded rings of lambda DNA sediment 1·14 times as fast in alkali as single-stranded linear molecules (Ogawa & Tomizawa, 1967; Kiger & Young, manuscript in preparation). Alkali-denatured component II and alkali-denatured phage DNA were sedimented through alkaline sucrose gradients and the infectivity and radioactivity in each fraction assayed, after reneutralization (Fig. 2). The infectivity present in denatured component II sediments 1·11 times as fast as the peak of radioactivity (most of which is due to component III contamination of the component II preparation) and 1·16 times as fast as the residual infectivity associated with the denatured phage DNA. The specific, normalized infectivity of the denatured component II DNA on the gradient is 6·1 and that of denatured phage DNA is 0·044.

We conclude that the infectivity of denatured component II is due to single-stranded rings. The infectivity of single-stranded rings but not single-stranded linear molecules of DNA extracted from phages ϕX174 (Fiers & Sinsheimer, 1962) and from fd

† The magnitude of this increase in infectivity is variable from assay to assay and depends upon the preparation of spheroplasts used in the assay.

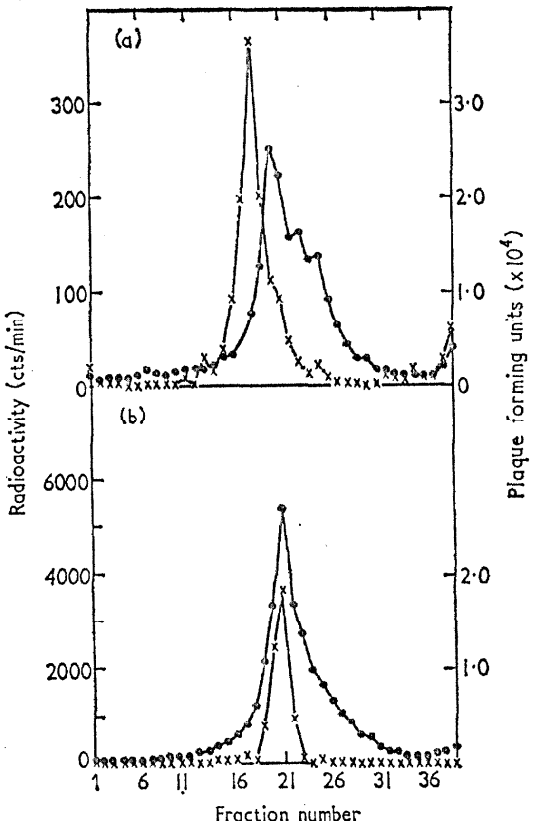


FIG. 2. Distribution of ^{32}P-labeled lambda DNA (—○—○—) and infectivity (—×—×—) after sedimentation through a 5 to 20% w/v sucrose gradient containing 0·05 M-K_3PO_4 and enough KOH to make the pH 12·2. The samples were denatured prior to centrifugation by incubation at 37°C for 15 min in 0·2 N-NaOH. Centrifugation was for 15 hr at 25,000 rev./min at 6°C in a Spinco SW25·3 rotor. Sedimentation is from right to left. Samples (a) and (b) were sedimented simultaneously in the same rotor; (a) is component II DNA, (b) is phage DNA.

(Marvin & Schaller, 1966) is well established. This now appears to be a general phenomenon, not restricted to DNA from single-stranded DNA phages. The infectivity of single-stranded rings of polyoma virus DNA is also suggested by the work of Dulbecco & Vogt (1963).

Increases in infectivity upon denaturation of component II of ϕX174 replicative form similar to those reported here for lambda have been observed by others and reviewed by Jaenisch, Hofschneider & Preuss (1966).

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CHAPTER 2

The Fractionation of Replicating Lambda DNA on Benzoylated-Naphthoylated DEAE Cellulose

1. INTRODUCTION

Circular forms of lambda DNA have been shown to be important intermediates in lambda DNA replication (41,42). Following infection of sensitive bacteria by C_I mutants, the infecting DNA is converted to covalently closed circles (component I) and open circles (component II). Component I has been shown to replicate repeatedly in a semi-conservative manner. Net synthesis of component I proceeds throughout the latent period until phage DNA synthesis begins. Pulse-labelling of infected cells has shown that component II is synthesized both before and after the end of the eclipse period. It has been suggested that component II is a precursor to both component I and to phage DNA. Short pulses have revealed the presence of a third, heterogeneous, component synthesized during phage DNA synthesis which is probably a precursor to phage DNA. This heterogeneous DNA has a range of sedimentation coefficients between 1.2 to 1.8 times the rate of phage DNA and may contain single-stranded rings that are released upon denaturation (16,42).

Some controversy exists about the nature of DNA pulse-labeled during lambda infection. Smith & Skalka (33) have reported pulse-labeled lambda DNA to sediment at twice or more the rate of phage DNA and to be very shear sensitive. Alkaline sedimentation of denatured pulse-labeled DNA shows most of the labeled DNA to sediment ahead of phage DNA single-strands. They interpret these observations as evidence for the existence of long linear concatenates of lambda DNA.

There is evidence which indicates that lambda DNA synthesis following induction of lysogenic cells is different (in degree at least) from lambda DNA synthesis following infection of sensitive cells by C_I mutants. Component I seems to be only a very minor component of the vegetative lambda DNA (21). Less than one component I molecule per cell is synthesized following induction of temperature-sensitive lysogens of lambda (Kiger & Ilg, unpublished experiments).

Salzman & Weissbach (31) and Weissbach & Salzman (37) have reported that DNA, pulse-labeled following ultraviolet light induction of lambda lysogens is very heterogeneous, some of the labeled DNA sedimenting twice or more the rate of lambda phage DNA. This DNA is shear-sensitive and is a precursor of phage DNA. They interpret these findings as evidence for linear concatenates of lambda DNA. Induction of lambda sus A and lambda sus D lysogens (defective in phage DNA synthesis (2,3,4, 31,36)) results in the accumulation of the heterogeneous lambda DNA intermediates. Alkaline sedimentation of DNA accumulated by induced lambda sus A lysogens shows single-strand DNA which sediments faster than phage DNA single-strands.

Chromatography on benzoylated-naphthoylated DEAE cellulose (BNC) has been shown to be an effective way of fractionating nucleic acids on the basis of their secondary structure (32). This technique has allowed fractionation of the replicative intermediates of the RNA bacteriophage MS2 (14). We have employed chromatography on BNC to fractionate the lambda DNA synthesized following induction of temperature-sensitive lysogens of lambda. This technique has allowed the isolation

of two unique species of intracellular lambda DNA. One of these species is composed of mature phage DNA and component II. The other species is the heterogeneous intermediate described by others (31,42). The structure of this latter species is investigated, and a general model for DNA replication is suggested as a result.

2. MATERIALS AND METHODS

(a) Media and Solutions

KG medium is described by Young & Sinsheimer (41).

TE is 0.01M Tris-HCl, 0.001 M EDTA, pH 8.1.

TM is 0.01M Tris-HCl, 0.01M $MgSO_4$, pH 7.1.

SSC is 0.15M NaCl, 0.015M Na_3 citrate. 1x, 2x etc. indicates the degree of concentration of the solution.

(b) Chemicals

Lysozyme, sarkosyl, mitomycin C and their use are described by Young & Sinsheimer (40,41) and in Chapter 1. ^{3}H-thymine is purchased from New England Nuclear Corp. and has a specific activity of 16.8 c/millimole. ^{3}H-thymidine is purchased from Schwartz Bioresearch Inc. and has a specific activity of 11.0 c/millimole. DEAE cellulose (Cellex D) for production of BNC is purchased from Biorad Corp. We would like to thank J.W. Sedat and A. Lyon for initial gifts of BNC.

(c) E. coli K12 strains

C600 and C600 (434hy) are used as plating bacteria and were obtained from Dr. J. Weigle. $159T^-$ is thymine requiring strain selected by Dr. W. Salivar from the strain 159 obtained from Dr. D. Pratt. 159 is a derivative of W 3102 and is $F^+Sm^+Su^-hcr^-$.

C600 (λC_{ts}), C600($\lambda susA_{11}C_{ts}$), C600 ($\lambda susRC_{ts}$) and C600 ($\lambda susP_{422}C_{ts}$) were obtained from J.S. Parkinson. The C_{ts} character is the C_It_1, temperature sensitive clear mutation of Lieb (19). A_{11} and R are the sus mutations of Campbell (2) and P_{422} is a sus mutation isolated by J.S. Parkinson.

$159T^-$ (λC_{ts}), $159T^-$($\lambda susA_{11}C_{ts}$), $159T^-$($\lambda sus\ RC_{ts}$) and $159T^-$ ($\lambda sus\ P_{422}C_{ts}$) were prepared essentially as described by Young & Sinsheimer (41) except that after the infection, cells were plated and colony formers tested for the presence of the prophage by streaking against λC_{26} and λb_2b_5c. The presence of A_{11} and P_{422} in the lysogens was confirmed by complementation tests.

(d) Phage strain

Lambda 434hy C_{51} was obtained from Dr. J. Weigle. Radioactively labeled lambda stocks are made as described by Young & Sinsheimer (40).

(e) Induction and Long Term Labeling of Lysogens

Lysogens are grown in KG medium supplemented with 10 μg/ml thymine to approximately 3×10^8 cells/ml. Uracil is added to 5 μg/ml and mitomycin C to 20 μg equivalents/ml. The culture is placed in a 45°C water

bath with aeration for 10 minutes in the dark. About 5×10^{-3} or less survivors are found at the end of 10 minutes.

Cells are collected by filtration on an HA Millipore filter and washed with 1 volume of TM. The bacteria are then resuspended in fresh KG medium supplemented with 10 μg/ml thymine, 5 μg/ml uracil and ^{3}H-thymine (10 μc/ml or more depending on the specific activity desired). The culture is returned to 45°C for 5 minutes and then shifted to 37°C. Time is measured from the beginning of incubation at 45°C and the time required for filtration and resuspension is not counted in measuring the time sequence of viral growth and maturation. Phage maturation is complete by 60-70 minutes. Induction is due to the C_{ts} character of the lysogen. Mitomycin C is used only to suppress E. coli DNA synthesis.

Eisen et al(5) have taken advantage of the fact that mitomycin C is also capable of inducing lysogens to study DNA synthesis in induced cells. They grow lysogenic bacteria in 10 μg/ml mitomycin C to both induce the prophage and suppress host DNA synthesis. We have employed temperature-sensitive lysogens to circumvent the comparatively long period of time necessary to obtain induction by mitomycin C.

(f) Pulse Labeling and Chase of Induced Lysogens

Three methods have been employed.

(i) Lysogens are grown to 3×10^{8} cells/ml in KG medium supplimented with 10 μg/ml thymine. The cells collected by centrifugation and resuspended in KG medium. The culture is starved of thymine at 37°C for 15 minutes with aeration. The thymine concentration is then brought

to 0.5 μg/ml and the culture induced and treated with mitomycin as described above. After filtration growth is continued in KG medium with 0.5 μg/ml thymine. Five minutes prior to the pulse uracil is added to 5 μg/ml. Labeling commences with the addition of 100 μc/ml ^{3}H-thymine to the culture.

(ii) Lysogens are induced and treated with mitomycin C as described in (d) above. The culture is not filtered until 5 minutes prior to the pulse but is shifted to 37°C at t = 15 minutes and incubated in the dark. Then the culture is filtered, washed with 1 volume of TM and resuspended in 1 vol. of KG medium supplemented with 5 μg/ml uracil. The culture is starved of thymine for 5 minutes at 37°C with aeration followed by the addition of 100 μc/ml ^{3}H-thymine.

(iii) Lysogens are grown to 3 x 10^{8} cells/ml, induced and treated with mitomycin C as described in (e) above. Following filtration at t = 10 minutes, the cells are resuspended in KG medium supplemented with 5 μg/ml thymine and incubated as described. Five minutes prior to the pulse uracil is added to 5 μg/ml. Labeling commences with the addition of 100 μc/ml ^{3}H-thymidine to the culture.

Pulses are terminated by pouring the culture into a one liter beaker on ice containing 1 mg/ml thymidine. If a chase is not to be performed the culture is made 0.01M in sodium azide and kept on ice. For a chase the culture is filtered and the bacteria collected on an HA Millipore filter, washed with one volume of cold KG medium containing 1 mg/ml thymidine and resuspended in KG medium supplemented with 1 mg/ml thymidine. Incubation is continued at 37°C and chases terminated by bringing the culture to 0.01 M in sodium azide.

Method (iii) results in the incorporation of about 10 times as much ^{3}H as either (i) or (ii). (See Young and Sinsheimer, 41).

(g) DNA Extraction

Phenol extraction of sarcosyl lysed spheroplasts is used to prepare DNA as described by Young & Sinsheimer (40). DNA preparations are generally dialysed overnight against 0.1 x SSC although ether extraction of the phenol is sufficient if the DNA is to be immediately adsorbed to BNC.

(h) Chromatography on Benzoylated-Naphthoylated DEAE Cellulose

BNC, prepared as described by Tener, Gilliam, von Tigerstromm, Millward & Wimmer (35, and personal communication) as amended by Sedat, Kelly and Sinsheimer (32) is made up as a water slurry, poured into a glass column and allowed to settle. A column 15 mm x 15 mm is washed with the passage of 30-40 ml of 0.3 M NaCl, TE.

DNA solutions are made 0.3M NaCl and slowly passed through the column. Usually the DNA is diluted until its viscosity is not noticably different than that of water. DNA from approximately 1.5 x 10^{9} cells is put on a 15 mm x 15 mm column. After the DNA is run onto the column, the column is washed with a volume of 0.3M NaCl, TE equal to that of the DNA solution.

Gradient elution of a column 15 mm x 15 mm is performed with a linear gradient from 0.3 M NaCl, TE (30 ml) to 1.0 M NaCl, TE (30 ml) at a flow rate of 0.5-1.0 ml/min. After this gradient is completed a

second gradient of 1.0 M NaCl, TE to 2% caffeine, 1.0 M NaCl, TE is run through the column at the same flow rate (32). (Caution: concentrated caffeine solutions are potentially dangerous.)

Stepwise elution of a 15 mm x 15 mm column is performed after washing with 0.3 M NaCl, TE as described above by passing 10-12 ml of 1 M NaCl, TE through the column followed by 10-12 ml of 2% caffeine (sometimes 1.8% caffeine is used), 1.0 M NaCl, TE. 2 ml fractions are collected. The eluted DNA appears in the first 2-3 fractions collected following the application of the buffer.

Fractions containing DNA are dialysed overnight against 0.1 x SSC before further use.

(i) Zonal Centrifugation

Sedimentation at neutral pH is through 5%-20% sucrose gradients in TE (autoclaved before use) in a Spinco SW 25.1 rotor at 25,000 rev/min, 6°C for 6 hr. 1.0 ml samples are layered onto 25 ml gradients.

