STRUCTURE AND SYNTHESIS OF BACTERIOPHAGE LAMBDA DNA

Thesis by

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ABSTRACT

The research in this thesis represents an attempt to study the replication of bacteriophage lambda in terms of the structure of the vegetative DNA. A spheroplast assay for lambda DNA is described in Part I and several parameters affecting the efficiency of the assay are investigated. The biological activity of intracellular lambda DNA is then studied using this assay. In contrast to the results obtained with the transformation or helper assay, no eclipse of infectivity of vegetative lambda DNA occurs in the spheroplast assay. The DNA species responsible for most of the infectivity in the spheroplast assay of DNA extracted from infected, immune bacteria, is the fast-sedimenting "super-coiled" form of lambda DNA.

In Part II the structure and replication of the fast-sedimenting species of vegetative lambda DNA is examined physically and biologically. Its physical properties are those of a twisted, circular molecule each of whose two strands is closed upon itself. Using a mitomycin C technique to inhibit host DNA synthesis, the accumulation and semi-conservative replication of closed-circular lambda DNA specifically early in the infection is demonstrated with radio-isotopes. Biological analysis using the spheroplast assay verifies this conclusion. Chloramphenicol inhibits the synthesis of viral DNA at a lower concentration than that needed to inhibit the synthesis of closed-circular DNA. Chloramphenicol does not prevent the conversion from viral to closed-circular DNA.

The data presented in Part III suggest that open-circular lambda DNA may act as a precursor to both closed-circular and viral DNA. The closed-circular species is not a major precursor to viral DNA. Sedimentation analysis also reveals the existence of as yet unidentified intermediates in the replication of viral DNA.

Part IV describes several methods for purifying intracellular lambda DNA and examines the biological activity of the purified DNA in the spheroplast assay. The circular DNA is infective before and after denaturation, whereas denaturation inactivates viral DNA. The increased infectivity of open-circular DNA after denaturation (relative to the native state) appears to be due to the appearance of biologically-active single-stranded rings of lambda DNA. The closed-circular DNA usually has the same infectivity before and after denaturation.

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GENERAL INTRODUCTION

Lambda is a temperate bacterial virus which was discovered while

studying bacterial recombination by Escherichia coli strain K12 (48).

Temperate bacteriophage had been described earlier (see 1, 52 for reviews)

but none have since received the attention accorded λ . This distinc-

tion is undoubtedly due to its natural symbiotic relationship with a

host, E. coli K12, whose genetics and physiology are so well character-

ized. As a typical temperate phage, λ can enter either a reductive or a

productive life cycle after infection of a sensitive bacterium (52).

The former response results in lysogenization of the infected bacterium.

The surviving lysogenic bacterium harbors the DNA of the virus, or a

replica thereof, as a prophage, which is the reduced state of the viral

genome. The alternative life cycle is a productive one, in which the

infection produces a lytic rather than a lysogenic response. Superfic-

ially at least, this response is the same as that elicited by any of

the virulent bacteriophage: the phage enters the vegetative phase,

multiplies, forms mature particles, and eventually lyses the host.

This thesis studies almost exclusively the productive life cycle of λ ,

although the conclusions drawn shall not be so restrictive as the work

performed.

Lysogenization

Lysogenization is the process whereby the DNA of the infecting

phage, or a replica thereof, becomes a genetic element of the bacterium.

The first genetic studies of lysogeny by Lederberg & Lederberg (49)

demonstrated that the prophage, as the reduced state of the phage chromo-

some became known, behaves as a chromosomal, not a cytoplasmic agent.

It is now known, of course, that the DNA of the phage is responsible

for its perpetuation as a prophage and that it usually (in λ always)

attaches at a specific chromosomal locus (44). The attachment is thought

to be mediated by a "pairing" region on the phage genome (b2+) which,

if deleted, allows the phage to undergo only abortive lysogeny. During

abortive lysogeny the phage DNA is carried along as a non-replicating

cytoplasmic factor which becomes partitioned to only one daughter cell

at each division (46). In addition to the b2+ region, a functional

int (for integration) locus is apparently required for integration of

the prophage (81).

Lysogeny requires the proper function of at least three cistrons,

cI, cII, and cIII (39). The phenotype of these mutants is the production

of clear plaques as opposed to the normal turbid plaques produced by

wild type temperate phages. The absence of bacteria from the plaque

is due to the inability of the mutants to lysogenize. Evidence for

sequential action of these cistrons was first reported by Kaiser (39).

Complementation occurs when infection with cI precedes infection by

cII or cIII by 6 to 20 minutes. Complementation does not occur, however,

if infection is carried out in the reverse sequence. Thus it appears
that cI mutants are blocked at a late stage in lysogenization, and cII
and cIII at an early stage. These results are verified by more recent
evidence that only the cI+ cistron functions in a lysogenic bacterium
(2, 30). The cI gene product is a repressor which prevents autonomous
replication of the prophage or an homologous phage (32). The repressor
has recently been isolated by Ptashne and shown to be at least primarily
protein in nature (60). The function of the cII and cIII cistrons of
λ are unknown. The locus analogous to λcII on phage P22 (P22c1), how-
ever, is responsible for repressing phage and host DNA synthesis early
in lysogenization by P22 (67).

Campbell (8) proposed that lysogenization is accomplished by a
recombination event between the chromosome of the bacteria and the
circularized genome of the phage. This would explain the observation
that the order of genes of the prophage is a circular permutation of
the gene order present in the vegetative pool (6). This mechanism would
produce a linearly inserted prophage. However, Jacob and Wollman (35)
proposed, on the basis of the distance between closely-linked bacterial
loci spanning the prophage region, that the presence of the phage chromo-
some did not increase the distance between the bacterial markers, and
hence the prophage must be "hooked" to the bacterial chromosome in an
unspecified manner, rather than being inserted into it. Recent experiments
using either transduction (64) or mapping of deletion mutants (19) has
provided unequivocal evidence for the linear insertion of the prophage
with a circularly-permuted gene order.

Initial experiments indicated that little, if any, of the prophage material originated from the infecting particle (68). However, this result has not been verified in recent, more direct experiments (B. Hoffman and I. Rubenstein, personal communication). The latter experiments suggest that phage DNA can be incorporated covalently into the chromosome of the bacterium as a once-replicated (semi-conserved) unit the length of the phage genome. The amount of semi-conserved phage material which becomes incorporated into the bacterial chromosome increases with increasing multiplicity of infection, suggesting that the genome copies used for lysogenization can originate either from infecting particles or from replication. Replication per se does not appear to be a prerequisite for lysogenization since certain conditional lethal mutants of λ, which are unable to synthesize DNA, are able to lysogenize the restrictive host (5). They do so efficiently, however, only at high multiplicities of infection, again suggesting that a large number of copies of the genome facilitate some phase of lysogenization. (Parenthetically, it should be noted that these mutants have not been shown to be absolutely blocked in DNA synthesis, eg by using density-labeled phage particles.)

Induction

The conversion from the reduced state of the phage genome to a productive life cycle is called induction and may occur spontaneously or be produced artificially (52). Spontaneous induction occurs with

a frequency of about 5×10^{-5} per division cycle per bacterium in λ

lysogens (76). Lwoff, Siminovitch, and Kjeldgaard discovered that

essentially all of the cells of a lysogenic culture could be induced

to produce phage by irradiation with ultraviolet light (53). Since

then a vast spectrum of agents have been shown to possess inducing

power. They have in common the ability to upset bacterial metabolism.

Those which affect the DNA metabolism have attracted the most interest.

Most of the latter are also carcinogens and/or mutagens, prompting

the once-popular idea that induction was a form of mutation on the

one hand, and on the other, that the lysogenic state is the microbial

counterpart of a primordial tumor in mammals (52). These forms of

induction are indirect in that they appear to act initially on bacterial

DNA metabolism rather than on the repressor which prevents autonomous

phage replication. Direct induction is possible by heat-inactivation

of a thermo-labile repressor made by some λ mutants (71, 50). Studies

of these mutants have revealed that the repressor may have a dual func-

tion: as a repressor under normal conditions and as an inducer after

it has been damaged (50, 21). Interpretations of the data without

invoking an inducer, however, are possible. In addition to a possible

phage inducer function, some bacterial enzyme is apparently necessary

for induction since certain bacterial mutants which are unable to re-

combine cannot be induced. They can be lysogenized, however (20). After

(or as) induction occurs, the prophage may be excised and converted

to a mature phage particle without replication (59).

Induction is usually manifest by killing the lysogen. However, certain defective prophages, which develop little if at all, are still capable of killing the host. Killing seems to be due to excision of the prophage, rather than some other event connected with induction since the defective prophages mentioned above do not kill the bacterium if they are induced while attached to a dispensable episome, but do if attached to the chromosome (L. Siminovitch, personal communication).

Phage structure, adsorption, and injection

The head of λ appears to be an isometric hexagon 550 ± 25 Å in diameter. The tail, which is often curved, is about 1500 A long. Empty phage heads often contain a core of proteinaceous material which is about 300 A in diameter (41). Kaiser has suggested that the core is a spool about which DNA could wind compactly and orderly within the phage head. The tails of phage ghosts contain a channel (not seen in phage containing DNA) through which the DNA presumably exits.

Lysates of λ contain in addition to normal phage particles, equal numbers of smaller "petit λ ", small, tail-less, DNA-less particles about 400 Å in diameter. They may also contain cores (45).

The tail of the phage has no visible fibers. Injection presumably occurs without a contractile mechanism since no contracted particles have been observed.

Adsorption to early log-phase E. coli is very poor (76). It can be enhanced by growing the bacteria to late log phase or stationary

phase and then starving the bacteria in Mg^{++}-H_2O (33). A better

method for enhancing adsorption, however, is to grow the bacteria with

maltose as the carbon source (60). The gene for maltose is near the

locus on the E. coli chromosome which specifies the λ receptor on the

cell wall (36).

Lambda will adsorb but not inject at 0 °C (3).

Viral DNA Structure

The hereditary material of bacteriophage λ can be isolated as a

single, linear, double-stranded DNA molecule with a molecular weight

of 31-33 x 10^6 daltons (10, 70). Biophysical and biochemical character-

ization of the DNA isolated from purified phage have revealed the fol-

lowing structure of the phage genome: single-stranded segments protrude

at each end of the molecule. These contain about 20 nucleotide residues

(Kaiser and Wu, personal communication) and the segments at opposite,

but not identical ends of the molecule are complementary and may cohere

to form intramolecular circular molecules (hereafter referred to as

hydrogen-bonded circles) or intermolecular linear dimers, trimers, and

higher aggregates (26, 27). The single-stranded segments are most

likely on opposite strands (69). The DNA may be broken by hydrodynamic

shear into two pieces of similar length (40, 28). The half-molecules

so produced may be separated on the basis of their different guanine

plus cytosine content by either CsCl equilibrium density gradient cen-

trifugation, Hg(II) binding and Cs_2SO_4 equilibrium density gradient

centrifugation, or column chromatography on methylated albumin-Kieselguhr (25, 75, 28). The more dense "left" half of the molecule is terminated by deoxyguanylic acid-5'-phosphate, the "right" less-dense half by deoxyadenylic acid-5'-phosphate (78). The individual poly-nucleotide strands (which are intact in the majority of the molecules (79, 73, 11) may be separated on the basis of their unique density in alkaline solutions of CsCl (13)). Separation occurs because the two strands differ in their content of guanine plus thymine, which bases bind Cs+ by displace-ment of a proton on a nitrogen atom (74). The two strands may also be separated after denaturation by annealing them with polyriboguanylic acid or polyriboIG (47). The heavy strand, defined by its buoyant density in alkaline CsCl, is terminated by deoxyguanylic acid-5'-phosphate, the light strand by deoxyadenylic acid-5'-phosphate (78). Hence, the heavy strand terminates in a 5'-phosphate in the "left" half of the molecule (vide ante), the light strand terminates in a 5'-phosphate in the "right" half.

It appears from this synopsis that the chemical and physical struc-ture of λDNA is more fully characterized than that of any other double-stranded DNA. This has prompted a number of investigators to study the transcription of the DNA in vitro and in vivo.

Transcription

In Vitro

Transcription in vitro by E. coli RNA polymerase occurs almost entirely from the right half of the intact DNA molecule, which can be

demonstrated by annealing the synthetic RNA with isolated right and left half-molecules. The same results obtain with either linear DNA or hydrogen-bonded circular DNA used as templates (Hurwitz, personal communication). The transcription is primarily from one strand, the heavy (pG-terminated) strand (72).