Alkaline sedimentation is through 5%-20% sucrose gradients in 0.1 N NaOH (freshly prepared every two days) in a Spinco SW25.3 rotor at 25,000 rev/min, 6°C for 13-15 hours as indicated. 0.5 ml-0.75 ml samples are layered over 16 ml gradients.

All fractions are collected by dripping through a hole in the bottom of the tube.

(j) Infectivity of Lambda DNA

Infectivity of lambda DNA is assayed by the spheroplast assay of Young and Sinsheimer (40) with the modification described in Chapter 1. All fractions are first dialyzed against 0.05 M PO_4 buffer pH 8 as described by Rüst (29). DNA samples are denatured in alkali at pH 12.2 at 37°C for 10 minutes followed by neutralization with HCl. Serial dilutions of DNA samples are assayed and data shown are from the linear portions of the assay expressed as PFU/spheroplast tube.

(k) Intracellular Phage Assay

Intracellular phage are assayed by dilution of 0.1 ml of culture into 1.65 ml of cold 25% sucrose in 0.04 M Tris pH 8.1 containing 200 µg/ml of lysozyme followed by the addition of 0.15 ml of 4% EDTA. After 30 minutes on ice 0.10 ml of 1 M $MgSO_4$ is added to stabilize the phage and the spheroplasts are lysed by freezing and thawing once.

(l) Plaque Assay

The plaque assay for lambda phage is described by Young & Sinsheimer (40). TM is used to dilute phage for assay.

3. RESULTS

(a) Mitomycin C Treatment of Bacterial Strains and the Induction of Lysogens

In order to specifically label radioactively, replicating viral DNA following induction of lysogenic bacteria it is necessary to suppress

concomitant bacterial DNA synthesis. Lindqvist & Sinsheimer (20) found that mitomycin C treatment of hcr$^-$ bacteria blocked bacterial DNA synthesis while not affecting the capacity of the bacteria to support ØX174 growth. Young & Sinsheimer (41,42) have successfully applied this technique to the study of lambda DNA synthesis following infection of sensitive hcr$^-$ bacteria by clear mutants of lambda. We have adapted this technique to suppress host DNA synthesis following heat induction of temperature-sensitive lysogens of the C_{It1} type studied by Lieb (19) and Green (12).

The incorporation of ^{3}H-thymine into the DNA of lysogenic and non-lysogenic cells following heat treatment and growth in various concentrations of mitomycin C is shown in Figure 1. It is evident that mitomycin C depresses the rate of incorporation of ^{3}H-thymine into both lysogenic and non-lysogenic bacteria. Increasing concentrations of mitomycin C are also seen to decrease the capacity of the bacteria to yield infective phage. The depression of the rate of DNA synthesis at all concentrations of mitomycin C is greatest for the non-lysogenic strain suggesting that bacterial DNA synthesis is preferentially inhibited compared to lambda DNA synthesis. However, even at mitomycin C concentrations that reduce phage yields to less than 10% of normal, bacterial DNA synthesis is not completely blocked.

Removal of the mitomycin C from the culture following 10 minutes of treatment at 45°C is observed to markedly decrease the rate of subsequent bacterial DNA synthesis (compare Figure 1f with 1d) but not to alter the effect of mitomycin C treatment on subsequent lambda DNA syn-

thesis or phage production. We have no ready explanation for this observation but have found it quite useful. All the work to be reported here is done with lysogens treated at 45°C with 20 μg equivalents/ml mitomycin C followed by removal of mitomycin C from the culture (see Methods). The kinetics of DNA synthesis and phage production following heat induction and mitomycin C treatment of two lysogens ($\lambda susRC_{ts}$) defective for lysozyme (4) and ($\lambda susP_{422}C_{ts}$) defective for DNA synthesis (4,13), is shown in Figure 2. ^{3}H-thymine incorporation by the induced $susP_{422}$ lysogen is a good indication of residual host DNA synthesis and indicates that no more than 10% of the ^{3}H-thymine incorporation into the induced susR lysogen can be into host DNA.

(b) Chromatography on BNC of DNA Synthesized Following Induction of a sus A Lysogen.

(i) Elution from BNC

Conditional lethal mutants of the A cistron are classed as tail donors under the restrictive condition in the *in vitro* complementation assay of Weigle (36) but are known to synthesize some form of lambda DNA (3,4,31), which is not infective in the assay of Kaiser & Hogness (3). ^{3}H-DNA labelled from 10-45 minutes following induction of 159T^{-} ($\lambda susA_{11}C_{ts}$) is chromatographed on BNC simultaneously with markers of native ^{32}P-lambda C_{ts} phage DNA and heat denatured ^{14}C-lambda C_{ts} phage DNA. The elution pattern obtained is shown in Figure 3.

Approximately 99% of the native ^{32}P-lambda DNA eluted is eluted in the salt gradient. No detectable denatured ^{14}C-lambda DNA is eluted in

the salt gradient but instead a large fraction is eluted in the caffeine gradient. In contrast, some of the ^{3}H-DNA extracted from induced cells is eluted with the native phage DNA and some is eluted by caffeine but by a lower concentration of caffeine than is required to elute denatured phage DNA. Following completion of the caffeine gradient 20 ml of 8M urea, 0.1 N ammonium formate, pH 3.1, 1.0 M NH_4 Cl, 1% SDS is passed through the column (at the suggestion of J.W.Sedat). This solution elutes some additional DNA. The recovery of each radioactive label in each of the three elution steps is shown in Table 1.

The data of Sedat, Kelly & Sinsheimer (32) and Kelly & Sinsheimer (14) suggest that fractionation on BNC is on the basis of the degree of single-strandedness of the nucleic acid, as indeed is suggested here by the behavior of native and denatured phage DNA. The ^{3}H-DNA eluted here in the caffeine gradient upon equilibrium CsCl density gradient centrifugation in the presence of native ^{32}P-lambda DNA is found to be completely isopycnic with native DNA. If this DNA contains single-strand regions they must represent a fairly small proportion of the molecule A lower limit is indicated by lambda phage DNA which possesses single-strand ends 20 nucleotides in length (38) but is still eluted in the salt gradient.

(ii) Sedimentation Analysis of sus A DNA Fractionated on BNC

Sedimentation through neutral sucrose gradients of unfractionated sus A DNA and sus A DNA eluted in the salt and caffeine gradients is shown in Figure 4. The unfractionated DNA is very heterogeneous in its sedimentation properties. Some material sediments behind the phage DNA

marker and a large portion of the material sediments up to twice as fast as phage DNA. The material eluted from BNC is much less heterogeneous. That eluted in the salt gradient sediments 1.2 times as fast as the marker phage DNA suggesting that it may be open-circular (component II) DNA. The DNA eluted from BNC in the caffeine gradient is somewhat more heterogeneous. A portion of it sediments more slowly than phage DNA and the rest sediments slightly ahead of the marker. Interestingly, the DNA observed to sediment at almost twice the rate of phage DNA present in the unfractionated preparation does not appear in the sedimentation patterns of the DNA eluted from BNC. This point will be discussed below.

(c) Chromatography on BNC of DNA Synthesized Following Induction of a Viable Prophage

(i) Spheroplast Infectivity of DNA Eluted from BNC.

^{3}H-DNA labeled from 10-45 minutes following induction of 159T^{-} (λC_{ts}) is eluted from BNC as shown in Figure 5. A marker of native ^{32}P-lambda 434hyC_{51} DNA is chromatographed simultaneously. Recovery of ^{3}H-DNA in the two gradients is 67% of that put on the column. Of the recovered DNA, 42% is eluted in the salt gradient and 58% in the caffeine gradient.

The fractions from both gradients are dialysed against 0.05 M PO_4, pH 8 and split into two portions. One portion of each fraction is alkali denatured as described in Methods, and the spheroplast infectivity of each fraction, native and denatured, is assayed and shown in Figure 5. The data are obtained by selectively plating the phage produced on C600 (λ 434hy) so as not to assay the phage DNA used as a marker on the column.

The DNA eluted in the salt gradient is approximately equally infective

before and after denaturation. We have previously shown that the infectivity present after denaturation of intracellular DNA is due to either single-strand rings or to closed-circular DNA (component I). Single-strand linear DNA whether arising from the denaturation of phage DNA or component II is not infective. Furthermore single-strand rings are more efficient in infecting spheroplasts than are a comparable number of double-strand molecules (16 and Chapter 1).

Thus the presence of equal amounts of infectivity before and after denaturation of the DNA eluted in the salt gradient suggests that this DNA is composed of phage DNA and a lesser amount of component II DNA or some other form of open-circular DNA containing covalently closed single-strand rings. The infectivity present after denaturation is most probably not due to component I for reasons discussed below.

In striking contrast many of the fractions containing DNA eluted in the caffeine gradient are virtually non-infective before denaturation but upon denaturation increase in infectivity by as much as a thousand-fold. This observation suggests the presence of a novel structure in induced cells consisting of predominantly native double-stranded DNA containing single-strand rings (16).

Some of the fractions from the caffeine gradient containing DNA also contain appreciable infectivity in the native state. This may indicate a heterogeneity of the material eluted from BNC by caffeine.

(ii) Sedimentation Analysis of DNA Eluted from BNC

The neutral sedimentation patterns of unfractionated ^{3}H-DNA and DNA eluted from the BNC column described in Figure 5 are shown in Figure 6. The unfractionated DNA is heterogeneous with material sedimenting from

behind the phage DNA marker to up to 1.9-2.0 times the rate of the marker. Again, as with the susA DNA, this fast material is not evident after fractionation on BNC. The ^{3}H-DNA found in the first fraction, on the bottom of the tube, upon re-sedimentation is found to sediment as the bulk of the DNA and not to represent some very large component. Likewise DNA observed to sediment at twice the rate of the marker is believed to be either an artifact of sedimentation or of drop collection. Such DNA upon re-sedimentation is observed to sediment at only 1.6 times the rate of the marker phage DNA. We cannot exclude the possibility that handling of the DNA during drop collection after centrifugation or by passage through the BNC column shears the DNA found in this region of the gradient. The good recovery usually obtained of material adsorbed to BNC makes it unlikely we feel that this material is not recovered in some form from BNC. However, that material appearing in the gradient at a position twice as far down the gradient as phage DNA is an artifact is confirmed by observation of the centrifuge tube during drop collection. At that point in the gradient considerable viscosity of the drops due to the presence of E. coli DNA has repeatedly been observed. The passage of these viscous drops through the small hole in the bottom of the tube results in considerable swirling throughout the lower half of the remaining gradient as evidenced by a schlieren effect. The presence of radioactivity in fractions 7-10 of Figure 6a and c we feel is due to the observed swirling during drop collection.

Virtually none of the material found in this region of the gradient can be component I. For in contract to infection by lambda phage (41,42) following induction of the lambda prophage less than 1% of the ^{3}H-

thymine incorporated is found to be in component I as determined by CsCl equilibrium density gradient centrifugation in ethidium bromide (25, Kiger & Ilg, unpublished data). This means that less than one component I molecule is synthesized per cell following induction.

DNA eluted from BNC by salt sediments as phage DNA with the leading edge of the ^{3}H-DNA peak suggesting the presence of component II as was also suggested by the infectivity of the denatured DNA. The DNA eluted by caffeine is more heterogeneous and resembles the caffeine fraction obtained with sus A DNA.

Alkaline sedimentation of the DNA eluted from BNC is shown in Figure 7. ^{14}C-lambda phage DNA is sedimented in the same tube as a marker for the position of phage-length single-strands. The ^{3}H-DNA eluted by salt sediments almost entirely as phage-length single-strands. The single-strand rings suggested to be present by infectivity must be a relatively small fraction of the mass of the DNA (as is also indicated by neutral sedimentation).

The DNA eluted by caffeine is very heterogeneous. A majority of the ^{3}H-DNA sediments either faster or slower than phage-length single-strands. The arrows in Figure 7b indicate the portion of the gradient in which material sedimenting x times the rate of phage length single-strands is found. The data presented in Table 2 show the relative sedimentation rates expected in alkali for various (some hypothetical) species of single-strand lambda DNA. These data are calculated from the data of Studier (34) and from the observation that single-strand rings of lambda sediment 1.14 times the rate of phage-length linear single-

strands (23, and Chapter 1). Abelson & Thomas (1) have shown experimentally that this is a valid approach to determining the size of single polynucleotide chains. We have assumed that Studier's data obtained in 1M Na^+ are valid at 0.1M Na^+, i.e. that α in the equation

$$S = KM^{\alpha}$$

where S = sedimentation coefficient
M = molecular weight
K = constant

does not vary as a function of ionic strength. The work of Rupp & Howard-Flanders (26) indicates that this is a good assumption.

(d) Stepwise Elution of Intracellular DNA from BNC

(i) Elution and Rechromatography of Fractions

DNA labeled from 10-45 minutes following induction of 159T$^-$ (λ susRC$_{ts}$) is adsorbed to BNC in 0.3M NaCl, TE and the column eluted first with 1.0 M NaCl, TE followed by 2% caffeine, 1.0 M NaCl, TE. Approximately 100% of the DNA adsorbed to the column was recovered in either the salt or caffeine fractions. These fractions were dialysed overnight against 0.1 x SSC and portions of each were re-adsorbed to fresh BNC columns in 0.3 M NaCl, TE followed by re-elution as just described. The results are indicated in Table 3. Each fraction is re-eluted as would be naively expected from its original elution characteristics.

(ii) Heterogeneity of the DNA Eluted by Caffeine

The heterogeneity (suggested above) of the intracellular DNA eluted by caffeine from BNC is confirmed in the following experiment. The DNA eluted stepwise by caffeine (d,i) is sedimented through neutral and

alkaline sucrose gradients as shown in Figure 8a & b. Fractions from the neutral sucrose gradient (indicated by arrows c-g) are re-sedimented through alkaline sucrose gradients as shown in Figure 8 c-g.

The molecules sedimenting faster than phage DNA at neutral pH are composed almost entirely of single-strands which sediment faster in alkali than phage-length linear molecules. Further heterogeneity is apparent. Fraction (e) seems to be composed of double-length linear molecules, single-length rings and possibly some strands as short as or shorter than phage DNA single strands. Fraction (d) appears to have approximately the same composition but in addition possesses some molecules which sediment as fast as 1.5 or more times the rate of the marker. Fraction (c) has about the same composition as (d). Molecules sedimenting behind or just slightly behind phage DNA at neutral pH are composed of phage-length linear molecules or shorter pieces as indicated by fraction (f) and (g). These last two categories represent only a small fraction of the total DNA eluted by caffeine.

(e) Chromatography of Pulse-Labeled and Chased DNA on BNC

The behavior of pulse-labeled DNA (15 seconds at 37°C) and of pulse-labeled and chased DNA on BNC is shown for several different experiments in Table 4. It is apparent that when replicating DNA is labeled for only a short time almost all of the label goes into molecules eluted by caffeine. This is true whether or not phage DNA synthesis is occurring at the time of the pulse (e.g. experiment 1).

When incorporation of ^{3}H is stopped by its removal from the culture and the subsequent fate of incorporated label followed as a function of time it is evident that at least some of the molecules eluted by caffeine are precursors for molecules that can be eluted by salt. This fact explains why some small fraction of the pulse-labeled DNA is eluted by salt; these molecules were evidently completed (in some sense) during the pulse. Even a 30 minute chase, however, does not result in complete transfer of the pulse-labeled material to the salt fraction. Evidently, the material eluted by caffeine is not solely molecules in the process of replication.

As indicated in Table 4 recovery of pulse-labeled DNA from BNC is noticably reduced compared to the chased DNA. The nature of pulse-labeled and chased DNA will be discussed further in Chapter 3.

4. DISCUSSION

The elution pattern of intracellular lambda DNA from BNC indicates that some large portion of this DNA binds more strongly to BNC than the rest. The pulse-labeling experiments indicate that at least some of the DNA binding strongly to BNC (eluted by caffeine) is DNA in the act of replication. The work of several authors on diverse organisms has indicated that replicating DNA has some properties of single-stranded DNA (6, 15,30). As is shown here and elsewhere (32) single-stranded DNA binds strongly to BNC and can be largely eluted by caffeine. This then would seem to be the basis on which BNC fractionates intracellular

lambda DNA. We do not, however, have any independent evidence to indicate that the DNA eluted by caffeine possesses single-stranded regions.

It is clear from infectivity and from alkaline sedimentation that the DNA fractions eluted by salt and by caffeine are qualitatively different. The caffeine fraction of intracellular DNA described here is most certainly the component X detected in pulse-labeled lambda infected cells by Young & Sinsheimer (42). A similar, heterogeneous fraction of intracellular DNA has also been detected by pulse-labeling of induced lysogens by Weissbach & Salzman (37) who interpret their results as evidence for linear lambda concatenates.

The data of Table 4 demonstrate that the caffeine fraction is not simply DNA in the process of replication. If this were true a short pulse during the period of phage DNA synthesis would be expected to all chase into phage DNA which is eluted by salt. It is evident that phage production and phage DNA synthesis are not directly coupled to the synthesis of the DNA eluted by caffeine. The data suggest that the DNA molecules eluted by caffeine form a pool of intermediates that are randomly matured to DNA eluted by salt. In the case of lambda C_{ts} lysogens the DNA eluted by salt is largely phage DNA. The presence of component II in the salt fraction is detectable only by the infectivity of single-strand rings produced by denaturation. We suggest that maturation of the DNA in the caffeine fraction is a rapid process and that component II is a transitory intermediate in this process. In contrast, the salt fraction formed following induction of sus A lysogens appears to consist only of component II. We suggest that sus A is blocked in the

conversion of component II to phage DNA and that maturation of the DNA molecules of the caffeine fraction to component II occurs normally in sus A. This hypothesis is subject to experiment.

It is evident that prior fractionation of intracellular lambda DNA on BNC allows sedimentation analysis of uniformly labeled intermediate and progeny DNAs without problems of cross-contamination. One interesting feature of the fractionated DNA is that DNA that sediments 1.8-2.0 times the rate of phage DNA in a neutral sucrose gradient before fractionation is lost upon fractionation on BNC. Indeed, as we pointed out above, the presence of lambda DNA in this region of the gradient seems to be an artifact of fraction collection. The presence of labeled DNA in this region following induction (31,37) or infection (33) has been cited as evidence for long linear concatenates of lambda. We find the presence of labeled lambda DNA in this region of the gradient to be due to the presence of viscous E. coli DNA at this point which results in some mixing of the gradient during fraction collection. Failure to effectively suppress the incorporation of label into E. coli DNA could also result in label in this part of the gradient. A purification of lambda DNA eluting in caffeine of 2-3 fold over E. coli DNA is obtained upon passage through BNC (unpublished data). We presume that this reduces the E. coli DNA concentration in the caffeine fraction enough so that the E. coli DNA does not interfere with fraction collection upon centrifugation of the caffeine DNA through neutral sucrose gradients.

Alkaline sedimentation of the DNA eluted by caffeine reveals very little DNA that is larger than twice the length of lambda phage DNA

(bearing in mind that as much as 10% of the incorporated radioactivity may be in E. coli DNA). We only mention in passing that quite similar alkaline sedimentation patterns of replicating T4 DNA have been presented by Frankel (7) to show that little single-stranded DNA sedimenting faster than 1.5 x phage DNA is present during T4 replication. To account for these observations we propose the following model for lambda replication in Figure 9, which with only the slight modifications noted can apply equally well to ØX174 replication.

DNA synthesis is initiated on an open-circular (component II) ring the existence of which has been demonstrated here and elsewhere (41,42). Synthesis of the nascent 5'→3' strand proceeds by addition of nucleotides to the 3'OH of the open component II strand. The requirement of oligonucleotides for in vitro initiation of DNA synthesis on a single-strand template suggests that this requirement may have to be met in vivo as well (10,11). Thus the nascent 5'→3' strand is covalently bonded to the identical parental strand. Synthesis of the complementary strand is grossly in the direction 3'→5' but this may be accomplished by a "back and fill" mechanism which would permit synthesis of short segments of the complementary strand in the direction 5'→3' (22) which are subsequently covalently bonded by the action of the ligase enzyme (8,9,24,43). This mode of synthesis provides a single growing point for each molecule and is in accord with the known polarity of synthesis by DNA polymerase (22).

The consequence of this mode of synthesis is evident from Figure 9. Upon completion of one round of synthesis a structure is formed consisting

of a double-length single-strand, a phage DNA-length single-strand ring and a phage-length single strand. This structure can be converted to several equivalent structures, as indicated, by action of the ligase enzyme and/or a specific "nicking" enzyme(s). One of these structures is a double-length ring which although we have no evidence for it for lambda, is known to be present during ØX174 replication (27,28). Specific "nicking" enzymes have been implicated in ØX 174 replication (17, 18) and may be presumed to be ubiquitous. One of these equivalent structures by a single-strand break can be converted to two daughter component II molecules. Subsequent replication can lead to formation of a pool of component II molecules.

As has been suggested here and by others (41) component II is a direct precursor of lambda phage DNA. A specific single-strand scission is sufficient for the conversion. Component II is also a precursor for ØX174 phage DNA strands (18).

A model for DNA replication in B. subtilis involving covalent addition of new strands to old has been independently proposed by Yoshikawa (39).

The nature of strand synthesis will be dealt with more closely in the next chapter.

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TABLE 1

Recovery of Native and Denatured Phage DNA and Intracellular Lambda DNA from a BNC column

	Salt	Caffeine	8 M Urea, 0.1N NH_4HCOO 1.0M NH_4CL, 1% SDS	Total
^{3}H-Intracellular lambda DNA	19%	61%	8 %	88%
^{32}P-Native lambda DNA	84%	-	6 %	90%
^{14}C-Denatured lambda DNA	-	78%	11 %	89%

TABLE 2

Alkaline Sedimentation Rates of Lambda DNA Species

Single-stranded Species of Lambda DNA	Relative Sedimentation Rate in Alkali
Phage-length linear	1.00
Phage-length ring	1.14
Double phage-length linear	1.30
Double phage-length ring	1.48
Triple phage-length linear	1.50

TABLE 3

Rechromatography of ^{3}H-DNA Eluted from BNC

First Elution of ^{3}H-DNA	Second Elution of ^{3}H-DNA	
	Salt	Caffeine
SALT		
45% of total adsorbed to BNC	97%	3%
CAFFEINE		
55% of total adsorbed to BNC	9%	91%

Lambda DNA labeled with ^{3}H-thymine from 10-45 min. after induction of $159T^{-}(\lambda RC_{ts})$ is adsorbed to BNC in 0.3 M NaCl, TE, and eluted stepwise first with 1.0 M NaCl, TE and then with 2% caffeine, 1.0 M NaCl, TE. The material eluted in each of these buffers is dialysed against 0.1 x SSC overnight and then brought to 0.3 M NaCl and chromatographed in the manner just described.

TABLE 4

Chromatography of Pulse and Chase ^{3}H-DNA

Experiment	Method	Duration of Pulse or Chase	PFU/cell	Recovery of Incorporated ^{3}H after Phenol Extraction	BNC		
					Salt	Caffeine	Recovery from BNC
1	1	15 second Pulse	-	.42	.12	.88	.61
		15 minute Chase	-	.55	.39	.61	.79
2	1	15 second Pulse	0.2	.24	.002	.998	.47
		15 minute Chase	2.3	.48	.37	.63	.56
3	2	15 second Pulse	1.2	.14	.07	.93	.63
		15 minute Chase	4.8	.36	.35	.65	.89
		30 minute Chase	15.5	.40	.53	.47	.85
4	3	15 second Pulse	13.9	.37	.06	.94	.59
		15 minute Chase	18.0	.72	.50	.50	.96
		30 minute Chase	48	.60	.62	.38	.96

^{3}H-lambda DNA is extracted from cells by phenol as described in Methods and the recovery of ^{3}H determined by acid precipitation of cells before extraction and acid precipitation of nucleic acids after extraction. ^{3}H-thymine was added 30 min. after induction in experiment 1 and 45 min. after induction in experiments 2 - 4. Elution is stepwise.

FIGURE 1. The Effect of Mitomycin C on Bacterial and Phage DNA Synthesis.

$159T^-$ and $159T^-(\lambda C_{ts})$ are grown to 3.3×10^8 cells/ml in KG medium supplemented with 10 µg/ml thymine at 37°C. Uracil is added to a concentration of 5 µg/ml and 5 minutes later ^{3}H-thymine is added (10 µc/ml) and each culture distributed to tubes containing a given amount of mitomycin C in a 45°C water bath (time = 0). Cultures are incubated at 45°C for 15 minutes and then shifted to 37°C. At the indicated times 0.5 ml of culture is precipitated in cold 5% trichloroacetic acid. Phage production is measured after lysis and $CHCl_3$.

(a)	0 µg/ml	Mitomycin C		yield =	74	PFU/cell
(b)	5 "	"		"	80	"
(c)	10 "	"		"	56	"
(d)	20 "	"		"	34	"
(e)	50 "	"		"	6	"

The effect of removing mitomycin C after treatment is shown in (f). After 10 minutes incubation at 45°C in 20 µg/ml mitomycin C the cells are collected by filtration, washed and resuspended in 1 volume of fresh medium containing 10 µg/ml thymine, 5 µg/ml uracil and 10 µc/ml ^{3}H-thymine. Incubation is continued at 45°C for 5 minutes and then the cultures are shifted to 37°C. Phage yield is 33 PFU/cell.

●——● $159T^-(\lambda C_{ts})$

X------X $159T^-$

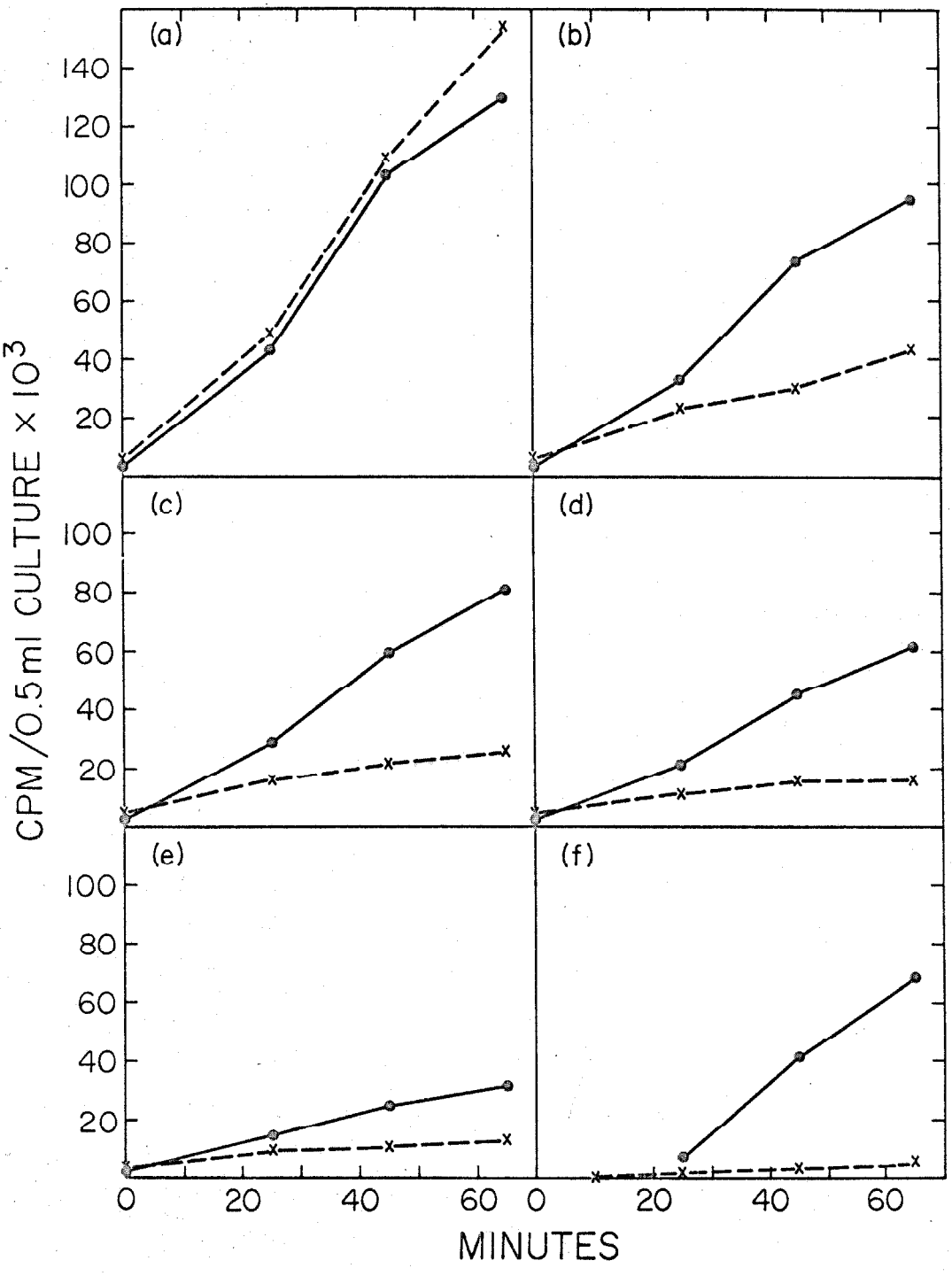
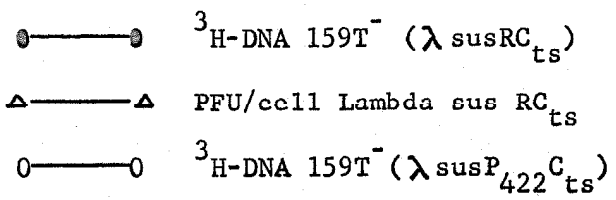


Figure 1.