In Vivo

Transcription of λ DNA occurs in at least three modes. 1. λ messenger RNA is transcribed only from the cI cistron of a repressed prophage (30). 2. After infection or induction there is an initial early phase (I) lasting about 15-20 minutes which is defined by a slow rate of λ messenger RNA synthesis. 3. A late phase (II) follows, terminating with lysis, during which the rate of λ messenger RNA synthesis increases some four-fold over the earlier rate. During lysogenization only phase I occurs. Phase II is also specifically repressed if protein synthesis is inhibited by the addition of 25 μg/ml of chloramphenicol five minutes before infection. However, even phase I is inhibited by chloramphenicol when a λ lysogen is infected with a virulent mutant of λ (which is able to multiply in the lysogen) (66). Thus it seems that protein synthesis is required to allow transcription once repression has been established but de novo (chloramphenicol sensitive) protein synthesis is not required to initiate phase I transcription in the absence of repression.

The early and late phases of transcription can also be distinguished qualitatively, on the basis of the regions of the λ genome transcribed early and late. During the early phase RNA is transcribed preferentially from the right half of the molecule, later, both halves are transcribed.

The content of purines in the λ messenger RNA is high at both times, indicating that the copied strand is pyrimidine-rich (65) as is true for the transcribed strand of several other phage (ØX174, 23; λ ,22). If DNA or protein synthesis is blocked, transcription occurs from the early regions but not the late (37). Because the two strands of λ are separable (<u>vide ante</u>) it has been possible to demonstrate that most of the early messenger RNA is made from the cytosine-poor strand; the late messenger RNA is made primarily from the cytosine-rich strand (72). Therefore, if the transcribed strand is rich in pyrimidines, the early strand must be rich in <u>T</u> since it is <u>C</u>-poor. This agrees with the right half being the early half and its being light in a CsCl density gradient, <u>ie</u> A T- rich. Genetic analysis of λ DNA heterozygotes suggests that the cytosine-poor strand which serves as the template for early messenger RNA synthesis is the strand which is high in guanine plus thymine content (that is, it is the heavy strand in an alkaline CsCl density gradient (29).

Genetics

The hereditary material of λ is a single DNA molecule. This was proved by demonstrating that one molecule of the phage DNA could infect bacteria (42). The genetic experiments done much earlier indicated that all of the genes known at that time constituted one linkage group with a length of about 15 map units. That is, the maximum amount of recombination between markers on opposite ends of the chromosome was about 15%

(38, 77). This amount of recombination can be increased by irradiating either one or both phage parents prior to infection (34). More recently recombination values of 25-30% have been routinely obtained without irradiation by using more favorable growth conditions (R. Huskey and J. Parkinson, personal communication). The genetic map of λ , unlike that of T4, is linear, not circular.

The order of genes on the vegetative phage (determined by recombination experiments) was shown by Kaiser (40) and Radding and Kaiser (61) to be congruent with the order of genes on the DNA isolated from the phage. Very roughly, the distances separating the loci are also in agreement. Thus, the order of genes in the phage DNA is the same as that of the vegetative DNA, not the prophage.

Recombination in λ takes place primarily by breakage and reunion and not by a copy-choice mechanism (54). This is also evidenced by λ's ability to recombine in the absence of DNA synthesis (during thymine starvation, R. D'Ari and J. Weigle, personal communication). Another manifestation of λ's infrequent mating, which made the above studies possible, is the dispersal to progeny phage of intact, semi-conserved sub-units of phage DNA, as opposed to the extensive breakage and dispersal of parental DNA to progeny T-even phage (55).

Some of the phenomena shown by the virulent phages are lacking in λ: Temporal exclusion, the rejection of an allelic phage from participation in the vegetative pool if it infects after the initial phage, does not occur in λ (31). Mutual exclusion (exclusion between different phages in a simultaneous infection) usually occurs to λ's detriment.

Virulent phages such as T5(76) or T4(24) exclude λ from growth (or

maturation) even if λ has a head start. A more balanced mutual ex-

clusion may exist between λ and ØX174, both of which depend more exten-

sively, and are less disruptive to, the host than the virulent phages

(W. Salivar, personal communication).

The number of rounds of mating which each λ genome undergoes is also

much smaller than in the case of the virulent phage T4, being about

0.5-1 compared to 5-7 (38). Similarily, the number of genomes able

to participate (at least in recombination) in the vegetative pool in

infected cells (in normal growth conditions) is only about 5-8 compared

to at least 30 in a T4 infected bacterium (33, 16).

Physiological Genetics

Structural and functional differentiation of the DNA molecule is

also revealed by genetic and physiological analysis of conditional lethal

mutants of λ isolated by Campbell (7). These sus (suppressor-sensitive)

mutants are analogous to T4 amber mutants.

The mutants can be divided into two classes: 1. Those whose defects

prevent DNA synthesis (early mutants) and 2. Those which synthesize DNA

but are blocked at a later stage of phage development or lysis (late

mutants) (5, 37, 14). The early mutants appear to be unable to perform

any late functions thus far measured, although the inhibition is probably

not quantitative. All of the sus mutants are able to lysogenize, but the

early (and not the late) sus mutants do so as efficiently as wild type

only at high multiplicities (5).

Cistrons governing early functions are located in the right-half of the genome, the late cistrons are primarily in the left-half.[1] The one obvious exception is cistron R which is known to be the structural gene for λ endolysin (9), a late function, but which maps on the right end of the chromosome. However, the late and early functions would occur as two contiguous groups if the genome of the phage were circularized during vegetative growth.

The division of the genome into two regions, one concerned primarily with late functions and one with early functions, is similar to the results obtained with T4 (18). Also analogous is the requirement for DNA synthesis in order to obtain normal amounts of the products of the late cistrons. A defect in cistron Q allows DNA synthesis but prevents normal functioning by any of the late cistrons. As with sus mutants in cistrons N, O and P (which are early mutants) very little late messenger RNA is made after infection or induction of sus Q (37). Thus sus Q appears to be a "maturation defective" mutant (14).

The early DNA-negative mutants do make early messenger RNA and at least one early protein (the λ exonuclease) is made by all of the early mutants except sus N and is made by wild type in the absence of DNA synthesis. Also, some late protein, the endolysin, is made in the

[1]The left-half is defined as the more dense (higher guanine plus cytosine content) half, the right as the less dense half. The gene content of the isolated half-molecules is determined in the helper assay (vide infra).

absence of DNA synthesis although it is not made in normal quantities

(58). Hence, DNA synthesis is necessary but not sufficient to insure

normal late transcription. On the other hand, DNA synthesis is not

necessary to allow transcription of the early regions of the λ genome.

Campbell isolated sus mutants representing 18 cistrons (A - R).

In addition to these there are the three classical clear-plaque mutants

of λ, cI, cII, and cIII, which control the establishment and maintenance

of lysogeny (these are in the early region but synthesize normal amounts

of DNA). Several other cistrons have recently been identified (L.

Siminovitch, personal communication, and 17), bringing the total number

of identified cistrons to 25-30. With a molecular weight of 30 mega-

daltons, and assuming a coding ratio of three nucleotides per amino

acid and an average λ protein size of 300 amino acids, λ could contain

60 cistrons. However, portions of the DNA amounting to about 25% of the

genome can be deleted and the only noticeable loss is an inability to

become integrated as a prophage. Thus 45 essential cistrons might be

a better estimate of the number required for the normal life cycle.

About half this number have been identified, indicating perhaps a 50%

saturation of the genetic map.

Although a large number of the λ cistrons have been identified, the

specific functions of very few are known. The structural gene for only

two proteins, endolysin and the λ repressor, are known with any certainty;

R is the structural gene for endolysin (9) and cI is the structural

gene for the prophage repressor (60). It seems very likely that gene

J determines the antigenic protein of the tail. Gene E may be the

structural gene for the main protein component of λ capsids, since no head structures are visible in electron micrographs of extracts made from induced, non-permissive lysogens for sus E (L. Siminovitch, personal communication). Besides the structural components of the phage particle (which have not been studied) and the endolysin and repressor, two other phage-specific proteins are known: the λ-exonuclease, a thoroughly-characterized early enzyme (51) whose structural gene is between N and J (63) and β-protein, which is another λ antigen regulated in parallel with the exonuclease (62).

It is tempting to hypothesize, as others have done (14), that gene Q, which controls late functions, codes for a λ-specific RNA polymerase. Thus the early region of the genome would be transcribed by the E. coli RNA polymerase and the late region by the phage enzyme. Chloramphenicol would prevent synthesis of the latter and would thereby prevent late function, as would defects in gene Q. This is consistent with the ability of E. coli RNA polymerase to transcribe only the early region from phage DNA in vitro. The requirement for DNA synthesis to obtain normal late function, however, is not explained by this simple scheme.

From this brief summary it seems safe to conclude that the λ genome will not be the first ".... independent bit of genetic material about which we can say -- we know and we understand." (Zinder, discussing the RNA phages, 80).

Biological Activity of λ DNA

The viral DNA[2] is infective in the transformation assay developed
by Kaiser and Hogness (42), hereafter referred to as the helper assay.
In this assay bacteria are preinfected with a helper phage and then
with DNA of a genotype which can be distinguished from that of the
helper phage. It is now apparent that to be infective in this assay
the DNA molecule must possess at least one free single-stranded end
(69, 43). However, to be infective the molecule need not be intact
since half molecules (40, 61, 28) and even terminal (but not internal)
sixth molecules (43) are able to donate markers to progeny phage, pre-
sumably by recombination with the genome of the helper phage.

Lambda phage DNA is also able to infect spheroplasts, as first
reported by Meyer, Mackal, Tao and Evans (56; see also Brody et al.,
4, and Dityatkin, 12). Osmotically shocked E. coli can also be infected
with λ DNA (57).

Dove and Weigle (15) showed that after infection of a sensitive
or lysogenic host with λ , the viral DNA which can be extracted from the
bacteria contains relatively little of the original infectivity, measured

[2]Mature or viral DNA is defined as λ DNA which is indistinguishable
physically or biologically (by the criteria presently available) from the
DNA extracted from purified phage, regardless of the milieu from which
it was obtained eg. infected bacteria. It will be understood that mature
implies the presence of free, single-stranded ends.

in the helper assay. Infective DNA is produced by a chloramphenicol-
sensitive "maturation" reaction during a sensitive infection at about
the same time that mature phage begin to appear in the cell, considerably
later than the onset of phage DNA synthesis.

The first section of Part I is a short note which was published
as a letter to the editor of the Journal of Molecular Biology and is
reprinted here with the permission of Academic Press. Preliminary
evidence is given that the structural basis for the loss of infectivity
after infection is circularization of λ DNA. In the second section
a spheroplast assay for λ DNA is described and the results obtained
using this assay to study the biological activity of λ DNA extracted from
phage or infected bacteria are presented. When the spheroplast assay
is used to measure the biological activity of injected λ DNA, there
is no eclipse of infectivity. Moreover, at least part of the newly
synthesized vegetative DNA which is noninfectious in the helper assay
is able to infect spheroplasts. The DNA species which is responsible
for most of the infectivity in the spheroplast assay after infection
of an immune lysogen (in which no phage DNA multiplication occurs) is
the fast sedimenting form of λ DNA described by Bode and Kaiser (3).
Further properties of this component are described in Parts II, III,
and IV.

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Part I

VEGETATIVE LAMBDA DNA:

INFECTIVITY IN A SPHEROPLAST ASSAY

Novel Intra-cellular Forms of Lambda DNA

The DNA complement of bacteriophage lambda is infective if certain conditions of bacteria and "helper" phage are satisfied (Kaiser & Hogness, 1960). However, after infection of sensitive bacteria by lambda phage, most of the injected DNA appears to lose its infectivity, at least as measured under these conditions. Little infective DNA can be recovered from the infected cell until mature phage appear or just prior to this time (Dove & Weigle, personal communication). The knowledge that the replicating form of the single-stranded DNA of ϕX174 is a circular duplex DNA molecule (Kleinschmidt, Burton & Sinsheimer, 1963; Chandler, Hayashi, Hayashi & Spiegelman, 1964) together with indications that the notched circular form of lambda DNA prepared *in vitro* (Hershey, Burgi & Ingraham, 1963) (the expression "notched circular form" is proposed to describe a double-stranded ring DNA in which each strand has one or more breaks) is much less infective than the linear form (our work and personal communication from Kaiser) prompted us to look for such a component in cells infected with bacteriophage lambda.

Centrifugation at a pH which denatures the DNA enhances the difference in rate of sedimentation between linear DNA molecules and *closed* circular DNA duplexes (the expression "closed circular DNA duplex" is proposed to describe a double-stranded ring DNA in which there are no single-strand breaks). The latter retain their original molecular weight but lose their highly ordered secondary structure at pH 12, forming a species sedimenting much faster than the denatured linear strands (Weil & Vinograd, 1963; Burton & Sinsheimer, 1964 *Abst. 8th Biophys. Soc. Meeting* WA2). The *notched* circular form of lambda DNA gives rise to denatured linear strands in alkali. Our experiments however indicate the presence, in sensitive cells infected with bacteriophage lambda, of a DNA component with the alkaline sedimentation properties to be expected of a double-stranded, *closed* circular molecule.