FIGURE 2. Phage Production and DNA Synthesis Following Induction and Mitomycin C Treatment of $159T^-$ (λ sus RC_{ts}) and $159T^-$ (λ sus $P_{422}C_{ts}$).

Lysogens are grown to 5.3×10^8 cells/ml in KG medium, induced and treated with mitomycin C as described in Methods. Lambda sus$P_{422}C_{ts}$ phage yield is not shown and is 9×10^{-3} PFU/cell.



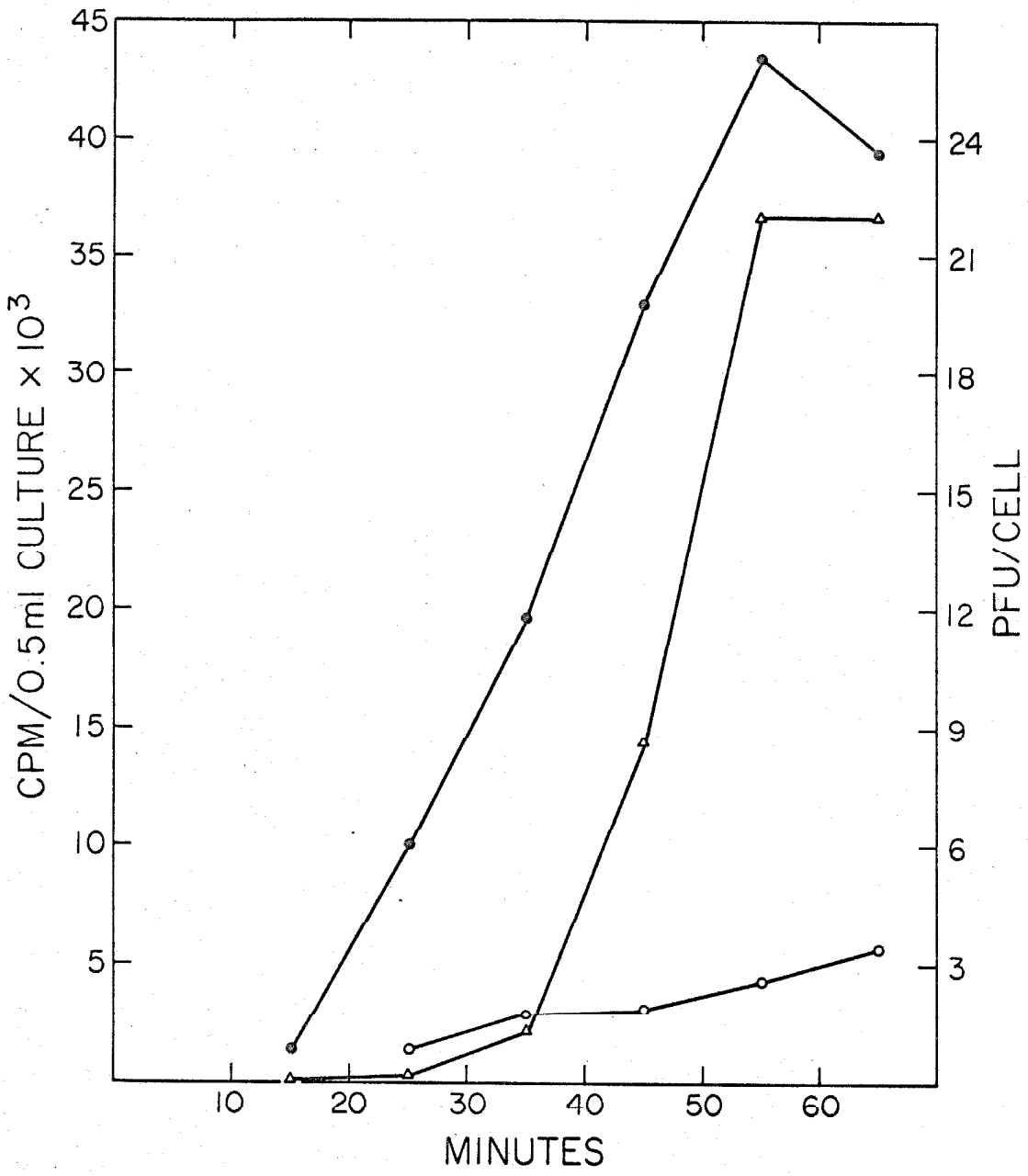

45
40
35
30
25
20
15
10
5
CPM/0.5ml CULTURE X 10^3
24
21
18
15
12
9
6
3
PFU/CELL
10
20
30
40
50
60
MINUTES

Figure 2.

FIGURE 3. Chromatography of DNA Synthesized after Induction of $159T^-$ ($\lambda susA_{11}C_{ts}$).

^3H-$159T^-$($\lambda susA_{11}C_{ts}$) DNA labeled from 10-45 minutes following induction and mitomycin C treatment is mixed with ^{32}P-lambda C_{ts} phage DNA and heat denatured ^{14}C-lambda C_{ts} phage DNA, and the mixture adsorbed to a BNC column (15 mm x 15 mm).

(a) Elution with a 0.3 M NaCl to 1.0 M NaCl gradient (total volume 60 ml). The shoulder on the peaks is due to a disturbance of the gradient at that point. No detectable ^{14}C is eluted in this gradient.

(b) Elution with a 0-2% caffeine gradient in 1.0 M NaCl (total volume 60 ml). Approximately 1% of the ^{32}P put on the column is eluted in this gradient but not shown.

●——● ^3H-$159T^-$($\lambda susA_{11}C_{ts}$) induced DNA

X——X ^{32}P-lambda C_{ts} phage DNA

0——0 ^{14}C-lambda C_{ts} denatured phage DNA

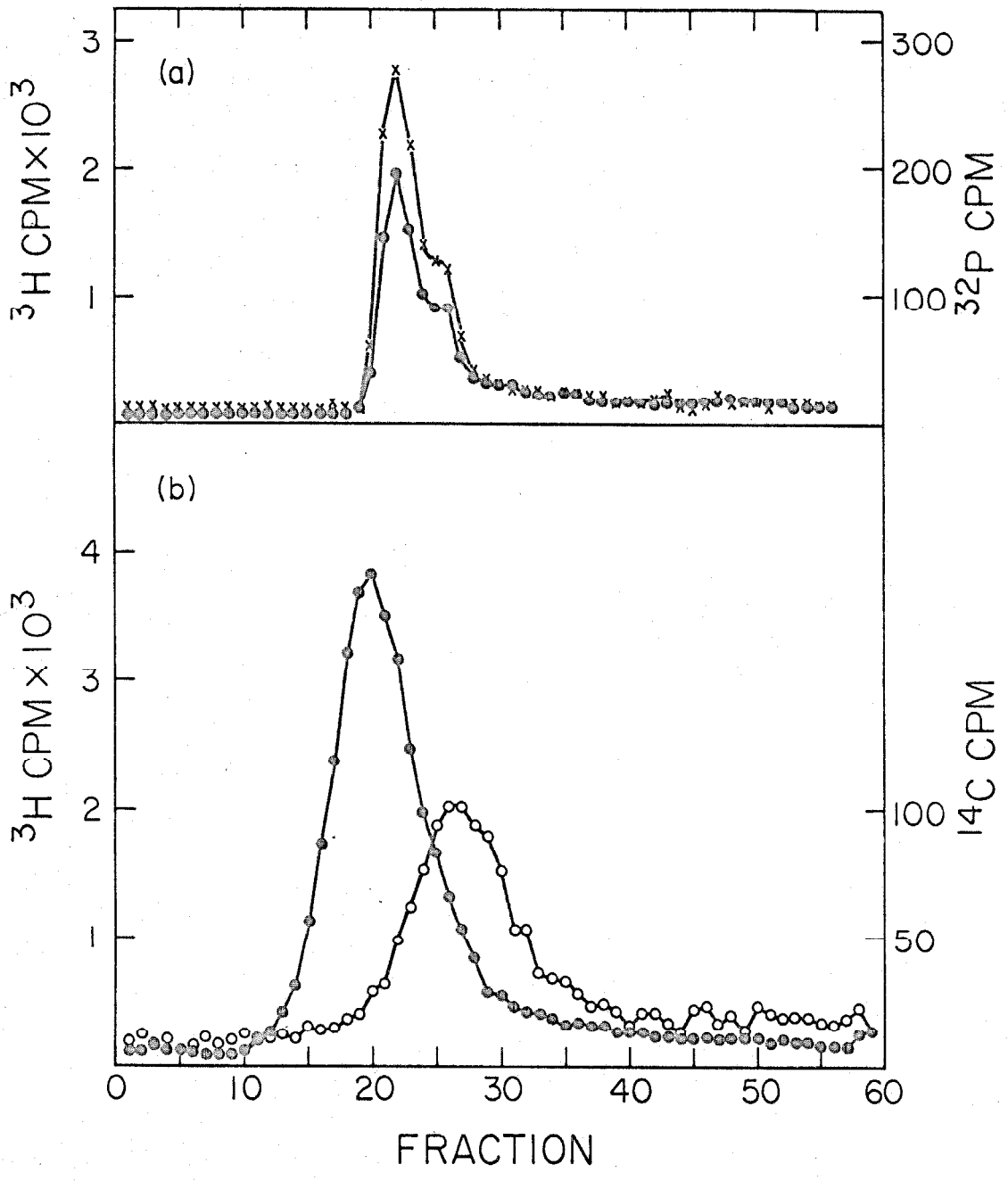

(a)
(b)
^{3}H CPM×10^3
^{32}P CPM
^{14}C CPM
FRACTION
0
10
20
30
40
50
60
1
2
3
4
100
200
300
50

Figure 3.

FIGURE 4. Sedimentation of DNA Synthesized after Induction of 159T$^-$ (λsus $A_{11}C_{ts}$).

Sedimentation is through a 5%-20% sucrose gradient in TE in a Spinco SW 25.1 rotor at 25,000 rev/min, 6°C, for 6 hours. Sedimentation is from right to left with a marker of ^{32}P-lambda C_{ts} phage DNA.

(a) ^{3}H-159T$^-$ (λ sus$A_{11}C_{ts}$) DNA labeled from 10-45 minutes following induction.

(b) ^{3}H-DNA eluted from BNC in the salt gradient of Figure 3.

(c) ^{3}H-DNA eluted from BNC in the caffeine gradient of Figure 3.

●——● ^{3}H-DNA

X——X ^{32}P-phage DNA

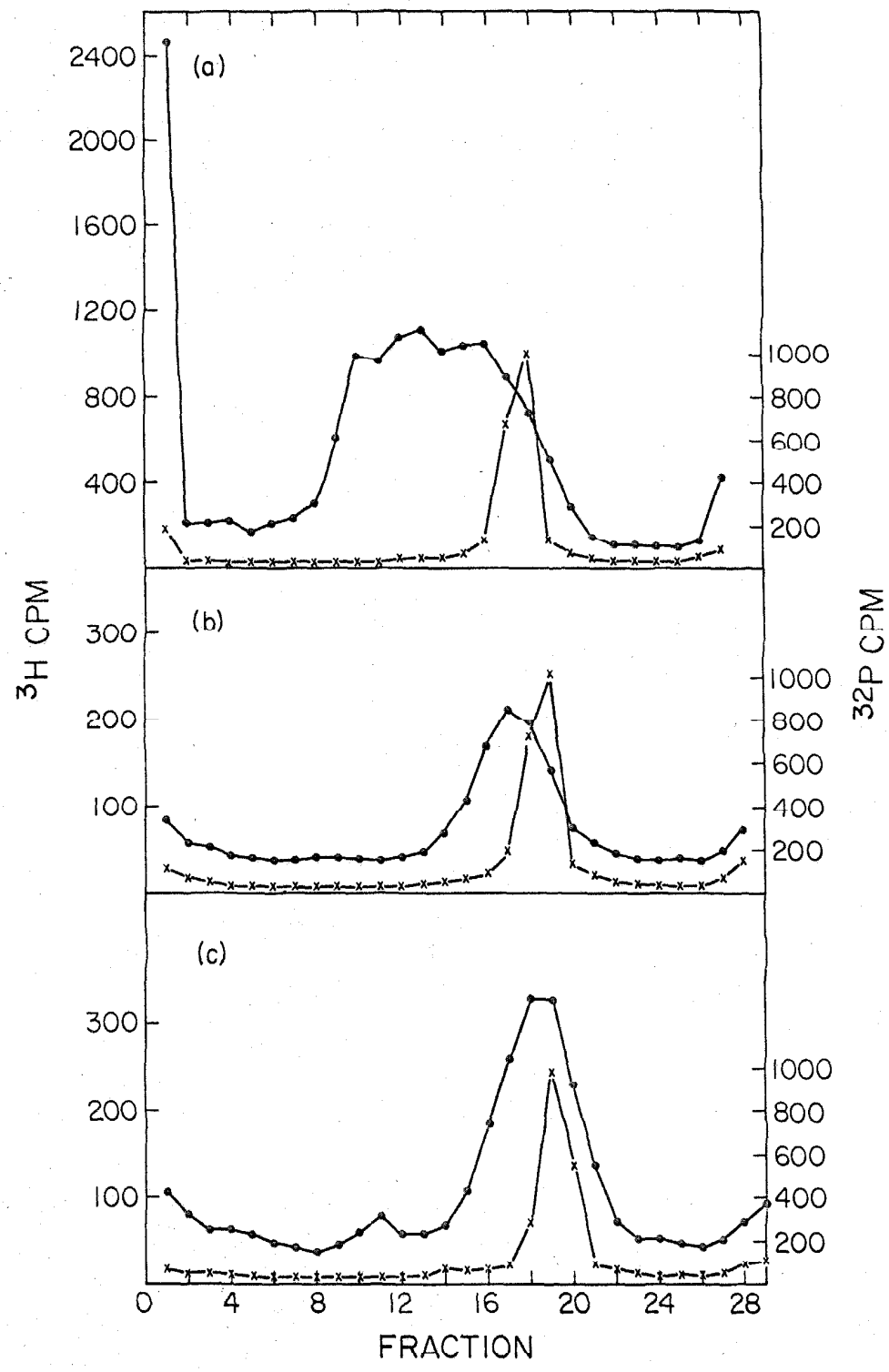

(a)
(b)
(c)
2400
2000
1600
1200
800
400
300
200
100
1000
800
600
400
200
0
4
8
12
16
20
24
28
FRACTION
^{3}H CPM
^{32}P CPM

Figure 4.

FIGURE 5. Chromatography of DNA Synthesized after Induction of $159T^-(\lambda C_{ts})$

^{3}H-$159T^-(\lambda C_{ts})$ DNA labeled from 10-45 minutes following induction and mitomycin C treatment is mixed with ^{32}P-$434hyC_{51}$ phage DNA and adsorbed to a BNC column (15 mm x 15 mm). Elution sequence is as described in Figure 3.

(a) salt gradient

(c) caffeine gradient

●———● ^{3}H-$159T^-(\lambda C_{ts})$ induced DNA

X———X ^{32}P-lambda $424hyC_{51}$ phage DNA

(b) spheroplast infectivity of lambda C_{ts} DNA eluted in the salt gradient

(d) spheroplast infectivity of lambda C_{ts} DNA eluted in the caffeine gradient

●———● infectivity of native DNA

X·····X infectivity of denatured DNA

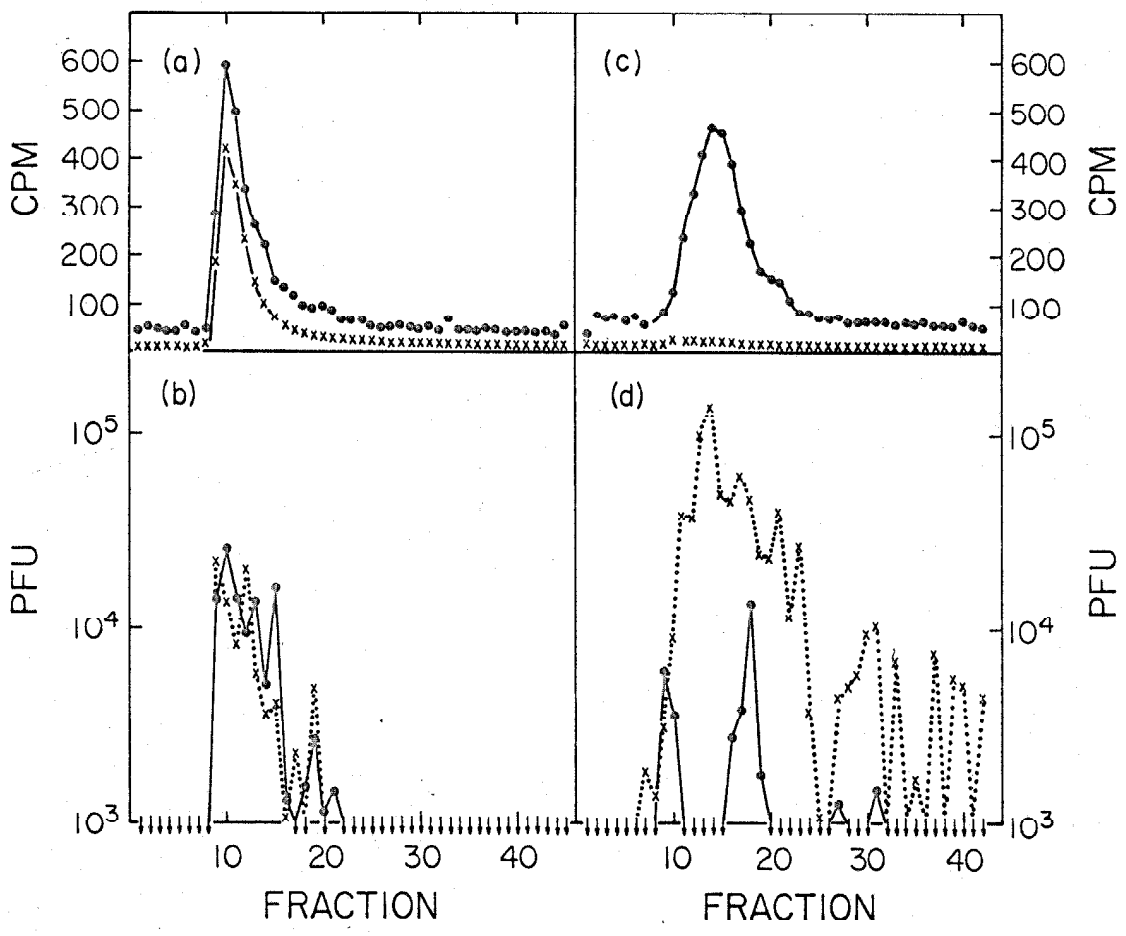

(a)
(b)
(c)
(d)
CPM
PFU
600
500
400
300
200
100
10^5
10^4
10^3
10
20
30
40
FRACTION

Figure 5.

FIGURE 6. Sedimentation of DNA Synthesized after Induction of 159T$^-$(λ C_{ts})

Sedimentation is through a 5%-20% sucrose gradient in TE in a Spinco SW 25.1 rotor at 25,000 rev/min, 6°C, for 6 hours. Sedimentation is from right to left with a marker of ^{32}P-434hyC_{51} phage DNA.

(a) ^{3}H-159T$^-$(λ C_{ts}) DNA labeled from 10-45 minutes following induction.

(b) ^{3}H-DNA eluted from BNC in the salt gradient of Figure 5.

(c) ^{3}H-DNA eluted from BNC in the caffeine gradient of Figure 5.

●——● ^{3}H-DNA

X——X ^{32}P-phage DNA

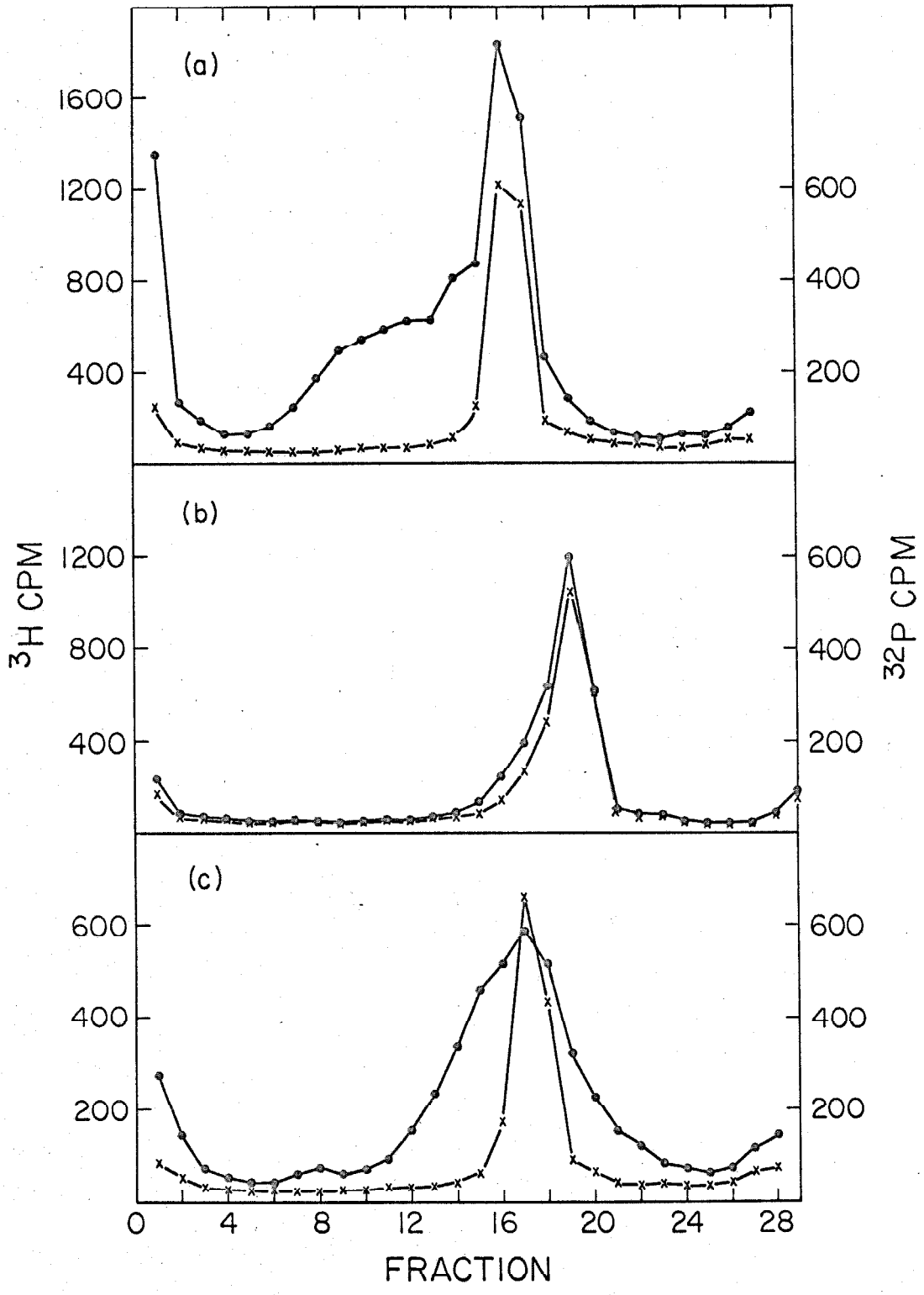

(a)
(b)
(c)
1600
1200
800
400
600
400
200
1200
800
400
600
400
200
600
400
200
600
400
200
0
4
8
12
16
20
24
28
FRACTION
3H CPM
32P CPM

Figure 6.

FIGURE 7. Alkaline Sedimentation of DNA Eluted from BNC.

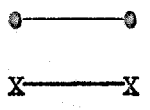
Sedimentation is through a 5%-20% sucrose gradient in 0.1 N NaOH in a Spinco SW 25.3 rotor at 25,000 rev/min, 6°C, for 15 hours. Sedimentation is from right to left with a marker of ^{14}C-lambda C_{ts} phage DNA. Prior to sedimentation the samples were denatured in 0.16 N NaOH at 37°C for 10 min, total volume 0.78 ml.

(a) ^{3}H-DNA eluted from BNC in the salt gradient of Figure 5.

(b) ^{3}H-DNA eluted from BNC in the caffeine gradient of Figure 5.

The fraction of material sedimenting in the indicated region is as follows:

Region	Fraction of ^{3}H	Ratio of ^{3}H in X to ^{3}H in region 1.14
ahead of 1.30	0.10	0.8
1.30	0.23	1.8
1.14	0.13	1.0
1.00	0.17	1.3
behind 1.00	0.37	2.9



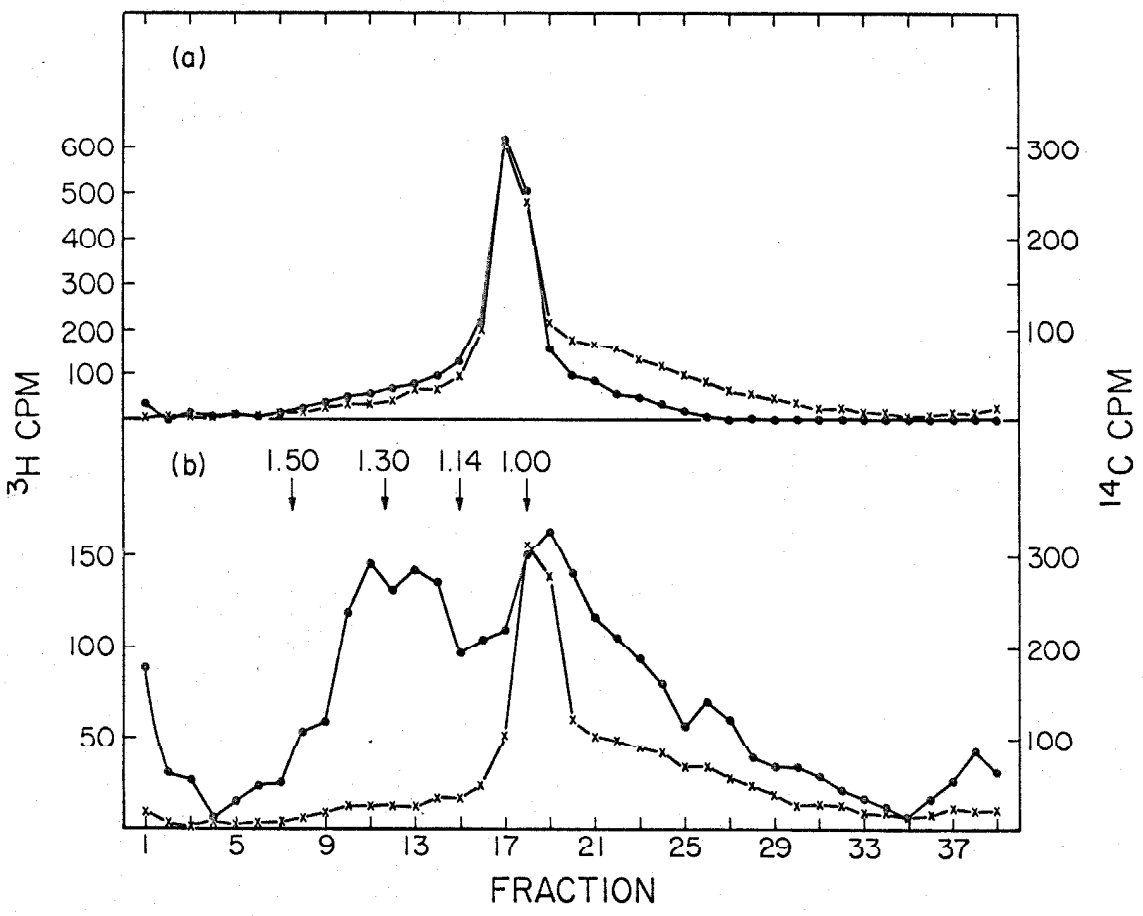

(a)
600
500
400
300
200
100
300
200
100
^{3}H CPM
^{14}C CPM
(b)
1.50
1.30
1.14
1.00
150
100
50
300
200
100
1
5
9
13
17
21
25
29
33
37
FRACTION

Figure 7.

FIGURE 8. Sedimentation Analysis of the DNA Eluted from BNC by Caffeine.

^{3}H-159T^{-}(λsusRC$_{ts}$) DNA labeled from 10-45 minutes following induction, mitomycin C treatment and growth in KG medium containing 10 μg/ml thymine, 75 μc/ml ^{3}H-thymine and 5 μg/ml uracil is adsorbed to BNC and eluted stepwise with 1.0 M NaCl, TE followed by 2% caffeine, 1.0 M NaCl, TE.

(a) ^{3}H-DNA eluted by caffeine is sedimented through a 5%-20% sucrose gradient in TE from right to left with a marker of ^{14}C-lambda C$_{ts}$ phage DNA consisting of linear molecules and annealed rings in a Spinco SW 25.1 rotor at 25,000 rev/min, 6°C, for 6 hr. 50 μl of each fraction is acid precipitated, collected on a filter and counted. The DNA on the bottom of the tube, fraction 1, upon resedimentation at neutral pH sediments as does the bulk of the DNA shown here.

(b) ^{3}H-DNA eluted by caffeine is sedimented through a 5%-20% sucrose gradient in 0.1N NaOH from right to left with a marker of ^{14}C-lambda C$_{ts}$ phage DNA. The position of the phage DNA is indicated by the arrow. Prior to centrifugation the sample is denatured in 0.23 N NaOH at 37°C for 10 min, total volume 0.52 ml. Centrifugation is in a Spinco SW 25.3 rotor at 25,000 rev/min, 6°C, for 13 hours. The arrows indicate the position of material sedimenting at the indicated rate times that of phage DNA.