CR34, a lambda-sensitive, thymidine-requiring strain of *Escherichia coli* was grown to 2 to 3 $\times 10^8$ cells/ml., collected, and washed with adsorption buffer (0·01 M-tris–HCl, pH 7·3, 0·01 M-MgSO$_4$) on a DA Millipore filter (Millipore Filter Co., Bedford, Mass.). The washed bacteria were resuspended in one-third volume of adsorption buffer and aerated 60 minutes at 30°C. They were then irradiated with twice the dose of ultraviolet light required to induce a strain of *E. coli* lysogenic for lambda. In our experiments this dose of ultraviolet light prevents synthesis of bacterial DNA in an uninfected cell for at least 60 minutes. The bacteria were then infected in adsorption buffer with lambda b2b5c at a multiplicity of five and adsorption allowed to continue 15 minutes at 30°C. After adsorption they were filtered and resuspended in one volume of prewarmed (30°C) media containing tritiated thymidine. Sixty minutes later the cells were harvested, disrupted by sodium dodecyl sulphate, and the nucleic acid extracted with phenol. The distributions of radioactivity and infectivity were analyzed on an alkaline sucrose density-gradient (5 to 20% sucrose, 0·02 M-Na$_3$PO$_4$, pH 12·1, 6°C). The results, shown in Fig. 1, indicate the presence of three centrifugal components.
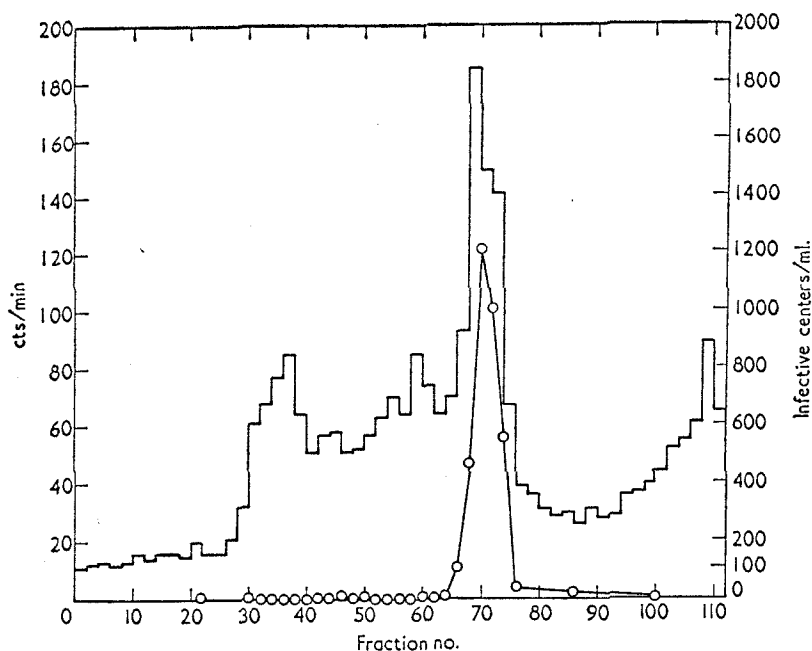
Fig. 1. Distribution of ^{3}H (————) and infectivity (—O—O—) after alkaline sucrose gradient sedimentation of the intracellular DNA extracted 60 min after infection with lambda b2b5c at a multiplicity of 5. The cells were irradiated with ultraviolet light prior to infection. A 5 to 20% w/v sucrose gradient containing 0·02 M-Na$_3$PO$_4$, pH 12·1, was used. The sample was denatured prior to centrifugation by 10 min incubation with 0·1 M-Na$_3$PO$_4$, pH 12·4, 25°C. Centrifugation was for 3 hr at 38,000 rev./min at 6°C in a Spinco SW39 rotor.

The slow peak has the same sedimentation coefficient as DNA prepared from purified lambda phage b2b5c, measured at this pH and ionic strength, either in the analytical ultracentrifuge or in a preparative sucrose gradient. The values of $S_{20,w}$ calculated by the procedure of Martin & Ames (1961) for the three components are 25, 32 and 46 s. Three similar components of S values 14 (single-strand ring), 23 and 33 s have been observed during ϕX infection.

The results of a similar experiment using, however, ^{32}P-labeled phage at a multiplicity of 3·5 are shown in Fig. 2. The treatment with ultraviolet light and the addition of tritiated thymidine were omitted. The DNA was extracted after 30 minutes at 30°C (the minimum eclipse period of the intracellular phage was 40 minutes) and analyzed on an alkaline sucrose gradient. The distribution of radioactivity in the gradient shows two of the same components as seen when the progeny DNA is labeled (Fig. 1); the peak of 32 s material is absent and apparently does not contain parental DNA.

These experiments suggest that the most rapidly sedimenting component arises from the non-infective, intracellular form of lambda DNA and is probably a circular structure. Its alkaline stability distinguishes it from the circular form prepared in vitro (Hershey et al., 1963).

The infectivity associated with the slow peak (see Fig. 1) in alkaline sucrose sedimentation is being investigated to determine whether it is a property of the denatured single polynucleotide strands or is a consequence of renaturation which occurred after centrifugation. We also find infectivity associated with the peak of
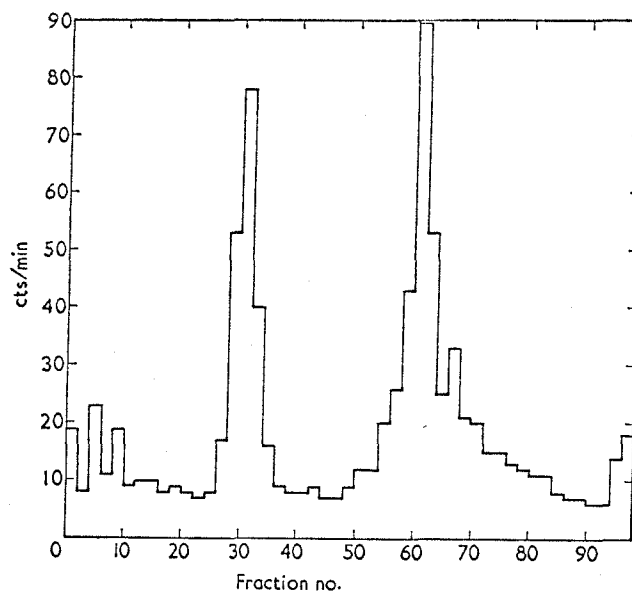
FIG. 2. Distribution of ^{32}P after alkaline sucrose gradient sedimentation of the intracellular DNA extracted 30 min after infection with ^{32}P-labelled lambda b2b5c at a multiplicity of 3·5. The minimum eclipse period of the intracellular phage was 40 min. Preparation of the sample and centrifugation were performed as in Fig. 1.

lambda DNA prepared from purified phage after a similar sedimentation in alkaline sucrose (however, for reports on the non-infectivity of denatured lambda DNA see Kaiser & Hogness, 1960, and Brody, Coleman, Mackal, Werninghaus & Evans, 1964).

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1. SUMMARY

The conditions for infection of spheroplasts of several strains of Escherichia coli by λDNA have been studied. An assay has been developed and used to compare the ability of λDNA, at various stages of infection, to infect either spheroplasts or helper-phage-infected whole bacteria.

In contrast to the results obtained with the helper assay, λ DNA extracted from an immune lysogen after superinfection is able to infect spheroplasts. The component from the infected cell which is responsible for most of the infectivity in the spheroplast assay is the twisted, circular form of λDNA. This component is noninfective in the helper assay.

Similarly, after infection of a sensitive bacterium by λ, there is no loss of DNA infectivity, measured in the spheroplast assay, although the ability of the extracted DNA to infect helper-infected bacteria decreases 85%. During infection, synthesis of λDNA which is able to infect spheroplasts precedes the synthesis of λ DNA which is able to infect helper-infected bacteria by at least 20 minutes at 30°C. The conclusion suggested by these and experiments presented in the following paper is that synthesis of the twisted, circular form of λDNA occurs until the time of phage maturation, at which time linear phage DNA, containing cohesive ends, is synthesized and encapsulated.

The "notched" circular form of λDNA, prepared in vitro, is also able to infect spheroplasts, although it has little infectivity in the helper assay.

2. MATERIALS AND METHODS

(a) Media and solutions

All solutions are made with distilled-deionized H_2O.

TBM broth contains 10 g Bacto Tryptone (Difco), 5 g KCl, and 2.4 g maltose per liter.

2XM9 medium contains 14 g Na_2HPO_4, 6 g KH_2PO_4, 2 g NH_4Cl, 10 ml. $1\underline{M}$ $MgSO_4$, 4 ml. 25% NaCl, and 40 ml. 10% glucose per liter. The glucose and $MgSO_4$ are added sterilely after autoclaving.

K medium contains 500 ml. of a 3% solution of vitamin-free Casamino acids (Difco) and 500 ml. of 2XM9. The two solutions are autoclaved separately and mixed after cooling to room temperature.

TPA medium (low phosphate) is described by Kelly, Gould and Sinsheimer (13).

Bottom agar contains 10 g Bacto Tryptone, 2.5 g KCl, 2.5 g NaCl, and 10 g Bacto agar per liter.

Top agar contains 10 g Bacto Tryptone, 2.5 g KCl, 2.5 g NaCl, and 8 g Bacto agar per liter.

The media (3XD, PA, and PAM) used in the spheroplast assay are the same as those described by Guthrie and Sinsheimer (8) except that PA and PAM contain only 1 g per liter of glucose.

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

λ dil is $0.01\underline{M}$ KPO_4, pH 7.0, $0.01\underline{M}$ $MgSO_4$, and 10 g/ml. of bovine plasma albumin (11).

$\underline{TM}$ is 0.01$\underline{M}$ tris, pH 7.2, 0.01$\underline{M}$ MgSO$_4$. Bacteriophage stocks are stored and diluted into TM.

$\underline{TE}$ is 0.01$\underline{M}$ tris, 0.001$\underline{M}$ EDTA, pH 8.1.

$\underline{EDTA}$ solutions are made with the disodium salt of ethylenediamine-tetraacetic acid (Sigma Chemical Company).

$\underline{Lysozyme}$ solutions of two grades are used: Grade 1 (Sigma Chemical Co., 32,000 units/mg) is used at 10 mg/ml. for DNA extractions; B grade (Calbiochem, 8,000 units/mg) is used at 2 mg/ml. to prepare spheroplasts for infection with DNA. They are dissolved in 0.25$\underline{M}$ tris, pH 8.1.

(b) $\underline{E.\ coli\ K12\ strains}$

$\underline{C600}$ (1) and its lysogenic derivatives are used as plating bacteria. C600 is used as the recipient for the helper DNA infectivity assay.

$\underline{CR34}$ is a thymidine-requiring derivative of C600 (17).

$\underline{W3110}$ and $\underline{W3110(\lambda)}$ are prototrophs.

$\underline{12/\lambda}$ is a λ-resistant, gal_1^-, gal_2^- derivative of 3101.

These strains were obtained from Dr. Jean Weigle.

$\underline{1100}$ and $\underline{1486-23}$ were obtained from Dr. H. Hoffmann-Berling. Their endonuclease I activity (14) is only a few per cent of that observed in the strain from which they were derived. Mutants resistant to λ were isolated by plating 10^7 bacteria with 10^6 λvir and testing the resultant colonies for their ability to plate λ.

AB2500 was obtained from Dr. Paul Howard-Flanders and is described

in Howard-Flanders, Boyce and Theriot (10).

(c) λ strains

All of the phage strains were originally obtained from Dr. Jean

Weigle.

Nonradioactive phage stocks are prepared as described previously (21).

Phage containing ^{3}H-thymidine are obtained either by inducing

CR34 with UV light or by infecting CR34 with the desired clear plaque

mutant. In either case the bacteria are first grown to 4 x 10^8 cells/ml.

in K medium supplemented with 10 μg/ml. ^{1}H-thymidine, harvested by cen-

trifugation and resuspended in 1/10 volume TM. They are then either

irradiated, in the case of CR34(λ) or infected with 5 pfu/cell. After

15 minutes at 37$^°$ to allow adsorption, the bacteria are diluted into

9 volumes of fresh, 37°C K medium containing 10 μg/ml. ^{1}H-thymidine

and sufficient ^{3}H-thymidine (New England Nuclear Corporation) to provide

a final specific activity of 0.12 c/mmole thymidine. After 80 minutes

growth (150 minutes for induction), $CHCl_3$ is added to complete lysis.

The phage are purified by differential centrifugation and CsCl density

gradient equilibrium centrifugation. The yield after lysis is 3-5 x 10^{10}

pfu/ml. and the stock contains approximately 3 x 10^{-6} cts/min/phage

particle measured on our scintillation counter which has a ^{3}H efficiency

of about 25% and based on the absorbance at 260 mμ. After purification

30-60% of the particles are able to form plaques.

Phage containing radiophosphorous are prepared by infecting 3110 which has been grown in TPA and infected as described above. 1 mc of carrier-free radiophosphorus (Nuclear Consultants, Inc.) is added per 40 ml. of TPA medium. The phage yield is 4-8 x 10^{10} pfu/ml. The virus is purified as described above. The stock contains approximately 6 x 10^{-6} cts/min/phage particle. At this specific activity (1.0 c/13.5 mmoles ^{31}P), there is 1 ^{32}P atom/2 phage particles.

(d) Preparation of nucleic acids

Phage DNA is extracted as described by Young and Sinsheimer (21) except that after addition of the phenol the tube is rolled horizontally around its long axis for 15 minutes at a rate of about 60 rev./min to produce an emulsion (5). The linear and circular forms of λDNA are prepared according to the method of Hershey, Burgi and Ingraham (9).

Bacterial nucleic acids are extracted by a procedure similar to that of Bode and Kaiser (2). The bacteria are converted to spheroplasts and lysed with Sarcosyl NL97 (Geigy Chemicals, Saw Mill River Road, Ardsley, New York). The use of Sarcosyl was suggested to us by Dr. Mervyn Smith. It is more soluble than sodium dodecyl sulfate in aqueous solutions at low temperatures and in concentrated salt solutions (such as CsCl). 2-4 x 10^9 bacteria are washed with cold TE by centrifugation or by filtration on DA Millipore filters (Millipore Filter Corp., Bedford, Massachusetts) and resuspended in 0.4 ml. 1.0 M sucrose in TE. 0.20 ml. grade 1 lysozyme, 10 mg/ml., and 0.20 ml. 0.2M EDTA, pH 8.1,

are added and the suspension is incubated for 20 minutes at $0°$. 1.2 ml.

of cold 0.75% Sarcosyl in TE is added with gentle stirring and the

mixture is allowed to sit at $0°$ for at least 60 minutes. During this

time the solution becomes transparent. 2.0 ml. of phenol (redistilled

and kept frozen until just prior to use) saturated with $0.1\underline{M}$ tris, pH

9.0, $0.1\underline{M}$ NaCl, $0.01\underline{M}$ EDTA are added and the mixture is warmed to $37°$.

An emulsion is produced by rolling as described above, and then the

phases are separated by centrifugation. The extraction is repeated

once. The combined phenol layers and interfacial material are extrac-

ted with 1.0 ml. of buffer. The combined aqueous phases are dialyzed

against TE to remove the phenol and Sarcosyl.

If the DNA is desired free of RNA, the procedure of Marmur (16)

consisting of RNase digestion and selective precipitation of the DNA

is used.

E. coli RNA essentially free of DNA is isolated by the Godson (6)

procedure. Residual DNA is removed by treatment with pancreatic deoxy-

ribonuclease.

The recovery of infecting phage DNA is usually between 60 and 100%.

Recovery of radioactive phage DNA from superinfected cells is consis-

tently higher than recovery from infected sensitive cells. In one exper-

iment reported here, SDS at $60°$ was used to lyse the cells. In this case

the recovery of radioactive phage DNA was only about 45%.

(e) <u>Plaque assay for λ phage</u>

Plating bacteria are grown to saturation in TBM, centrifuged, resuspended in TM, and aerated for an hour at 37°. Phage particles, usually in 0.1 ml. TM, are adsorbed to 0.30 ml. of plating bacteria for 15 minutes at 37°. Top agar is added and the infective centers are poured onto plates. This procedure assures uniform plaque morphology.

(f) <u>Intracellular phage assay</u>

0.10 ml. of the infected culture is mixed with 1.65 ml. of cold 0.05$\underline{M}$ tris, pH 8.1, containing 200 μg lysozyme, and 0.15 ml. 4% Na_2EDTA is added. The tube is kept cold and at the end of the experiment 0.1 ml. of 0.1$\underline{M}$ $MgSO_4$ is added to stabilize the phage. Phage are assayed after freezing and thawing once.

(g) <u>Helper infectivity assay of λDNA</u>

Recipient bacteria (C600) are prepared, frozen until desired, and infected with DNA with only trivial deviations from the procedure given by Bode & Kaiser (2) and Kaiser & Inman (12).

(h) <u>Spheroplast infectivity assay of λDNA</u>

Bacteria are grown with aeration at 37°C in 20 ml. of 3XD to an absorbence at 600 mμ of 0.4 (about 3 x 10^8 cells/ml.). After centrifuging for 5 minutes at 5,000 x g at room temperature, the pellet is resuspended in 0.7ml of 1.5$\underline{M}$ sucrose. The following solutions are added in order with gentle mixing: 0.34 ml. of a 30% sterile solution of bovine albumin (Armour Pharmaceutical Co., Kankakee, Ill.), 0.10 ml. of 2 mg/ml. grade B lysozyme, 0.08 4% EDTA, and 10 ml. of PA medium.

The suspension is incubated 15 minutes at 25 °C and 0.40 ml. of 1.0$\underline{M}$

$MgSO_4$ is added. This is the spheroplast stock. More than 95% of the

bacteria are converted to spheroplasts as judged by the phase microscope.

The stock should be used immediately after preparation as a decline in

competence is sometimes observed after an hour at 0°.

For infection, 0.30 ml. of spheroplast stock is added to 1.20 ml.

of a DNA solution in 0.05$\underline{M}$ tris, pH 8.1, and incubated 15 minutes at

30°. 1.50 ml. of PAM is then added and the suspension is incubated at

30° without aeration for 6 hours. Mature phage are measured after shak-

ing the solution with $CHCl_3$ and freezing and thawing.

Infective centers can be measured as described by Guthrie and

Sinsheimer (8). However the efficiency of plating the infected sphero-

plasts appears to be low and erratic. In some experiments no infective

centers are recovered by this procedure until free phage appear. In

one step growth experiments in spheroplasts the burst size determined

from the total phage and the number of infective centers measured im-

mediately after infection are 10-20 times higher than the burst size

determined in a single-burst experiment (see Results, sections (a,v)).

In order to obtain an estimate of the number of biologically

active DNA molecules in a sample, it is essential to assay several

dilutions, preferably in duplicate or triplicate, and compare this curve

to the curve obtained with known concentrations of phage DNA.

Only 5-10% of the added viral DNA (measured by radioactivity) will

sediment with the spheroplasts after adsorption. However, the infec-

tivity of the DNA remaining in the supernatant after centrifugation

of the infected spheroplasts is only 10-15% of the infectivity initially

present. This is observed with spheroplasts of either 1486-23/λ or

$\overline{12}$/λ.

(i) Sucrose density gradient centrifugation

Preparative zone centrifugations are performed in the SW 25.1 rotor

of the Spinco L1 centrifuge at 25,000 rev./min and 6°. Three 25 ml.

sucrose gradients are poured and collected simultaneously using a

Technicon peristaltic pump (15). Approximately 30 0.8 ml. fractions

are collected by a time-actuated fraction collector. The sucrose

solutions are in TE. Recrystallized sucrose (18), Mann sucrose density

grade (Mann Research Laboratories, Inc., New York, N.Y.) and Analar

sucrose (Gallard Schlesinger Chem. Co., Carle Place, N.Y.) have been

used. Unautoclaved 20% solutions do not inactivate λDNA during 5 hours

at 37°. The gradients deviate slightly from linearity in the upper

and lower 10% of the column.

(j) Radioactivity assays

Samples containing radiophosphorus are counted either on planchets

in a Nuclear Chicago low background counter or in a scintillation counter

(Nuclear Chicago 720 or Beckman). Samples containing ^{3}H are precip-

itated with an equal volume of cold 10% trichloroacetic acid and 50 μg

of denatured calf thymus DNA as carrier. The precipitate is collected

after 30 minutes at 0° by filtering through a Whatman glass filter

(2.4 cm GF/A) and washing with ice-cold H_2O. After drying in a glass

vial, 10 ml. of toluene containing Liquiflour (New England Nuclear

Corp.) is added and the sample is counted in the scintillation counter.

The counting efficiency is determined from a sample containing precip-

itated DNA and a known amount of ^3H-thymidine on the filter.

3. RESULTS

(a) Some characteristics of the spheroplast assay

(i) Effect of bacterial strain, age of bacterial culture, concentration

of spheroplasts, and ionic strength in the adsorption tube

E. coli C600λ, CR34/λ, $\overline{12}$/λ, and later 1486-23/λ and 1100/λ were

all used as hosts in the spheroplast assay. Spheroplast preparations

of strains not resistant to λ are still able to adsorb some of the phage

produced in the infection and hence can not be used in the assay. $\overline{12}$/λ

and 1486-23/λ are the most efficient hosts (efficiency is defined as the

number of phage produced per DNA molecule). The latter lacks endo-

nuclease I and in most experiments is more efficient than $\overline{12}$/λ (see

section vi).

The age of a bacterial culture at the time it is converted to

spheroplasts (after concentration to the same number of cells/ml.) has

no significant effect on the efficiency of the DNA assay. Increasing

the number of spheroplasts in the incubation tube by concentration prior

to infection does not increase the efficiency of the present assay.

The effect of various concentrations of NaCl in the incubation
tube is similar to that found in the ØX174 DNA spheroplast assay (8).
NaCl concentrations become inhibitory in the range 0.1-0.5$\underline{M}$.

(ii) <u>Dilution</u> <u>of</u> <u>spheroplasts</u>

If the spheroplasts are mixed with an equal volume of DNA in 0.05$\underline{M}$
tris, pH 8.1, the efficiency is about ten times lower than is obtained
if two volumes of DNA are added (Figure 1(a)). It can be seen that
increasing the volume of DNA relative to spheroplasts above 2:1 increases
the number of phage produced approximately linearly with the increasing
amount of DNA. No further stimulatory effect is observed. A similar
result is shown in Figure 1(b) in which the ratio of volume of DNA to
volume of spheroplasts is constant and increasing amounts of tris are
added. There is no increase in phage production after one volume of
tris has been added.

(iii) <u>Linearity</u> <u>of</u> <u>the</u> <u>assay</u>

Figure 2 shows the effect of various concentrations of DNA in the
assay. The number of phage produced is approximately linearly propor-
tional to the amount of DNA added between 5×10^6 and 10^{10} DNA molecules/
spheroplast tube. The slight deviation from linearity in this exper-
iment may reflect a small but consistent dilution error since it is
not usually observed. The implication from the linearity of the assay
is that one DNA molecule is sufficient to initiate an infection.

The efficiency is approximately 4×10^{-5} pfu/DNA molecule after

6 hours at 30°. On some occasions when the efficiency is less, a

saturation value lower than 10^{10} molecules/tube is observed.

(iv) Intracellular phage growth in spheroplasts

Figure 3 shows the production of intracellular and total phage

after infection of $\overline{12}/\lambda$ spheroplasts (the spheroplast preparation was

the same as that used to obtain Figure 2). 4.8×10^{10} DNA molecules

in 4.8 ml. of $0.05\underline{M}$ tris, pH 8.1, were mixed with 1.2 ml. of spheroplasts

and samples taken for assay of intracellular phage at the times indicated.

PAM was added 15 minutes after infection. The small arrows indicate

a phage titer less than the corresponding value on the ordinate (no plaques

were observed at the concentration plated). The points at 10 and 15

minutes represent a very small number of plaques and probably are due

to a small fraction of the infected spheroplasts which survived the

lysing procedure.

The continued phage production for up to at least 6 hours in sphero-

plasts contrasts with the results obtained in spheroplasts infected with

ØX174 DNA (7) in which phage production almost ceases after 60 minutes.

This extended period of phage production is not due to infection of

surviving bacteria by phage produced in the initial DNA infection since

no plaques are observed if 2×10^8 phage are plated on the bacteria before

conversion to spheroplasts. Also, if spheroplasts of sensitive bacteria are used

as hosts, the rate and extent of phage production is much greater. Ap-

parently, a significant fraction of the infected spheroplasts do not

lyse in the liquid medium and continue to produce phage beyond the normal

latent period of infected bacteria.

(v) <u>Single-burst experiment in spheroplasts</u>

Since the results obtained by plating infective centers in a one-step growth curve are erratic, an estimate of the burst size was made by performing a single-burst experiment. A dilute solution of DNA (2×10^6 molecules/ml.) was mixed with spheroplasts of 1486-23/λ as usual and after adding PAM, 1 ml. aliquots were immediately dispensed into 162 small tubes. After 6 hours incubation the tubes were frozen and thawed, seed bacteria and top agar were added, and the entire contents were plated. The results are shown in Figure 4. One hundred-twenty-five tubes contained no phage. The mean burst size was 38 and the median burst tube contained 27 phage. Assuming a Poisson distribution of infected spheroplasts among the tubes, approximately 10% or 4 tubes contained phage particles originating from more than one infected spheroplast.

The efficiency was 1×10^{-5} phage/DNA molecule. In terms of infected spheroplasts/DNA molecule the efficiency was 3×10^{-7}. If the saturating concentration of DNA in the assay (Fig. 2) represents the fraction of spheroplasts which can be infected with DNA, this fraction is about 0.003%.

The burst size of phage-infected <u>E. coli</u> 1486-23 is about 300.