Fractions from the neutral sucrose gradient in (a) are resedimented in alkaline sucrose gradients as described in (b) and are shown in (c) - (g).

●——● ^{3}H-DNA

X——X ^{14}C-lambda phage DNA

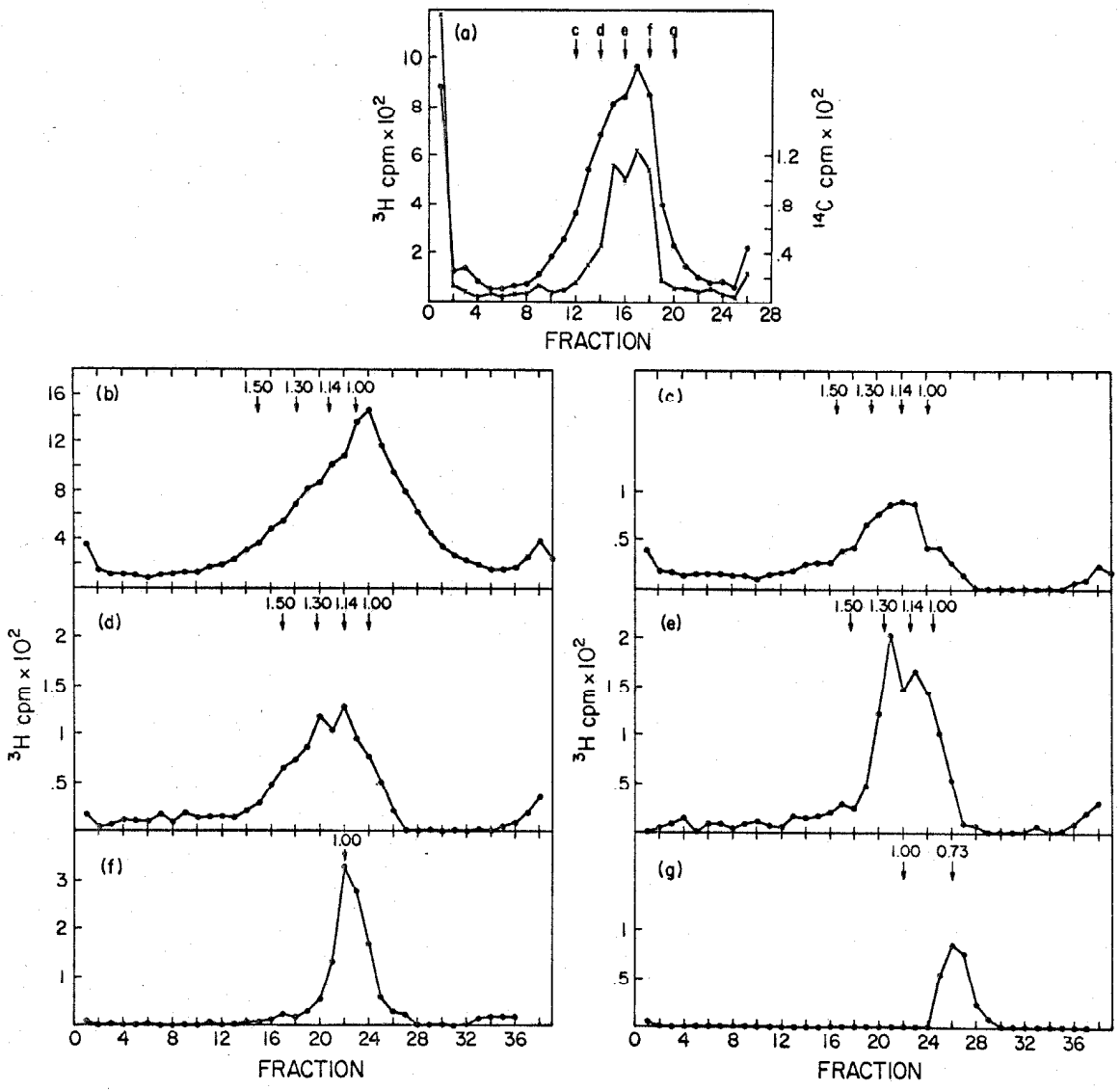

(a)
c d e f g
^{3}H cpm x 10^2
^{14}C cpm x 10^2
FRACTION
(b)
1.50 1.30 1.14 1.00
(c)
(d)
(e)
(f)
1.00
(g)
1.00 0.73

Figure 8.

FIGURE 9. A Model for the Replication of Circular DNA Molecules

Previously known molecular species of either intracellular lambda or ØX 174 DNA are so labeled. The strands of the parental component II molecules are represented by either filled or empty double lines. The filled dot at one end of an open strand represents a 3'-OH. Daughter strands are represented by either partially filled or shaded double lines. Arrows represent either the process of strand synthesis or the action of the ligase enzyme or of "nicking" enzymes as indicated.

The free end produced as an intermediate in the replication of component II might be bound to a cellular site of some kind (not shown).

Synthesis of a daughter 5' → 3' strand is seen to be through covalent addition to the identical parental strand. Synthesis of the complementary strand in a 3' → 5' direction might be by a "back and fill" mechanism by which short segments are synthesized in the direction 5'→ 3' and subsequently covalently linked by the ligase or by an unknown mechanism involving continuous growth in the direction 3' → 5'.

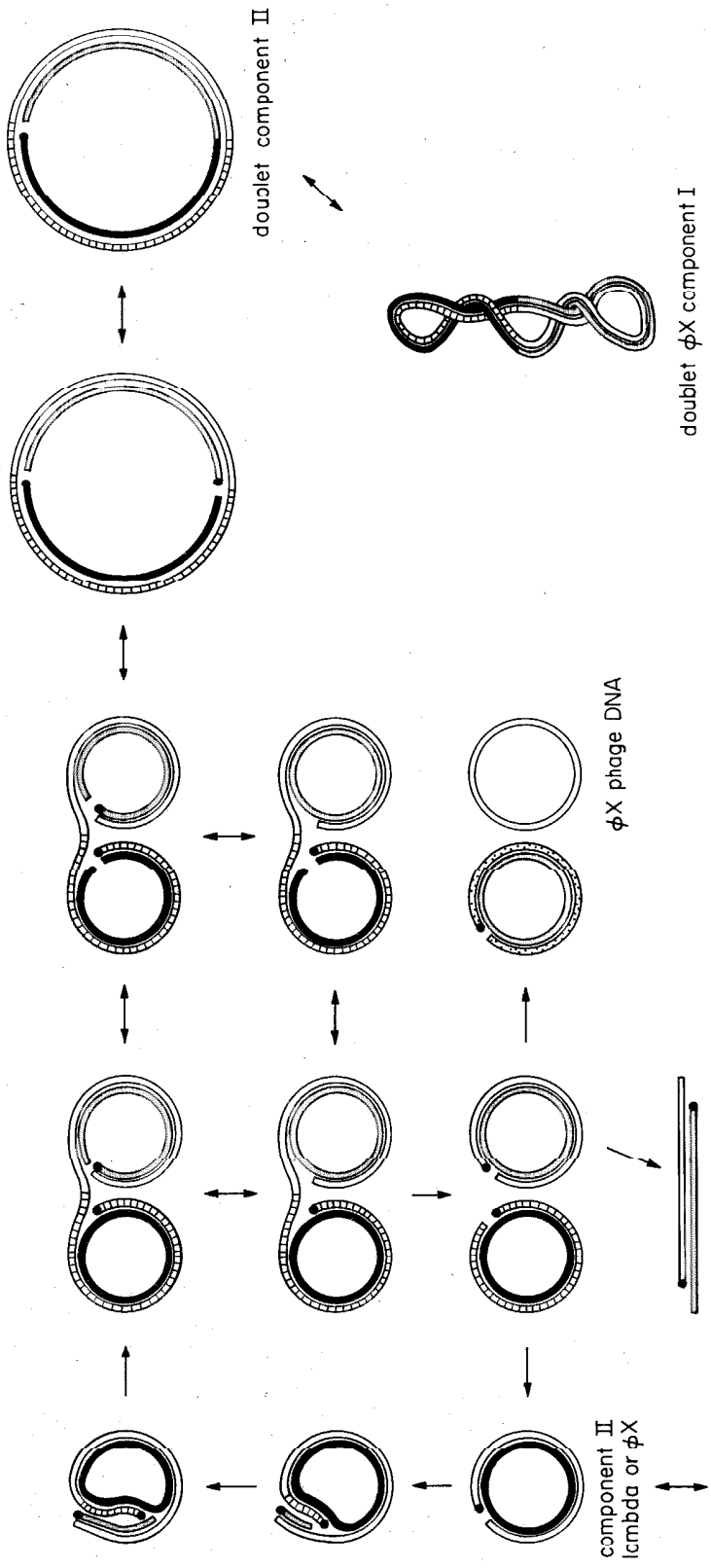

doublet component II
doublet ϕX component I
ϕX phage DNA
lambda phage DNA
component II
lambda or ϕX
component I
lambda or ϕX

Figure 9.

Chapter 3

The Nature of Replicating Intracellular Lambda DNA Labeled for Short Periods of Time

1. INTRODUCTION

The DNA labeled during short pulses of ^{3}H-thymine in cells infected by clear mutants of bacteriophage lambda has been studied by Young & Sinsheimer (5). They concluded that both early and late in infection component II was the first species to be labeled. In addition, pulses late in infection were observed to label a heterogeneous component which they designated component X. This component appeared to be a precursor to mature phage DNA.

DNA labeled during five minute pulses in ultraviolet-induced lysogens of lambda has been studied by Weissbach & Salzman (3). They found the labeled DNA to sediment at the rate of linear phage DNA but concluded that this labeled species lacks the single-stranded ends of mature lambda DNA. During a chase this pulse-labeled DNA was observed to assume a sedimentation rate faster than that of linear phage DNA and to sediment in a heterogeneous manner. This latter species has the sedimentation characteristics of the component X identified by Young & Sinsheimer (5). Weissbach & Salzman believe this heterogeneous component to be linear concatenates of lambda DNA.

We have previously discussed the isolation of uniformly-labeled DNA by chromatography on benzoylated-naphthoylated DEAE cellulose (BNC) from induced lysogens of lambda which behaves as the component X of Young & Sinsheimer (5). DNA labeled by very short pulses of ^{3}H-thymine or ^{3}H-thymidine also chromatographs on BNC as does component X indicating that this species is the replicating form of lambda DNA. A model of

replicating lambda DNA has been proposed based on the single-strand composition of the replicating DNA (Chapter 2).

Here we report the sedimentation properties of DNA labeled during very short pulses of ^{3}H-thymine and ^{3}H-thymidine during phage DNA synthesis following induction of lambda lysogens. The fate of pulse-labeled lambda DNA during chases with unlabeled thymidine is also investigated. We confirm the finding of Young & Sinsheimer (5) and of Chapter 2 that component X is a precursor to mature phage DNA. The nature of DNA strand synthesis is discussed in the light of these experiments and of a model of DNA synthesis previously proposed. Finally, very short pulses reveal the existence of a structure which is labeled very rapidly and which sediments at one-third the rate of lambda phage DNA at neutral pH.

2. METHODS

The techniques and materials employed here have been described for the most part in Chapter 2. We have employed a sus R lysogen (defective in lysozyme synthesis) to avoid the problem of premature lysis encountered by Young & Sinsheimer (5).

The pulse-chase experiments have been described in Chapter 2. For the pulse experiments described here, incubation is at 37^{o}C and incorporation of label is stopped by pipetting 5 ml of culture into a beaker on ice containing 0.05 ml of 1.0 M sodium azide. Incorporation of ^{3}H-thymidine into acid-precipitable material is linear with increasing length of pulse. Extrapolation of a plot of incorporation versus length of pulse

to zero incorporation suggests a 4 second delay after addition of ^{3}H-thymidine before incorporation begins.

Pronase treatment of lysates is carried out by adding 0.1 ml pronase (Calbiochem; 2 mg/ml dissolved in TE and incubated at 37°C for 90 minutes prior to use) to 0.4 ml of lysate prepared as described in Chapter 2, followed by incubation at 37°C for 2.5 hours. Samples can then be immediately analyzed by sedimentation. Young & Sinsheimer (4) have shown that pronase treatment of DNA does not alter its sedimentation properties compared to those of DNA extracted by phenol.

3. RESULTS

(a) Pulse-Chase Labeling of Replicating Lambda DNA

(i) Labeling of an Induced Lysogen During Replication of Lambda DNA

As noted previously, intracellular lambda DNA labeled for a brief period of time is adsorbed strongly to BNC and is eluted from BNC by caffeine somewhat less efficiently than DNA which is pulse-labeled and then chased for some period of time (Chapter 2).

Sedimentation of DNA labeled for a period of 15 seconds but not chromatographed on BNC is shown in Figure 1 a & b. The sedimentation pattern at neutral pH of pulse-labeled DNA resembles the pattern previously observed for uniformly labeled intracellular lambda DNA eluted from BNC by caffeine (Chapter 2) except that approximately 20% of the label sediments as a peak of about 10-12 S. This material is not hydrolyzed by

alkali. 64% of the label sediments faster than linear phage DNA.

Sedimentation of the pulse-labeled DNA at alkaline pH reveals a somewhat different pattern than that observed for uniformly-labeled DNA eluted from BNC by caffeine. While a considerable portion (34%) of the label sediments faster than phage DNA single-strands, the majority of the label sediments more slowly in a very heterogeneous manner.

The fate of the pulse-labeled DNA following chases of 15 minutes and 30 minutes is shown in Figure 1 c-f. The neutral sedimentation patterns indicate that as replication continues the pulse-label accumulates in material sedimenting at the rate of phage DNA. Even after 30 minutes, however, some label is still present in either component II or component X. This is in agreement with the chromatographic behavior of this material on BNC (Chapter 2). After a 15 minute chase the material sedimenting at 10S has disappeared. Alkaline sedimentation confirms these observations. After a chase of 15 minutes 31% of the pulse labeled material sediments as phage DNA single-strands and by 30 minutes, 41% of the label so sediments. Surprisingly, after a 30 minute chase 25% of the pulse-label still sediments more slowly than phage DNA single-strands and 34% sediments faster. We thus cannot adequately explain the sedimentation characteristics of a large fraction of the label.

(ii) Labeling of an induced lysogen starved of thymine

When an induced thymine-requiring lysogen is pulse-labeled subsequent to 5 minutes of thymine starvation and then chased for 15 minutes with unlabeled thymidine, the labeled DNA has a quantitatively different

sedimentation pattern than that observed above. Sedimentation of the pulsed and chased DNAs is shown in Figure 2.