(vi) <u>Competition experiments with E. coli DNA and RNA</u>

When <u>E. coli</u> DNA is added to the incubation tube with λDNA there is an inhibitory effect which depends on the concentration and secondary

structure of the E.coli DNA. As shown in Figure 5, native E. coli DNA inhibits the assay when its concentration in the incubation tube exceeds 1 μg/ml. This is about the concentration at which the assay saturates in the absence of added heterologous DNA (Figure 2).

Heat-denatured (10 minutes at 100° in SSC/10) E. coli DNA inhibits the assay at concentrations as low as 0.05 μg/ml.

Addition of total E. coli RNA produces quite a different effect. There is an increase in the number of phage produced when 1-50 μg/ml. of RNA are present. The maximum increase occurs when 5-10 μg/ml. of RNA are added and amounts to an increase of only 2-3 fold over the number of phage produced in the absence of added RNA.

The competition experiments were performed using spheroplasts of $\overline{12}/\lambda$. One possible explanation for the enhanced efficiency with added E. coli RNA might be that the RNA inhibits the action of endonuclease I (14) which is released upon conversion of the bacteria to spheroplasts (3). The data in Table 1 support this suggestion. The two strains lacking endonuclease I, E. coli 1486-23 and 1100, were kindly supplied by Dr. H. Hoffmann-Berling and selected for resistance to λ. Two separate cultures of each and of $\overline{12}/\lambda$ were grown and converted to spheroplasts as usual. E. coli RNA at 8 μg/ml. was added to one series of incubation tubes at the time of infection. The results shown in Table 1 are the average values from the two preparations. Spheroplasts of $\overline{12}/\lambda$ show approximately the same increment of infectivity with added RNA as was obtained in Figure 5. However, neither of the strains lacking endonuclease I exhibit this effect.

(b) Infectivity of superinfecting λDNA

Dove and Weigle (4) observed a loss of most of the infectivity of λ DNA after superinfection of a homo-immune lysogenic host. They could recover from the infected cell only about 10% of the infectivity which was present in the phage prior to infection. Bode & Kaiser (2) extended these observations and found that concomitant with the loss of infectivity of the injected radioactive DNA two new species of labelled DNA could be extracted from the infected cell. Evidence was presented that this radioactivity had not been incorporated into host DNA but neither of the two new radioactive components was infective, which would have been a more conclusive identification. The residual infectivity sedimented through a sucrose density gradient at the rate of added marker phage DNA, while the two new species sedimented 1.15 and 1.9 times the rate of the marker. In fresh lysates most of the label sedimented at the higher rate.

In these studies the infectivity of the DNA was determined by the helper assay. We have studied the infectivity of the intracellular DNA from superinfected lysogens in the spheroplast assay just described. Table 2 compares the specific infectivity of DNA extracted from superinfected cells measured in the helper and spheroplast assays. The specific infectivity is the number of infective centers (helper assay) or phage (spheroplast assay) produced per ^{3}H cts/min in the extracted DNA. This value is then normalized to the specific infectivity of the zero-minute sample, which is equivalent to phage DNA. The zero-minute

sample was taken after mixing ^{3}H-λ with 3110(λ) for 10 minutes in λdil

at 0°. This allows adsorption but not injection (2; personal observations).

The other samples were taken 20 and 40 minutes after raising the tem-

perature to 37° for 10 minutes to allow for injection, blending in a

Waring blendor to remove uninjected phage, centrifuging, and resuspending

in warm TBM. About 50% of the cts/min sedimented with the bacteria

after blending.

The expected eclipse of infectivity occurs when the infectivity of

the extracted DNA is measured in the helper assay. The relative specific

infectivity falls to about 2% by 40 minutes. In contrast, the infec-

tivity measured in spheroplasts does not decrease significantly after

infection and, within the error of the assay, is the same at 20 or 40

minutes after infection as it was prior to injection.

(c) <u>Fractionation</u> <u>of</u> <u>the</u> <u>infectivity</u> <u>by</u> <u>sedimentation</u> <u>through</u> <u>a</u>

<u>sucrose</u> <u>density</u> <u>gradient</u>

In an attempt to determine which of the components observed by

Bode & Kaiser, if either is responsible for the infectivity measured

in the spheroplast assay, DNA was extracted from an immune lysogen after

superinfection with ^{32}P-phage and centrifuged through a sucrose density

gradient. The radioactivity and helper infectivity were measured and then

each fraction was dialyzed to remove the sucrose and assayed in sphero-

plasts. The results are shown in Figure 6. Two components contain

most of the injected radioactivity with the faster, component I, con-

taining about 70% and component II about 30%. Component I, however,

has very little, if any, infectivity in the helper assay, nor does component II. All of the residual infectivity measured in the helper assay sediments slightly slower than component II, and is assumed to be due to a small number of intact phage DNA molecules.

However, the main peak of infectivity in the spheroplast assay cosediments with component I and a small amount of infectivity appears to be associated with component II. The specific infectivity of II appears to be lower than I but this may only reflect the small number of phage obtained from these fractions. The number of infective DNA equivalents per fraction was calculated in each assay from a standard curve of phage DNA. The specific infectivity of component I relative to phage DNA in the spheroplast assay was calculated from the ^{32}P content of fraction 12, the specific activity of the radioactive DNA, the number of phage produced from fraction 12 in the assay, and the standard curve. The results indicate that component I has approximately the same infectivity per molecule in the spheroplast assay as phage DNA, as can also be inferred from Table 2 and Figure 6.

The failure to find any infectivity in the spheroplast assay at the position of component III is simply due to the absence of a sufficient quantity of DNA.

The results shown in Figure 6 were obtained with DNA extracted from a λ-lysogen after infection with a cI mutant (c26). Similar experiments have been performed after infection of the same strain with wild-type λ and identical results were obtained.

Apparently the structural modifications of the DNA which produce

an increased sedimentation rate and a loss of infectivity in the helper

assay do not prevent the intracellular λDNA from successfully infecting

spheroplasts. The loss of activity in the helper assay is, therefore,

not explicable merely in terms of biological inactivation during infec-

tion or extraction from the cell, and must reside in the properties of

the helper-infected cells rather than a loss of function of the DNA.

(d) Infectivity of λ DNA during vegetative development

After infection of a sensitive cell by λ, there is an eclipse of

DNA infectivity, measured in the helper assay, which is comparable to

the eclipse observed after superinfection (4). Moreover, most of the

DNA which can be extracted from the infected cells is not infective

until approximately the time at which mature phage can be released.

This is true despite the presence of phage-specific DNA in the cell

earlier in the latent period (4; 23). At the end of the eclipse period,

synthesis of mature phage and DNA infective in the helper assay begins.

The results obtained using the spheroplast assay to measure the

biological activity of the vegetative DNA differ strikingly from those

observed in the helper assay. As shown in Figures 7A and 7B, there is

no eclipse of infectivity measured in the spheroplast assay, although

the expected decline of infectivity is observed in the helper assay.

The data shown in Figure 7A are from an experiment in which E. coli

CR34 was infected with ^{3}H-λb2b5c at a multiplicity of about one and

aerated at 30°C. To obtain the data shown in Figure 7B, E. coli

AB2500 was infected with ^{32}P-λc26 at an adsorbed multiplicity of five

phage per bacterium and aerated at 37°C. Other experimental details are

given in the legend to Figure 7.

A sample of the phage used in this infection was mixed with the

bacteria at 0°C and the DNA extracted with phenol as with the infected

bacteria. A standard curve of the biological activity of this sample

versus dilution was used to calculate the number of infective units per

bacterium (Figure 7A) or the relative infectivity (Figure 7B) of the

vegetative DNA. The DNA infectivity data in Figure 7B, but not 7A,

have been corrected for recovery of radioactivity in the dialyzed phenol

extract. A relative infectivity of 1.0 in Figure 7B corresponds to

2.0 x 10^9 and 2.3 x 10^9 infective DNA molecules per ml. in the phenol

extract, measured in the helper and spheroplast assays, respectively.

The number of DNA molecules expected from the multiplicity of infection

and the recovery of radioactivity is about 1.5 x 10^9 per ml., indicating

that the precision and accuracy of the two assays are similar in this

experiment.

The most important result of these two experiments is the demon-

stration that immediately after infection λDNA is apparently synthesized

in a form which is able to infect spheroplasts but not helper-infected

cells. The simplest and most attractive hypothesis is that both the

injected phage DNA (as in the case of superinfection, Table 2 and Figure

6) and the vegetative DNA (all or some) made immediately after infection

are able to infect spheroplasts but not helper-infected cells. The
synthesis of , or conversion to, DNA which can infect helper-infected
cells occurs much later, at, or shortly before, the synthesis of mature
phage.

The synthesis of biologically active DNA well before the end of the
phage eclipse period makes it seem unlikely that the early DNA is non-
infective in the helper assay because the molecules are only one-half
synthesized, for example. If the DNA synthesized during this period
were one long λ chromosome, containing duplications of the individual
genome (19) it is doubtful that the infectivity would increase as much
as is observed (unless fragmentation which might occur during extrac-
tion produced structures which retained their biological activity).

The molecular basis for the initial eclipse of infectivity of the
injected DNA, measured in the helper assay, seems to be explicable by
the conversion of the parental chromosome into a twisted, circular
form of DNA. This molecule is able to infect spheroplasts but not
helper-infected whole bacteria. That the infectivity of the vegetative
DNA is observed only with spheroplasts may be related to the observation
that most of the DNA which is synthesized before the end of the eclipse
period has sedimentation properties identical to those of the twisted,
circular form of λDNA (22).

(e) Infectivity of circular λDNA prepared in vitro

Another form of λDNA was also tested for its ability to infect spheroplasts. Hershey, Burgi & Ingraham (9) showed that λDNA can form dimers, trimers and circular molecules if the DNA is heated to below the melting temperature and slowly cooled in high salt. Heating and then quenching in ice converts the aggregates and circles to linear monomers. The circularization reaction competes with intermolecular aggregation, and is favored by low DNA concentrations. Most of the infectivity in the helper assay is lost if the ends are joined to form circles or are damaged enzymatically (12; 20).

Phage DNA was either heated at 75° for ten minutes and then quenched in an ice bath to form linear molecules, or cooled over a three-hour period from 75° to 35° to form circular molecules, as described in Methods.

The results of three experiments are shown in Table 3, in which the infectivity of the circular DNA preparation in the respective assay is compared to the infectivity of linear DNA, which has been assigned a relative infectivity of 1.0. A dilution curve was obtained for each sample and the infectivity measured in the range for which the number of phage or infective centers produced was directly proportional to the DNA concentration.

As Strack and Kaiser showed, most of the helper infectivity is lost when the DNA is circularized. This is most apparent at low DNA concentrations (experiment 1). At high DNA concentrations aggregates

are probably formed which are either infectious themselves or are de-
graded to infectious linear monomers during the assay. However, if

measured in the spheroplast assay, the circular DNA appears to be at least

as infective as the linear DNA. The values greater than one obtained

in experiments 1 and 2 may be an artifact caused by forming the linear

reference DNA from this circular DNA by an additional step (heating to

75° for 10 minutes and quenching in ice). Conceivably, the additional

heat treatment inactivated a significant fraction of the linear molecules

in these experiments. For experiment 3 the preparations of linear and

circular DNA were made independently from the same batch of phage DNA.

4.   GENERAL CONCLUSIONS

The spheroplast assay has proved to be a valuable tool for studying

the biological activity of several forms of λDNA.

(a) DNA extracted from phage

The linear monomeric form of λDNA is infective in both the sphero-

plast assay and the helper assay. The "notched" circles prepared in

vitro by annealing phage DNA at low concentration are at least as infec-

tive as the linear monomers in the spheroplast assay. In the helper

assay the circular molecules retain only slight infectivity.

(b) Intracellular DNA

(i) From an immune lysogen

There is no eclipse of infectivity of the superinfecting DNA if the spheroplast assay is used to measure the biological activity. W. Dove (personal communication) has also noted that injected λ DNA is infective in spheroplasts. The extracts from which the spheroplast infectivity is determined retain only a few per cent of their initial infectivity in the helper assay. The residual helper infectivity sediments through a sucrose density gradient at the position expected for intact, linear phage DNA. The majority of the activity in the spheroplast assay sediments about 1.9 times as fast as the helper infectivity, and cosediments with component I, the main peak of radioactivity of the injected DNA. A smaller amount of spheroplast-infective DNA sediments with the minor peak of radioactivity, component II, which sediments about 1.15 times faster than the helper infectivity.

From these observations and calculations of the specific infectivity of these components relative to phage DNA, it appears that (a) component I, the purported supercoiled form of λDNA, has the same specific infectivity in the spheroplast assay as phage DNA, and (b) component II, probably an open circle with a scission in one strand, has at least one-half the specific infectivity in this assay. Neither of these forms of DNA have significant biological activity in the helper assay.

(ii) After infection of a sensitive bacterium

In analogy with the results obtained after infection of an immune host, no eclipse of DNA infectivity is observed if spheroplasts, rather

than helper-infected whole cells, are used to measure the biological
activity. Shortly after infection, synthesis of DNA which is infective
in the spheroplast assay but not the helper assay begins. An increase
of infectivity in the helper assay does not occur until considerably
later, at the onset of intracellular phage production. That the in-
crease in infectivity observed in the spheroplast assay shortly after
infection is due to synthesis of more infectious molecules rather than
an increase in the specific infectivity of the injected phage DNA is
supported by the studies reported in the following paper.

The reasons for the failure of circular molecules to be infective in
the helper assay remain obscure. The availability of the free single-
stranded ends is obviously one prerequisite, but whether they are
required for penetration or for some function inside the helper-infected
bacterium is uncertain. The infectivity in the spheroplast assay of all
the circular forms of λDNA presently known demonstrates that free ends
are not required to initiate an infection. Therefore, there must be
some function which the ends perform during infection of a helper-
infected cell that is not required in a normal infection. Although
this function might be recombination with the helper phage, it seems
simpler to assume that penetration of the cell wall by λDNA requires
a free, cohesive end. Complementation studies could resolve this question.

TABLE 1

Efficiency of the spheroplast assay

in endonuclease I^+ and endonuclease I^- strains

(pfu/DNA x 10^6)

Spheroplasts	Minus RNA	Plus RNA
$\bar{1}\bar{2}$/ (+)	3.3	8.3
1100/ (-)	1.9	1.4
1486-23/ (-)	11.0	8.8

Two separate cultures of each strain to be tested were grown and converted to spheroplasts independently. E. coli RNA at a concentration of 8 µg/ml. was either present or absent during the DNA adsorption and thereafter. The results are the average values obtained with the two cultures.

TABLE 2

Specific infectivity of ^{3}H-λDNA after infection of 3110(λ)

Time (min)	Helper assay	Spheroplast assay
0	1.0	1.0
20	.046	1.5
40	.022	0.5

E. coli 3110(λ) was grown to 3 x 10^8 bacteria/ml., centrifuged, and washed with λ dil at 0°. ^{3}H-λ was added to a multiplicity of 20 plaque-formers per bacterium and allowed to adsorb for 10 minutes at 0°. An aliquot of the culture was taken and kept at 0° and the remainder was brought to 37° for 10 minutes. At ten minutes the culture was blended to remove uninjected phage, centrifuged, and resuspended in TB at 37°. The 20 and 40 minute samples refer to the time after resuspension in TB. About 68% of the cts/min adsorbed at 0° and about 50% of the added cts/min were resistant to blending after 10 minutes at 37° in λ dil. The infectivity in the extracts is corrected for recovery of ^{3}H cts/min and is normalized to the infectivity of the extract containing adsorbed but uninjected phage (at 0°C).

TABLE 3

Infectivity of circular phage DNA

Experiment	DNA concentration during circularization (μg/ml.)	Relative infectivity of circular DNA	
		Helper assay	Spheroplast assay
1	.072	.02	5.0
2	1.0	.29	1.4
3	8.0	.20	1.0

Phage DNA was heated at the concentration stated for ten minutes at 75°C in 0.05 M phosphate buffer, 0.1 M NaCl, and quenched in ice to form linear molecules. To form circular molecules the DNA in the same buffer plus 1.0 M NaCl was placed in a water bath at 75°C, the bath turned off and allowed to cool to 35°C over a three hour period.

For experiments 1 and 2 the linear DNA was made by heating ten minutes at 75°C and quenching in ice a solution which had previously been converted to circular DNA as described above.

Dilutions into the low ionic strength buffer required for the biological assays were performed just prior to infection.

The number of phage or infective centers produced by a given amount of linear DNA is given the value of 1.0 and the number of phage or infective centers produced by an equivalent amount of circular DNA is normalized to this value to obtain the relative infectivity of the circular DNA. The

values in all cases are taken from the range of the assay in which the

number of phage or infective centers produced is directly proportional

to the DNA concentration.

Figure 1. <u>Effect</u> <u>of</u> <u>dilution</u> <u>of</u> <u>the</u> <u>spheroplasts</u> <u>on</u> <u>the</u> <u>efficiency</u>

<u>of</u> <u>the</u> <u>assay</u>. Spheroplasts of $\overline{12}\lambda$ were prepared as described in

<u>Methods</u>. λb2b5c DNA at a concentration of 4×10^9 molecules/ml. in

.05 M tris, pH 8.1, was mixed with 0.1 ml. of spheroplast stock for

15 minutes and a volume of PAM equivalent to the volume of DNA plus

spheroplasts was added. Incubation at $30°$ was continued for 6 hours.

After $CHCl_3$ addition the culture was frozen and thawed and assayed for

phage. The two points at each ratio indicate duplicate assays. The

total number of active phage in each assay tube is plotted as a measure

of the efficiency of the assay.

(a) To 0.1 ml. of spheroplasts, 0.1, 0.2, 0.4, 0.6 and 0.8 ml., res-

pectively, of DNA solution was added.

(b) To 0.1 ml. of spheroplasts, 0, 0.1, 0.3, 0.5 and 0.7 ml., respec-

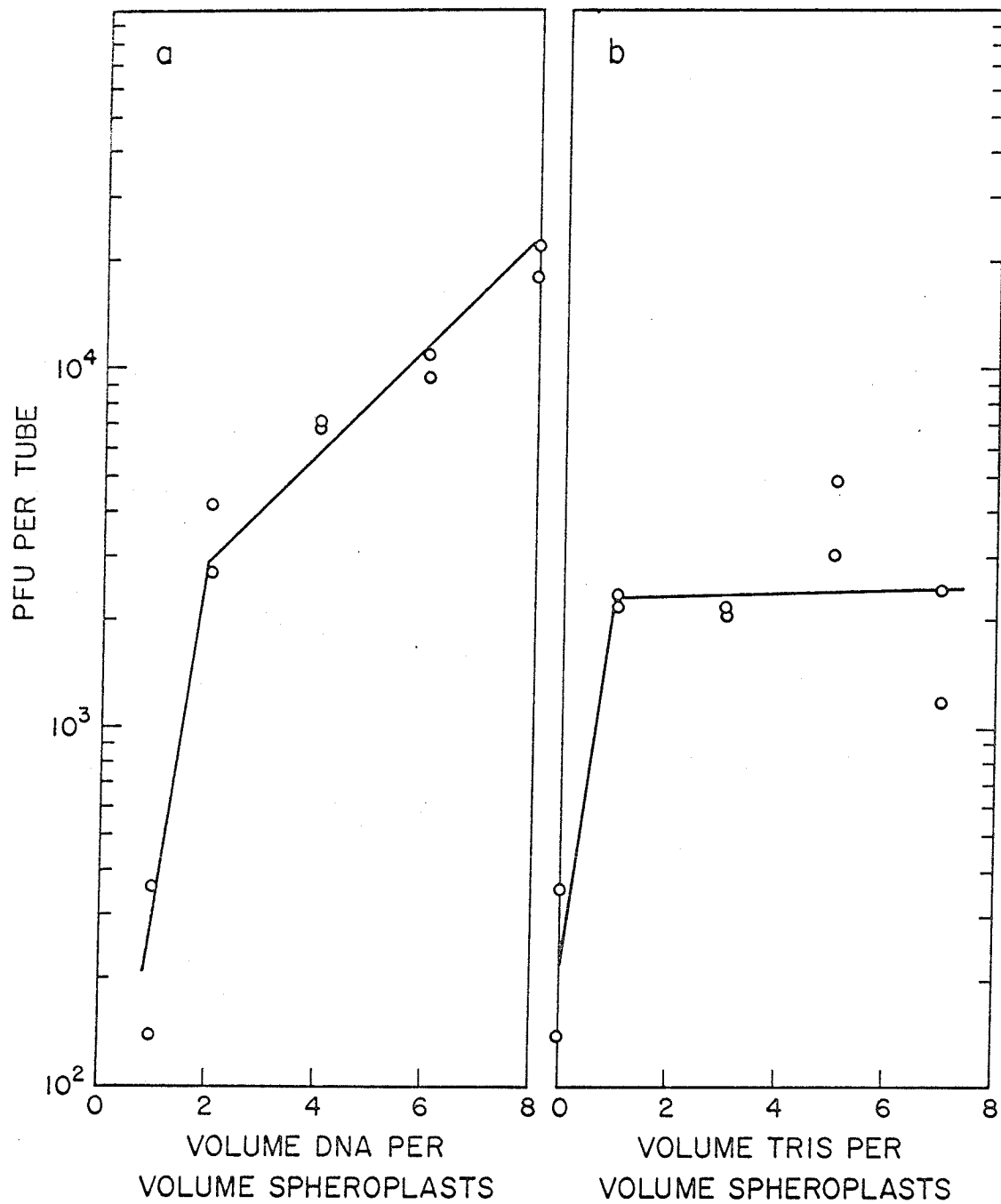tively, of 0.05 M tris, pH 8.1 was added, followed by 0.1 ml. of DNA

solution.

Figure 1

Figure 2. <u>Linearity</u> <u>of</u> <u>the</u> <u>spheroplast</u> <u>assay</u> <u>in</u> $\overline{12}\Lambda$ <u>spheroplasts</u>.

λb2c DNA at a concentration of 4×10^{10} molecules/ml. was diluted

5^1-5^7 times in .05 M tris, pH 8.1. Three 1.2 ml. portions of DNA at

each concentration were mixed with three 0.3 ml. portions of sphero-

plasts prepared as described in <u>Methods</u>. After 15 minutes at 30°, 1.5 ml.

of PAM was added and the incubation was continued for 6 hours. Phage

were assayed as described in <u>Methods</u> and the total number of phage per

assay tube is plotted for each DNA concentration. The efficiency of
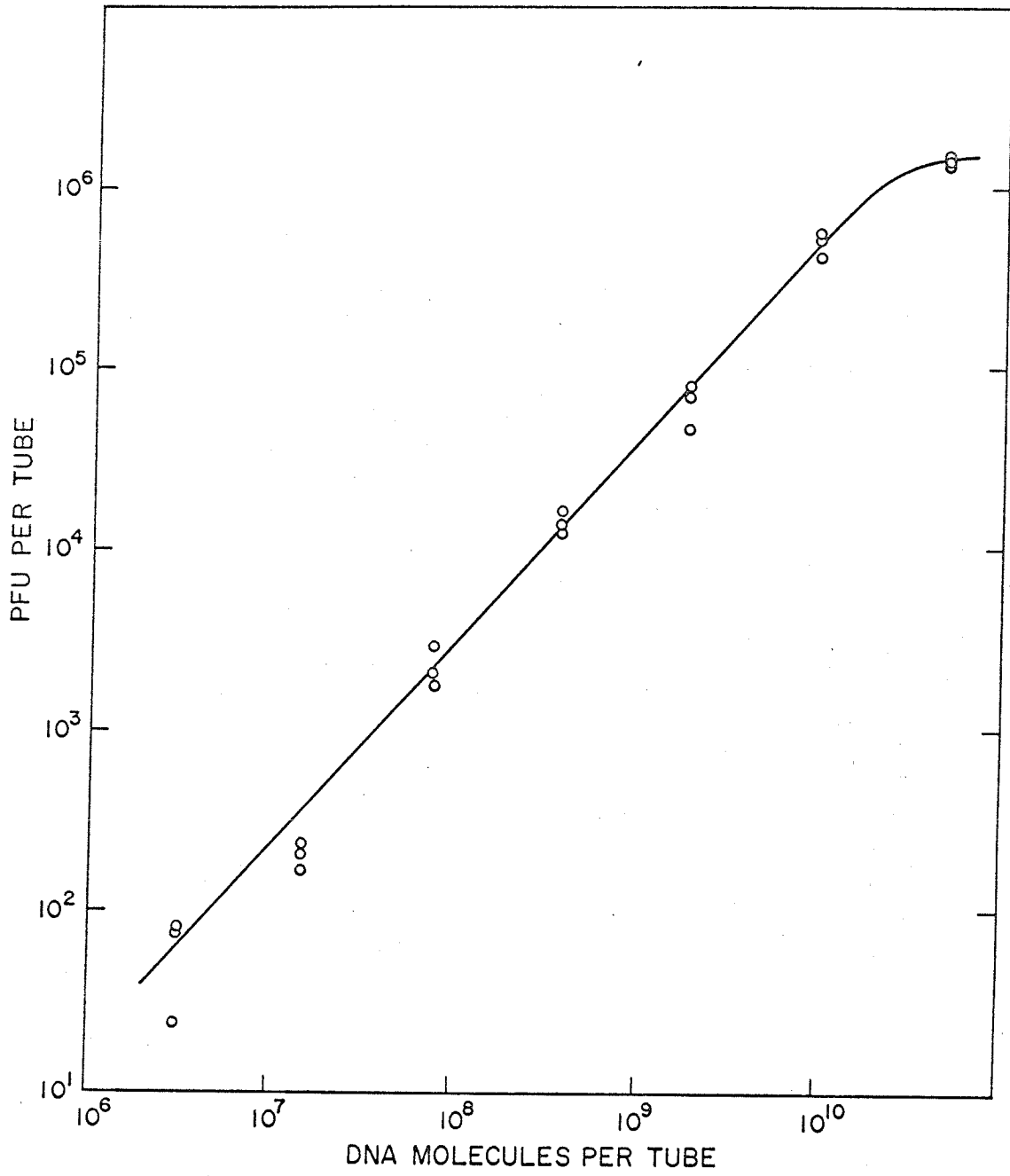
the assay (phage produced/DNA molecule) was about 4×10^{-5}.