Sedimentation of the pulse-labeled DNA at neutral pH reveals that much more of the label (30%) sediments as 10S material. Only 43% of the label sediments ahead of linear phage DNA. Digestion of each of these fractions by alkali (0.3N NaOH, 12 hours room temperature) followed by acid precipitation results in no change in the pattern. Sedimentation of this DNA at alkaline pH reveals most of the DNA to be considerably smaller than phage DNA single-strands. Only 17% of the label sediments faster than phage-length single-strands.

If this pulse-labeled DNA is first adsorbed to BNC and then eluted with caffeine all of the material sedimenting faster than phage DNA single-strands in alkali is lost. For this reason we have not used BNC chromatography to study pulse-labeled DNA from starved cells.

After a chase of 15 minutes the pulse-label sediments at neutral pH with a distinct peak at the position of phage DNA. 10S material is still present. Sedimentation of this DNA at alkaline pH demonstrates great heterogeneity of the single-strands with peaks at the position of phage DNA single-strands and at about 8S.

(b) Sedimentation of DNA Labeled for Increasing Lengths of Time

An induced, mitomycin C treated lysogen is grown in 5 μg/ml thymine as described in Chapter 2 and labeled for various lengths of time beginning at 45 minutes after induction. The recovery of incorporated ^{3}H-thymidine following extraction by phenol from cells labeled for increasing periods of time is shown in Table 1.

(i) Sedimentation of DNA extracted with phenol

The sedimentation patterns of DNA labeled for 10, 35 and 120 seconds followed by extraction with phenol are seen in Figure 3. Sedimentation at neutral pH of DNA labeled for 10 seconds reveals that about 40% of the label is in material that sediments at 10S. Another 40% of the label sediments faster than linear phage DNA. Alkaline sedimentation of the DNA labeled for 10 seconds shows that most of it is much smaller than phage DNA single-strands. Only 15% of the label sediments faster than phage DNA.

Sedimentation at neutral pH of the DNA labeled for 35 and 120 seconds shows that much less of the label is in the 10S component. The label incorporated over these longer periods goes into component X and the size of the 10S component seems to remain approximately constant. Sedimentation in alkali of the DNA labeled for 120 seconds shows a pattern very similar to that obtained for uniformly labeled DNA eluted from BNC by caffeine. Alkaline sedimentation of DNA labeled for 35 seconds demonstrates a pattern intermediate between the 10 and 120 second pulses.

The relatively large amount of slow 10S component present in short pulses might be due to preferential recovery of this component during phenol extraction as the data in Table 1 indicate that only a minority of the labeled DNA is recovered after phenol extraction. To determine if this is the case we have digested lysates with pronase as described in Methods and examined them with no further treatment. This procedure assures that all of the labeled material will be observed.

(ii) Sedimentation of lysates treated with pronase

Portions of the same lysates that were examined after phenol extraction in the above section were also digested with pronase as described in Methods. The sedimentation behavior of these lysates is shown in Figure 4.

Whether DNA is prepared by phenol extraction or pronase digestion the same fraction of incorporated ^{3}H-thymidine is observed to sediment ahead of linear phage DNA as does the great majority of component X (Chapter 2) at neutral pH. In contrast to the phenol extracted DNA, the DNA prepared by pronase digestion does not exhibit a discrete 10S component. Instead a large fraction of the label appears to either sediment very slowly or diffuse down from the top of the gradient. This material is acid precipitable. Since the same fraction of the label is in component X regardless of the method of preparation, we must conclude that the method of preparation greatly affects the properties of the component which yields 10S material upon extraction with phenol.

A quantitative comparison of the fraction of label sedimenting faster than linear phage DNA at neutral pH and the fraction sedimenting faster than phage DNA single-strands at alkaline pH for the pulses presented in Figure 1-4 is given in Table 2.

4. DISCUSSION

Interpretation of the data presented here is complicated by the presence of an unexpected small component which is rapidly labeled and

which following phenol extraction sediments at about 10S at neutral pH and upon denturation sediments in alkali at about 9S. This component appears to turn over rapidly as it is detectable only when the culture is labelled for less than 2 minutes; at longer times its presence is most probably obscured by the much larger amount of faster sedimenting DNA which has been labeled. This material remains acid precipitable following treatment with alkali sufficient to hydrolyze RNA.

A similar component labeled by very short pulses has been detected during T4 infection and in uninfected E. coli by Okazaki, Okazaki, Sakabe, Sugimoto & Sugino (2). These workers find that this component is degraded by pancreatic DNase and by E. coli exonuclease I suggesting that it is single-stranded DNA.

There is no evidence to indicate at present whether this 10S component is a precursor to component X and to replicated DNA in general or whether it is some other metabolic feature of the cell which is not directly related to DNA synthesis. One obvious approach to this question would be an attempt to anneal it to denatured lambda and E. coli DNA. It is doubtful a priori that such a small single-stranded molecule of DNA (if it is DNA) is the bearer of the genetic information of lambda, T4 or E. coli. This component obviously requires further investigation.

We will for the time concern ourselves only with those DNA molecules the size of lambda phage DNA or larger. In Table 2 we see that roughly half of the label found in component X for pulses of less than one minute appears to be covalently bonded to strands larger than phage length single strands. At this temperature no more than one phage is being matured per

minute. As the cells are not synchronized it is not surprising that the DNA labeled for short times sediments in as heterogeneous a manner as does uniformly labeled component X (Chapter 2).

We interpret these results to be evidence supporting a model for DNA synthesis in which the strand synthesized in the direction 5'→3' is extended by covalent joining of each nucleotide to the 3'-OH of the previously incorporated nucleotide. We have previously suggested that synthesis of this strand begins by joining to the 3'-OH of the open strand of a component II molecule (Chapter 2).

The data in Table 2 for the 10 second pulses and the 15 second pulse of Figure 2 in which the lysogen was starved of thymine prior to labeling (slowing the rate of DNA synthesis) suggest that covalent joining at the initiation of synthesis may not be obligatory but may occur sometimes after synthesis of a new strand has begun. In Table 2 the ratio of label in component X to label in single strands sedimenting faster than phage DNA single strands is observed to fall well below 2:1 after 120 seconds of labeling. This would be expected if one round of synthesis has been completed and another round begun by a component II molecule completed during the pulse.

It is clear that for short pulses at least half of the label in component X is in strands which are of phage-length or smaller. This is the observation that we would expect for label in the complementary strand (the strand synthesized 3'→5') if the general scheme of replication is as proposed in Chapter 2. The presence of the small single strands arising from the 10S component makes it impossible to determine

if the strand synthesized 3'→5' is synthesized as one growing chain or by a "back and fill" mechanism (Chapter 2 and (1)).

The pulse-chase experiments here confirm that component X is a precursor to mature phage DNA as is suggested in Chapter 2 and by Young & Sinsheimer (5). It is noteworthy that after a fifteen second pulse and a 30 minute chase that as much as 25% of the label incorporated in the pulse sediments in alkali at a rate slower than phage-length single strands.

As is pointed out in Chapter 2, the mitomycin C treatment employed to block host DNA synthesis also reduces the yield of phage. The mechanism of this is not at all clear. The possibility cannot be excluded that at least some of the label introduced into intracellular lambda DNA goes into aborted molecules. This, however, cannot explain the origin of the 10S material.

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3. Weissbach, A. & L.A. Salzman (1967). Proc. Nat. Acad. Sci., Wash. 58, 1096.

4. Young, E.T., II & R.L. Sinsheimer (1967). J. Mol. Biol. 30, 165.

5. Young, E.T., II & R.L. Sinsheimer (1968). J. Mol. Biol. 33, in press.

TABLE 1

Recovery of Incorporated ^{3}H-Thymidine Following Phenol Extraction of Pulse-Labeled, Induced Lysogen

Duration of Pulse seconds	Recovery of ^{3}H
10	37 %
20	29 %
35	30 %
65	32 %
120	41 %

TABLE 2

Pulse length		A fraction of label sedimenting faster than linear phage DNA at neutral pH	B fraction of label sedimenting faster than phage-length single strands at alkaline pH	$\frac{A}{B}$
Figure 1	15 sec.	64 %	34 %	1.9
Figure 2	15 sec.	43 %	17 %	2.5
Figure 3	10 sec.	41 %	15 %	2.7
	35 sec.	55 %	34 %	1.6
	120 sec.	75 %	61 %	1.2
Figure 4	10 sec.	43 %	12 %	3.6
	35 sec.	58 %	25 %	2.3
	120 sec.	77%	55 %	1.4

FIGURE 1. Sedimentation of Pulse-Labeled and Chased Lambda DNA

An induced lysogen of 159T$^-$(λ susRC$_{ts}$) is labeled for 15 seconds with ^{3}H-thymidine, 45 minutes after induction during phage DNA synthesis as described for method 3 (Chapter 2). The ^{3}H-thymidine is chased with unlabeled thymidine and the culture sampled at 15 minutes and 30 minutes after the pulse. At the time of the pulse there were 13.9 PFU/cell. 15 minutes later there were 18.0 PFU/cell and after 30 minutes there were 48 PFU/cell. DNA is extracted with phenol as previously described. All samples are precipitated with cold 5% trichloroacetic acid and collected on filters, dried and counted.

(a) Sedimentation of native ^{3}H-pulse-labeled DNA through a 5%-20% sucrose gradient in TE. Sedimentation is from right to left in a Spinco SW25.1 rotor at 25,000 rev/min, 6^{o}C, for 6 hours. A marker of ^{14}C-lambda susRC$_{ts}$ phage DNA consisting of linear phage DNA and annealed rings is cosedimented with the ^{3}H-DNA.

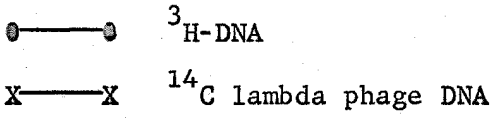
(b) Sedimentation of denatured ^{3}H-pulse-labeled DNA through a 5%-20% sucrose gradient in 0.1N NaOH. Sedimentation is from right to left in a Spinco SW 41 rotor at 30,000 rev/min, 6^{o}C for 9 hours. A marker of denatured ^{14}C-lambda susRC$_{ts}$ phage DNA is cosedimented with the ^{3}H-DNA. A 0.37 ml sample is layered over an 11 ml gradient.

(c) Sedimentation of native ^{3}H-DNA extracted from cells chased for 15 minutes following the pulse. Sedimentation is described in (a).

(d) Sedimentation of denatured ^{3}H-DNA extracted from cells chased for 15 minutes following the pulse. Sedimentation is described in (b).

(e) Sedimentation of native ^{3}H-DNA extracted from cells chased for 30 minutes following the pulse. Sedimentation is described in (a).

(f) Sedimentation of denatured ^{3}H-DNA extracted from cells chased for 30 minutes following the pulse. Sedimentation is described in (b).



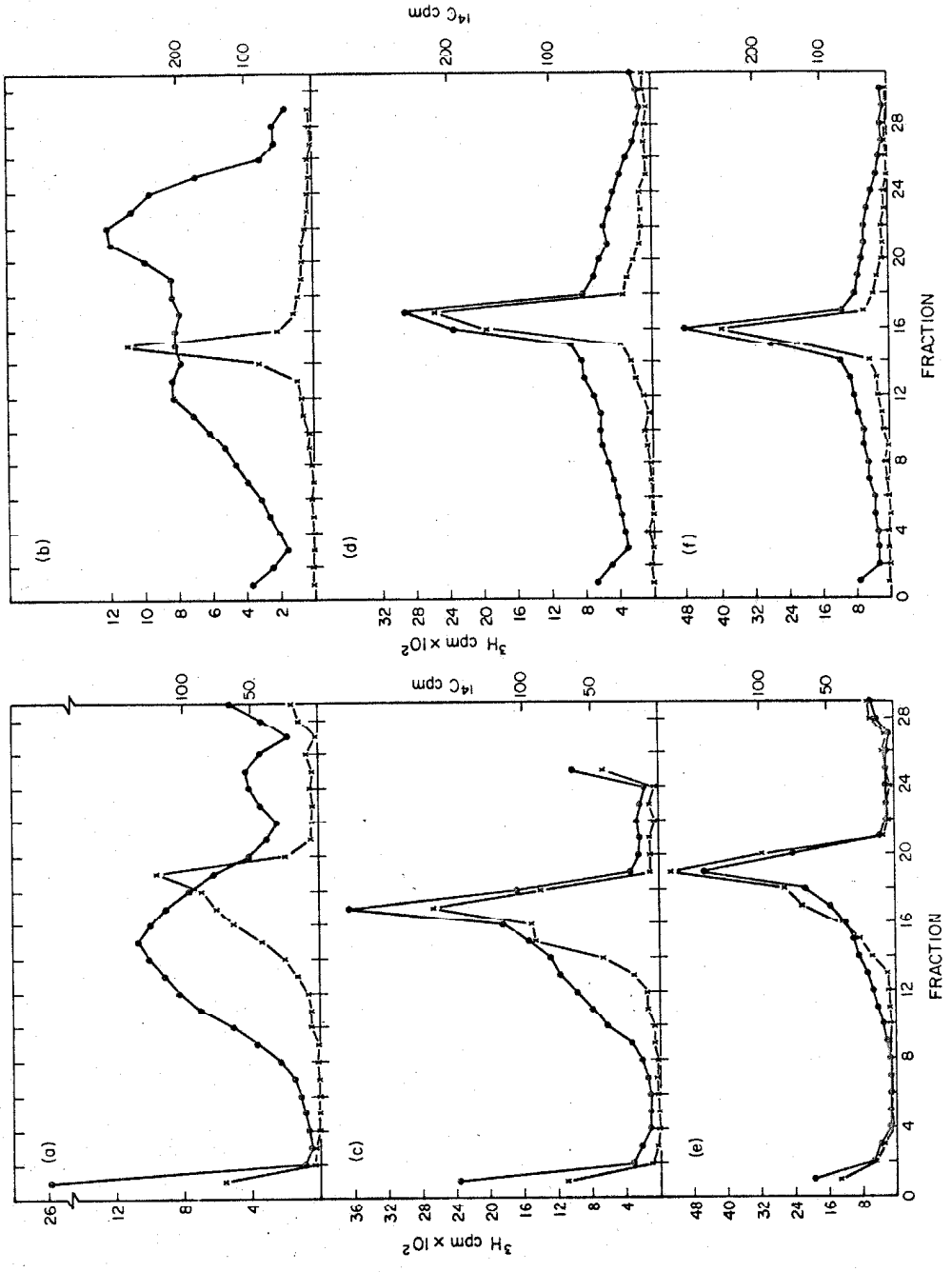


Figure 1.

FIGURE 2. Sedimentation of Pulse-Labeled and Chased Lambda DNA Extracted from Cells Starved Prior to the Pulse.