Figure 2

Figure 3. <u>Growth</u> <u>of</u> <u>phage</u> <u>after</u> <u>infection</u> <u>of</u> $\overline{12}/\lambda$ <u>spheroplasts</u>.
This assay was carried out with the same spheroplasts and assay conditions
described in the legend to Figure 2. The concentration of the DNA added
to the spheroplasts was 1×10^{10} molecules/ml. At 0 minutes the DNA
was mixed with the spheroplasts. Aliquots were withdrawn at the indi-
cated times, lysed, and assayed for phage. The arrows (↓) indicate
values <u>less</u> <u>than</u> their corresponding position on the ordinate; that is,
no phage were detected at the dilution plated. The efficiency of the
assay (phage produced/DNA molecule) was about 4×10^{-5} at 6 hours.

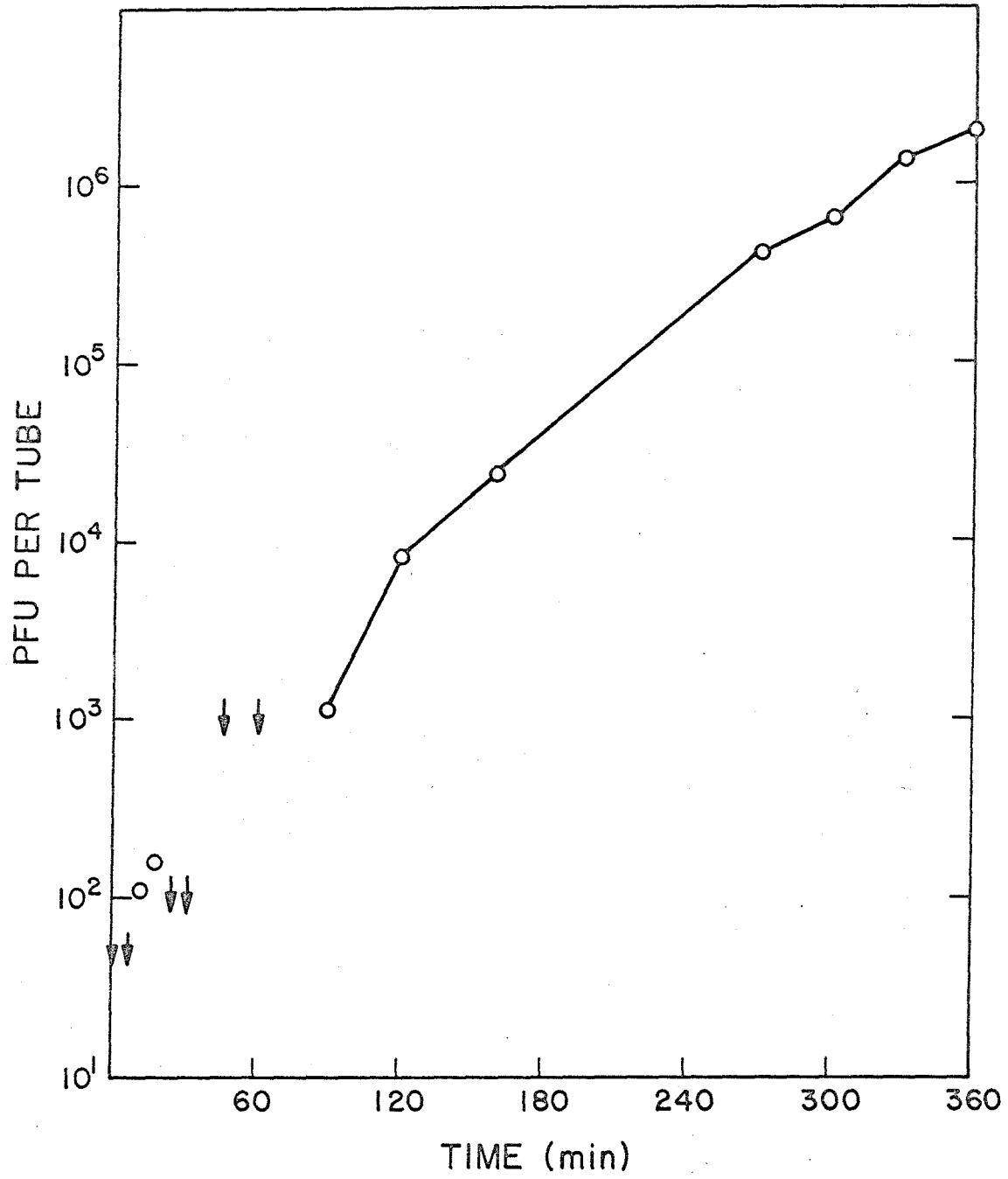Figure 3

Figure 4. <u>Single-burst</u> <u>experiment</u> <u>in</u> <u>spheroplasts</u> <u>of</u> <u>12/λ</u>.

Spheroplasts were prepared as in <u>Methods</u>. λb2b5c DNA, 60 ml., at a

concentration of 2 x 10^6 molecules/ml. was mixed with 15 ml. of sphero-

plasts for 15 minutes at 30° and 75 ml. of PAM was added. Aliquots of

about 1.0 ml. were dispensed with an automatic pipette to 162 small

tubes. After 6 hours at 30°, the tubes were frozen and thawed, 0.25 ml.

of seed bacteria (C600) was added, and 15 minutes later the entire con-

tents were mixed with top agar and plated. Of the 162 tubes plated,

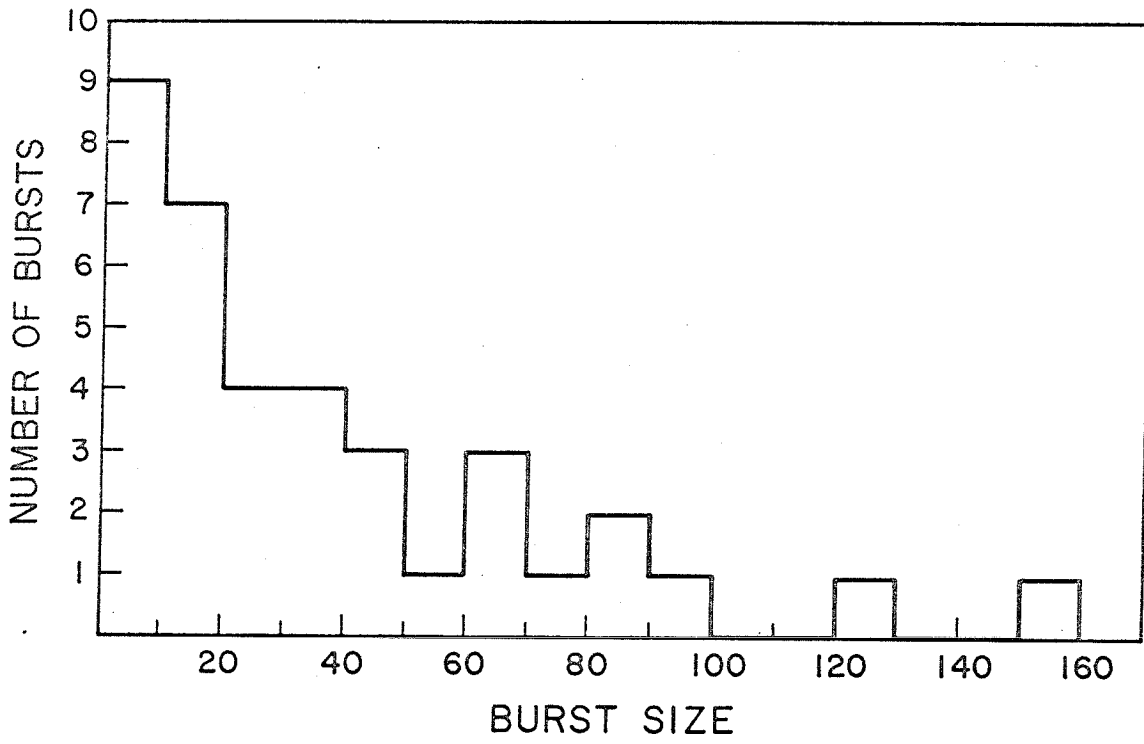37 contained phage. The distribution of phage among the single-burst

tubes is plotted.

Figure 4

Figure 5. <u>Effect</u> <u>of</u> <u>added</u> <u>E. coli</u> <u>RNA</u> <u>or</u> <u>DNA</u> <u>on</u> <u>the</u> <u>efficiency</u> <u>of</u> <u>the</u>

<u>spheroplast</u> <u>assay.</u> The incubation tube contained 0.3 ml. of $\bar{1}\bar{2}/\lambda$ sphero-

plast stock and 1.2 ml. of 0.05 M tris, pH 8.1, containing 1×10^{10}

λb2b5c DNA molecules and <u>E. coli</u> RNA, native DNA, or denatured DNA. The

concentration of the RNA and DNA was calculated from the absorbance at

260 mμ assuming A_{260} = 1 corresponds to 50 μg/ml. The infectivity data

were normalized to the value obtained in the absence of added heterologous

nucleic acid.

    0——0   Native DNA

    □——□   Denatured DNA
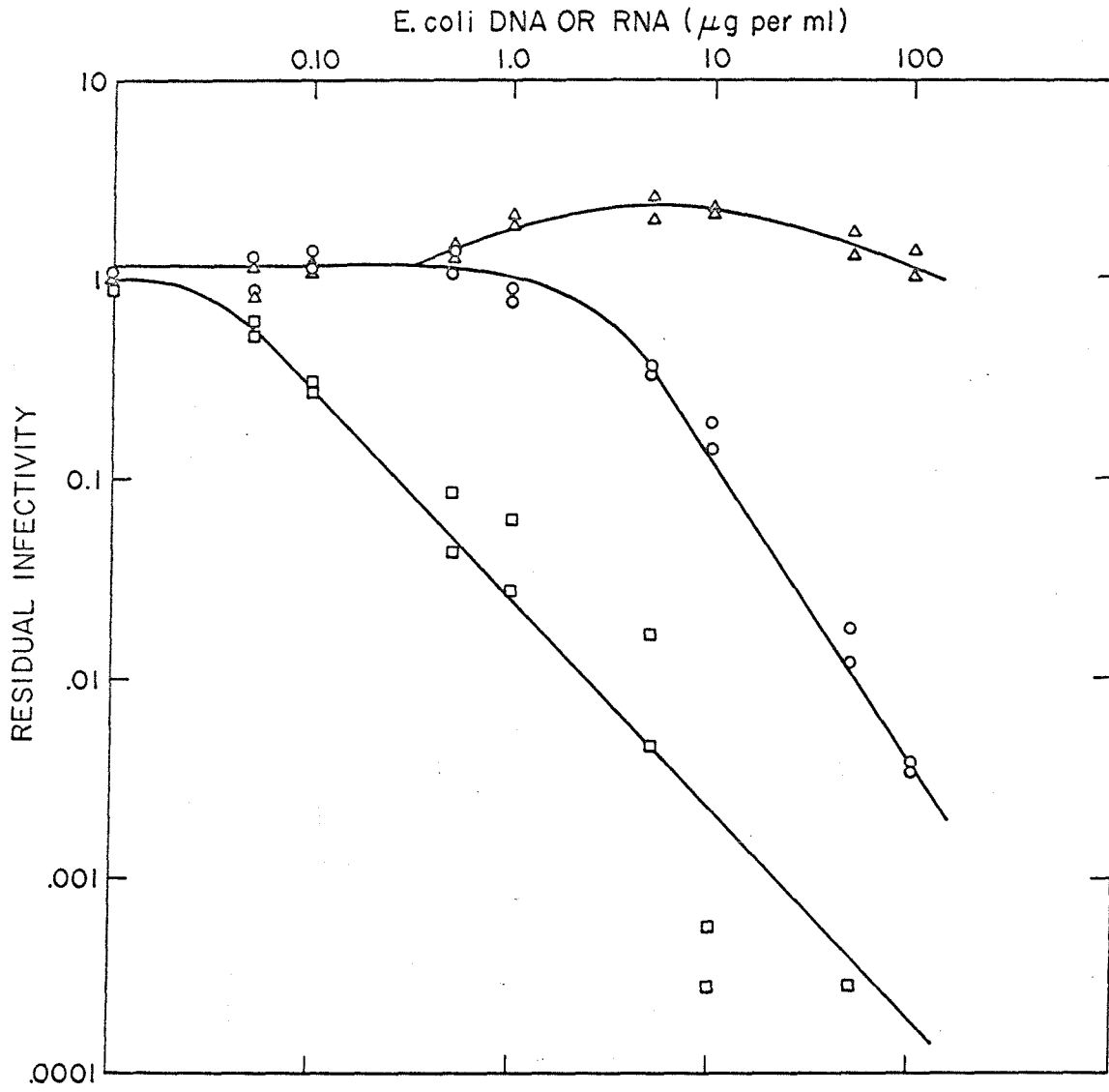
    △——△   RNA

Figure 5

Figure 6. Sedimentation of ^{32}P-λc26 DNA extracted 60 minutes after

infection of 3110(λ). 3110(λ) was grown in K medium and infected at

1 x 10^9 bacteria/ml. with about one plaque-former per bacterium. After

60 minutes the bacteria were chilled to 0°, blended for 2 minutes, and

centrifuged. A sample of the extracted DNA was layered on a 25 ml.