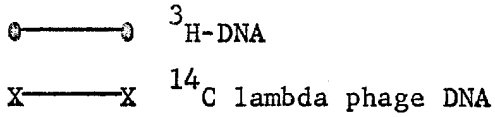
An induced lysogen of 159T$^-$(λ susRC$_{ts}$) is labeled for 15 seconds with ^{3}H-thymine at 45 minutes after induction subsequent to 5 minutes of thymine starvation as described for method 2 (Chapter 2). The ^{3}H-thymine is chased for 15 minutes with unlabeled thymidine. At the time of the pulse there were 1.2 PFU/cell. At the end of the chase there were 4.8 PFU/cell. DNA is extracted with phenol as previously described.

(a) Sedimentation of native ^{3}H-pulse-labeled DNA with a marker of ^{14}C-lambda susRC$_{ts}$ phage DNA consisting of linear phage DNA and annealed rings. Sedimentation is described in the legend to Figure 1a.

(b) Sedimentation of denatured ^{3}H-pulse-labeled DNA with a marker of ^{14}C-lambda phage DNA. Sedimentation is described in the legend to Figure 1b.

(c) Sedimentation of native ^{3}H-DNA extracted from cells chased for 15 minutes. Sedimentation is described in the legend to Figure 1a.

(d) Sedimentation of denatured ^{3}H-DNA extracted from cells chased for 15 minutes. Sedimentation is described in the legend to Figure 1b.



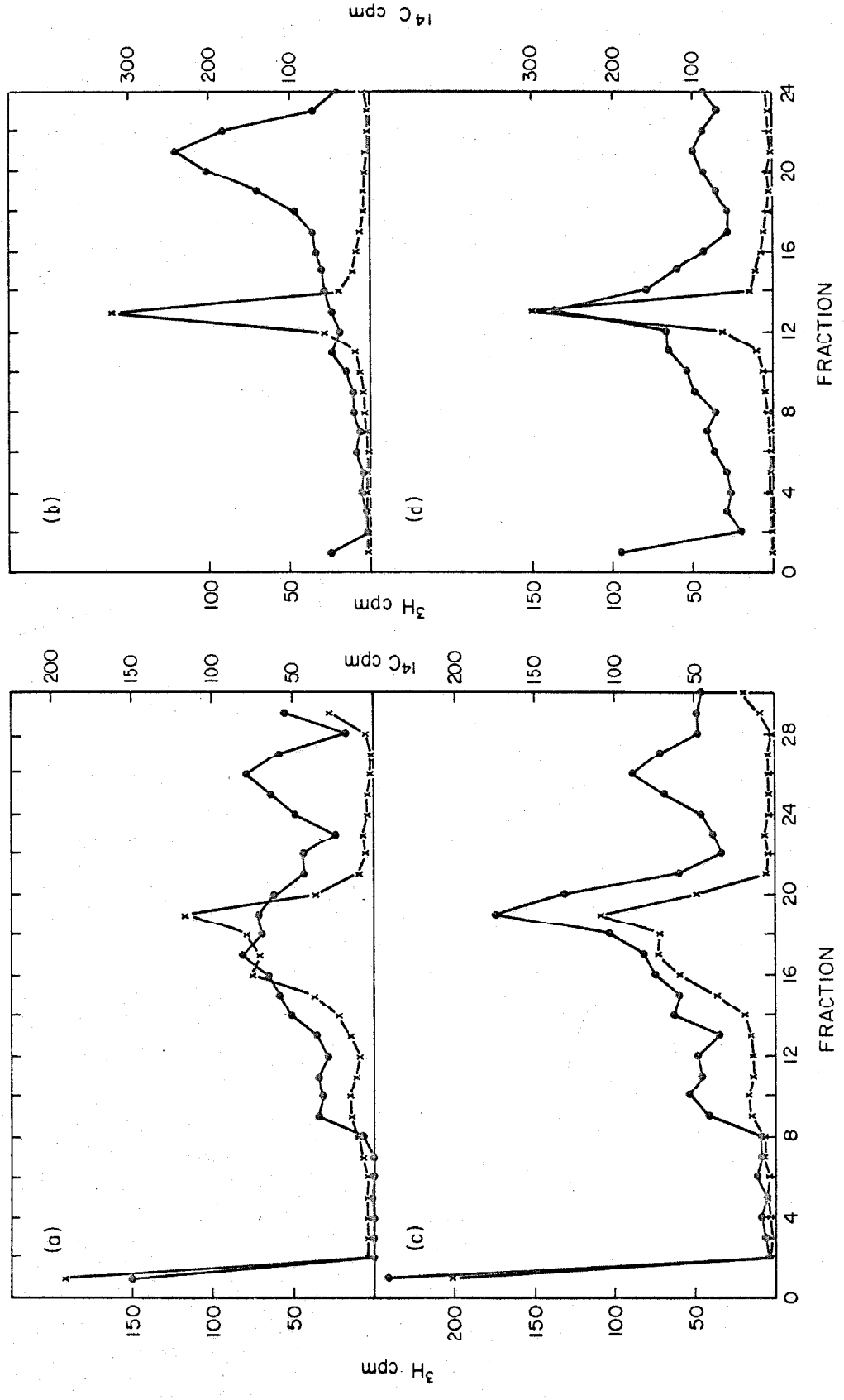


Figure 2.

FIGURE 3. Sedimentation of ^{3}H-Lambda DNA Labeled with Pulses of Various Duration and Extracted with Phenol.

An induced lysogen of 159T^{-} (λ susRC$_{ts}$) is labeled with ^{3}H-thymidine 45 minutes after induction as described in the text. At the time of labeling there were 5 PFU/cell. Final burst size for the culture was 32 PFU/cell.

Native ^{3}H-DNA is sedimented through 5%-20% sucrose gradients in TE. Sedimentation is from right to left in the presence of a ^{14}C-lambda susRC$_{ts}$ phage DNA marker consisting of linear phage DNA and annealed rings. Sedimentation is in a Spinco SW25.1 rotor at 25,000 rev/min, 6°C, for 5.5 hours.

(a) 10 second pulse

(c) 35 second pulse

(e) 120 second pulse

Denatured ^{3}H-DNA is sedimented through 5%-20% sucrose gradients in 0.1N NaOH in the presence of a ^{14}C-lambda phage DNA marker. Sedimentation is from right to left in a Spinco SW 41 rotor at 30,000 rev/min, 6°C, for 9 hours.

(b) 10 second pulse

(d) 35 second pulse

(f) 120 second pulse

●——● ^{3}H-DNA

X——X ^{14}C lambda phage DNA

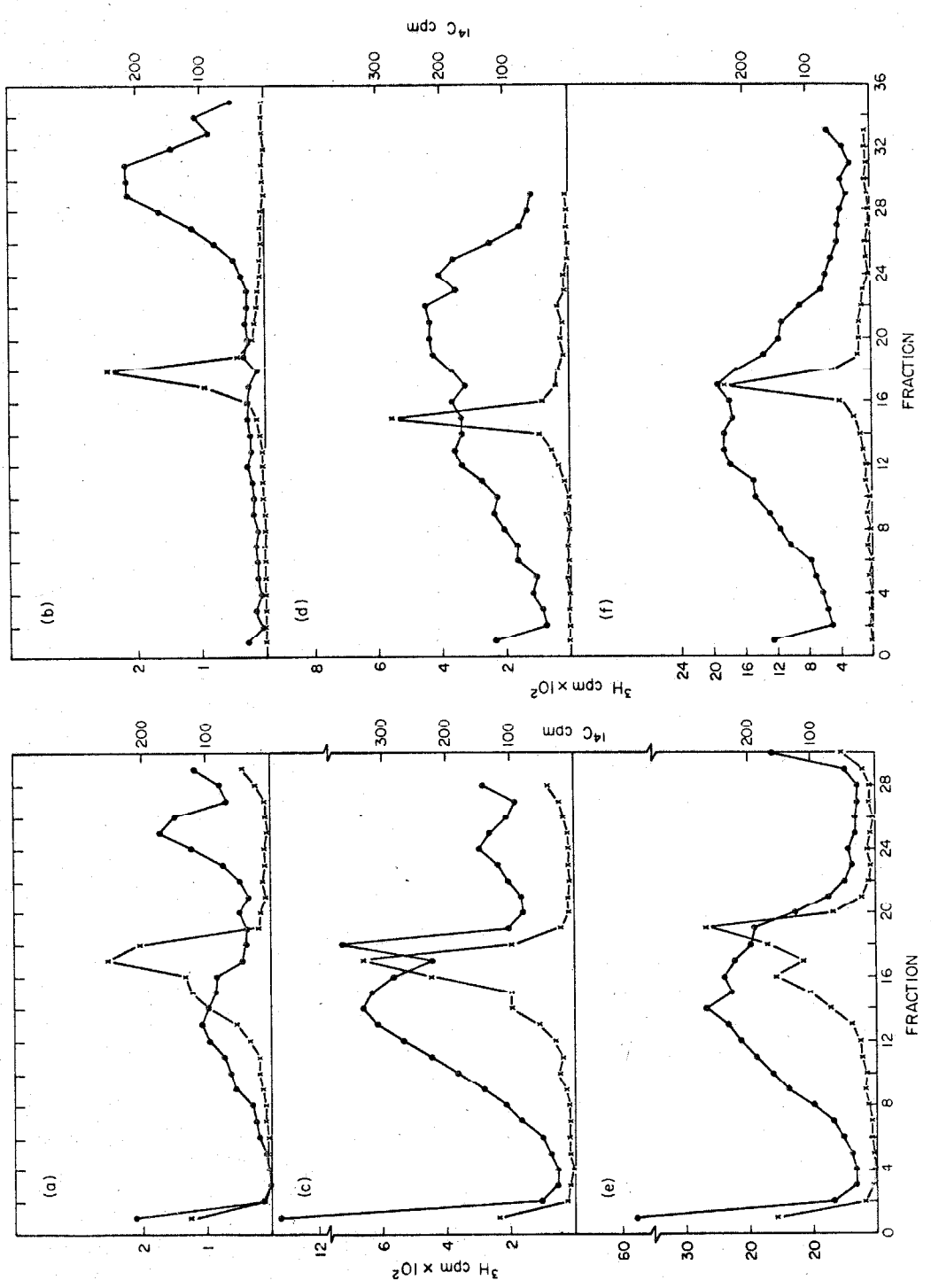


Figure 3.

FIGURE 4. Sedimentation of ^{3}H-Lambda DNA Labeled with Pulses of Various Duration and Digested with Pronase.

Native ^{3}H-DNA is sedimented through 5%-20% sucrose gradients in TE in the presence of a ^{14}C-lambda susRC_{ts} phage DNA marker consisting of linear DNA and annealed rings. Sedimentation is from right to left in a Spinco SW 25.1 rotor at 25,000 rev/min, 6°C for 6 hours.

(a) 10 second pulse

(b) 35 second pulse

(c) 120 second pulse

Denatured ^{3}H-DNA is sedimented through 5%-20% sucrose gradients in 0.1N NaOH in the presence of a ^{14}C-lambda phage DNA marker. Sedimentation is from right to left in a Spinco SW 41 rotor at 30,000 rev/min, 6°C for 9 hours.

(d) 10 second pulse

(e) 20 second pulse

(f) 35 second pulse

(g) 65 second pulse

(h) 120 second pulse

●——● ^{3}H-DNA

X——X ^{14}C lambda phage DNA

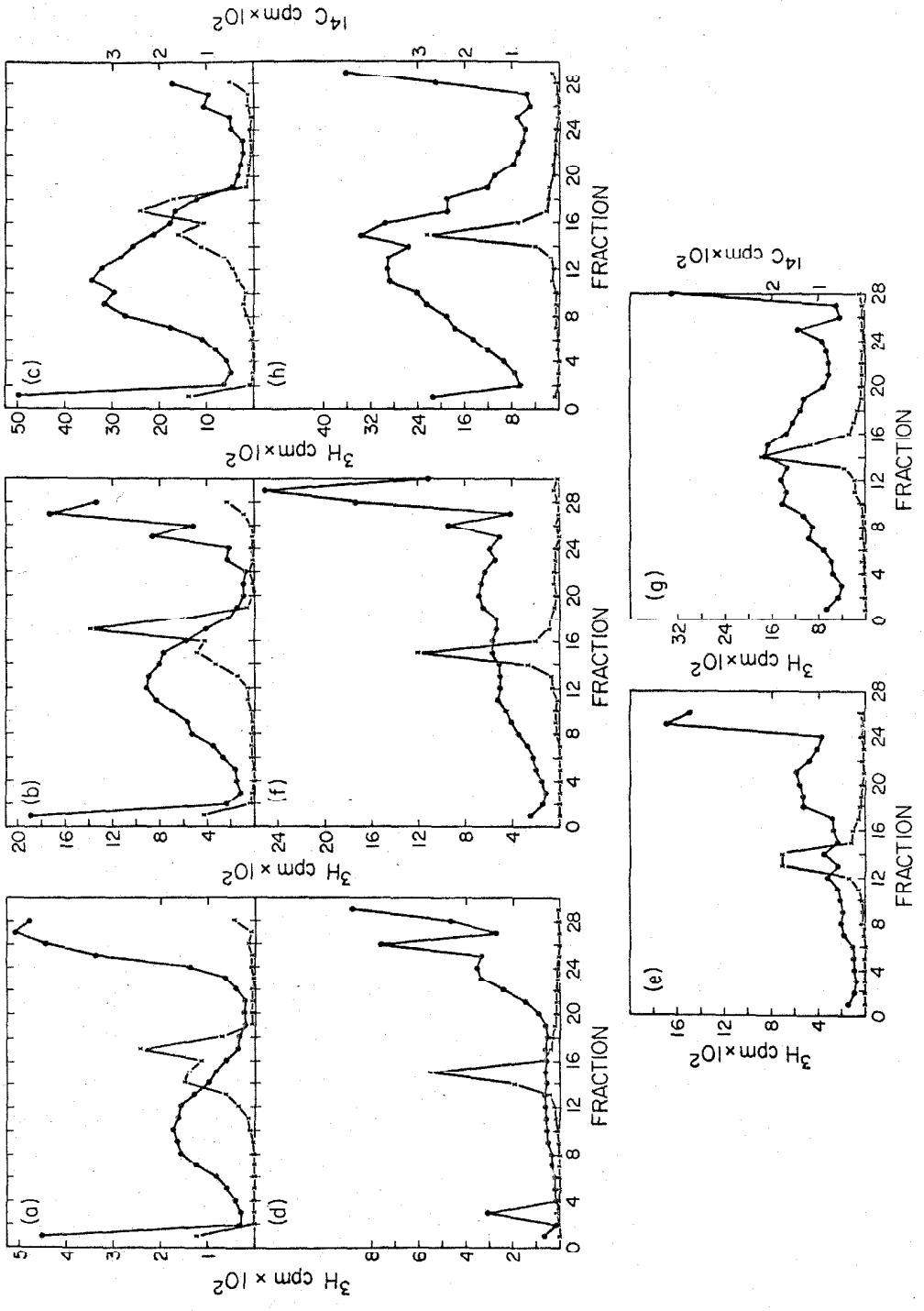


Figure 4.