5-20% sucrose gradient containing 10 mM tris, pH 8.1, 1 mM EDTA, and

0.1M NaCl. Centrifugation was for 5 hours at 25,000 rev./min, at 6°,

in the SW25.1 rotor. The 30 fractions were analyzed for ^{32}P and infec-

tivity in the helper assay and then dialyzed; the solution remaining

was used to infect spheroplasts. Sedimentation proceeds from right to

left. The tube containing fraction number 11 was broken during dialysis.

x———x   cts/min

0———0   spheroplast assay

△———△   helper assay

Figure 6

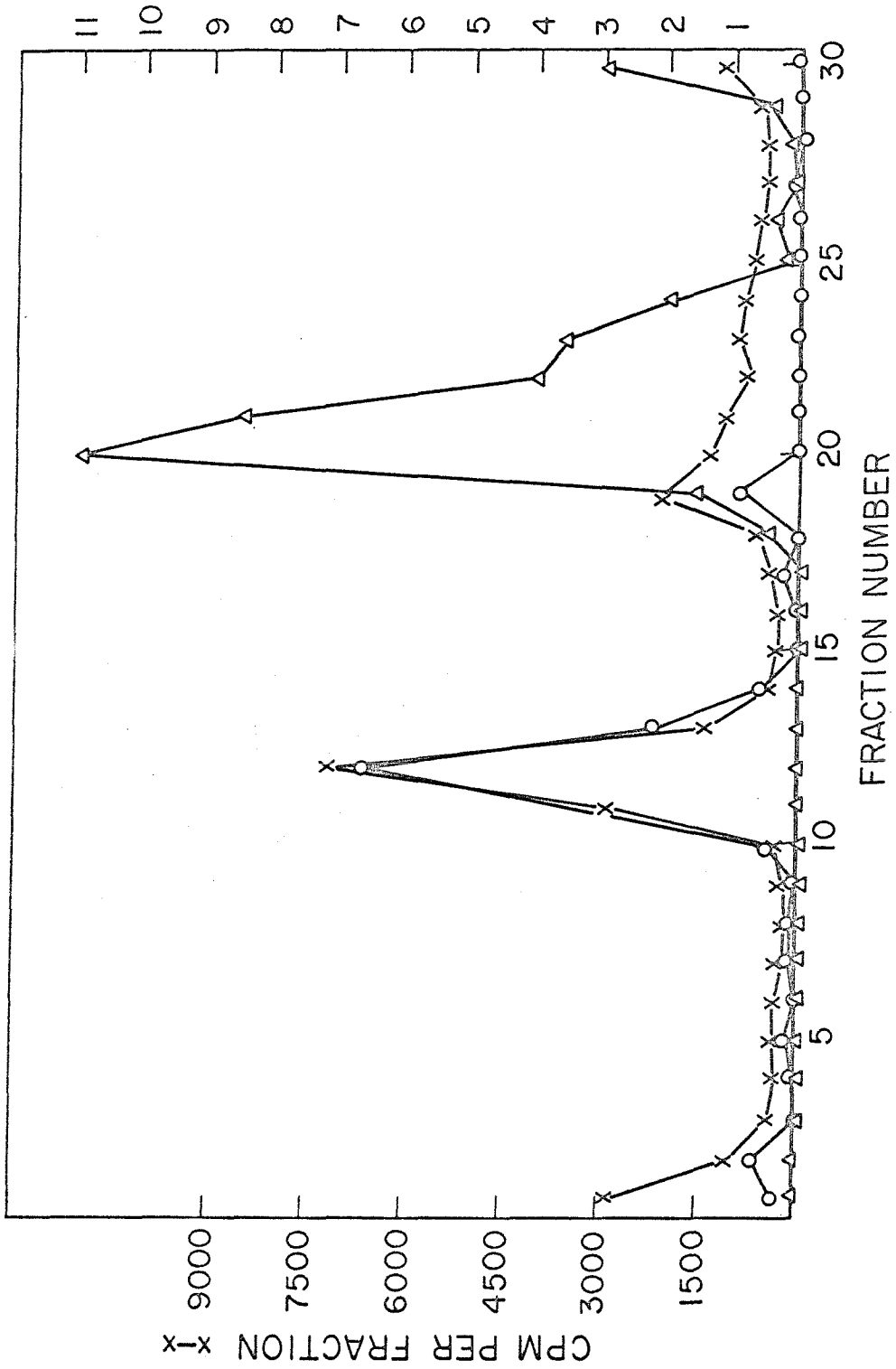Figure 7. <u>Infective</u> <u>DNA</u> <u>and</u> <u>intracellular</u> <u>phage</u> <u>during</u> <u>a</u> <u>sensitive</u>

<u>infection</u>.

<u>A</u>. <u>E</u>. <u>coli</u> CR34 was starved by aeration in TM for 60 minutes at 37°

and then infected with about one ^{3}H-λb2b5c per bacterium. After 10 min-

utes for adsorption the bacteria were centrifuged, resuspended at 37°C in

K medium plus 20 µg/ml. thymidine and portions were taken and chilled

at various times. The DNA was extracted by lysis with SDS at 60°.

Intracellular phage were measured as described in Methods. About 50%

of the radioactivity was recovered in the dialyzed phenol extracts. The

number of infective DNA molecules per cell (about 75% of the cells became

infective centers) is calculated from a standard dilution curve of

phage DNA (extracted from the phage used in the experiment) and is not

corrected for ^{3}H recovery.

<u>B</u>. AB2500 was grown to 6×10^8 cells/ml. in K medium plus thymidine,

centrifuged, resuspended in 1/5 volume TM at 37°, and infected with

^{32}P-λc26 at a multiplicity of 5 pfu/bacterium. After 5 minutes at 37°

the bacteria were blended to remove uninjected phage, centrifuged, and

resuspended in one volume of 37 K medium plus 5 µg/ml. thymidine. At

the indicated times samples were withdrawn for intracellular phage assay

or DNA extraction, as described in <u>Methods</u>.

More than 99% of the pfu adsorbed during 5 minutes at 37°, and

47% of the cts/min sedimented with the bacteria after blending.

A relative infectivity of 1.0 corresponds to the number of phage

or infective centers produced by an extract of the radioactive phage

used in the experiment. The number of phage or infective centers pro-
duced by an equivalent number of ^{32}P cts/min in the extracts of infected
bacteria are normalized to the value obtained with the radioactive phage
DNA. Duplicate samples were taken at 60 minutes and the relative infec-
tivity of each sample is recorded.

A relative infectivity of 1.0 corresponds to 2.0 x 10^9 and 2.3
x 10^9 infective DNA molecules per ml. in the phenol extract, measured
in the helper and spheroplast assays, respectively.

      ●———●   intracellular phage

      △———△   helper assay

      O———O   spheroplast assay

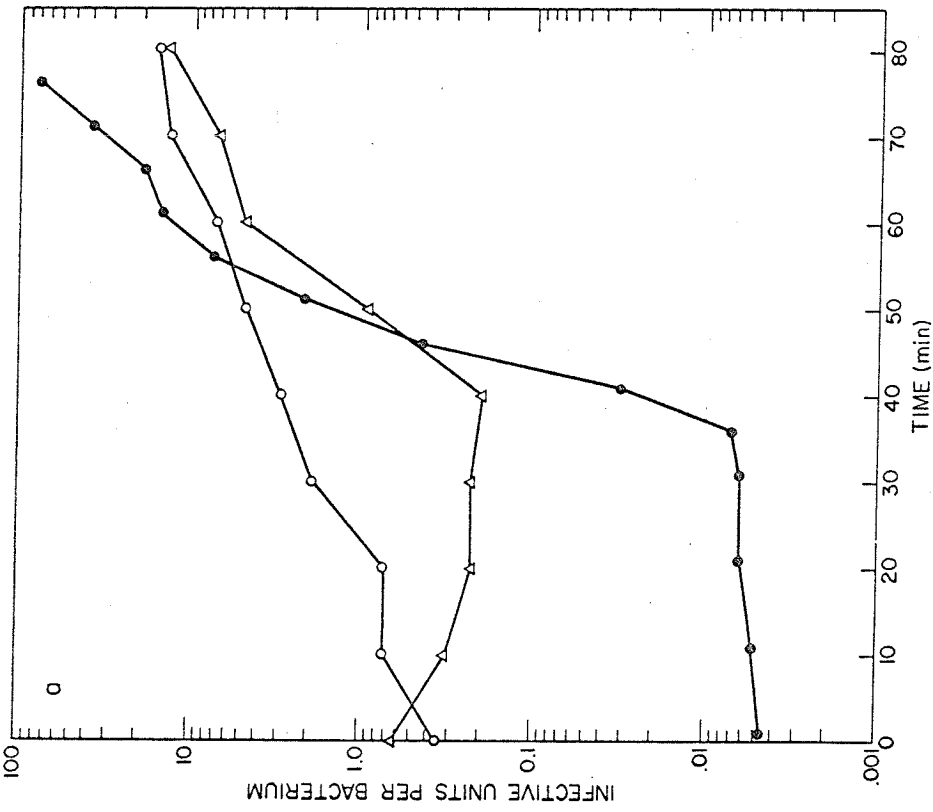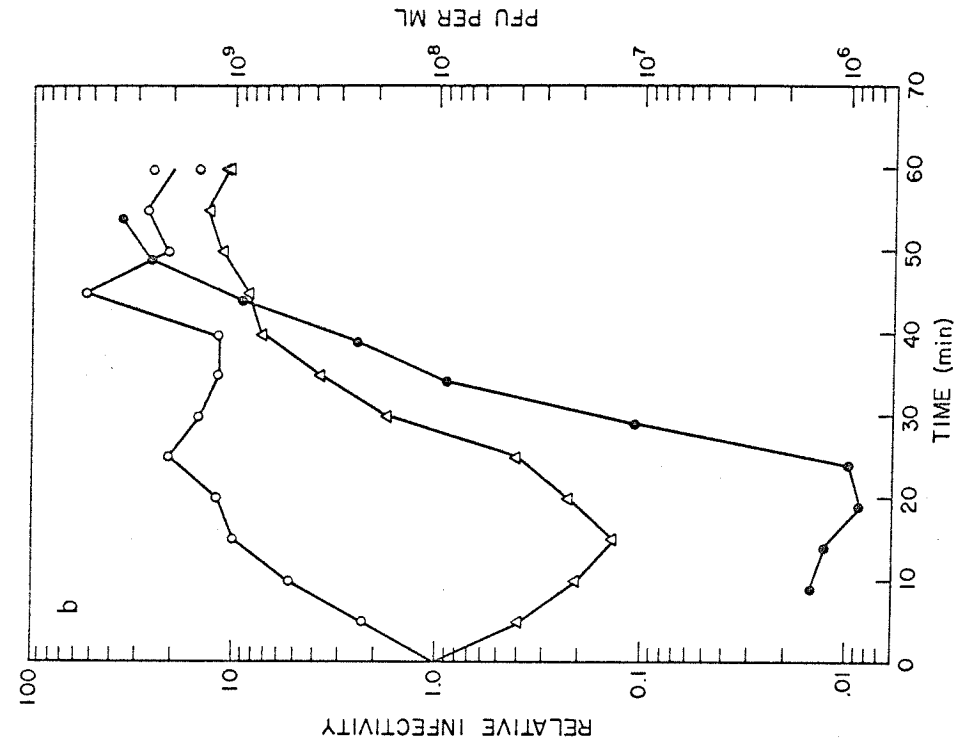Figure 7

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Part II

VEGETATIVE LAMBDA DNA:

PHYSICAL CHARACTERIZATION AND REPLICATION

1. INTRODUCTION

In contrast to the considerable effort that has been expended study-
ing phage DNA structure (55), relatively little is known about the details
of its replication. Creatures containing double-stranded DNA replicate
their DNA semiconservatively and the conserved subunits have been iden-
tified as single polynucleotide strands in a number of cases (54).
Other general principles governing DNA replication and its regulation will
undoubtedly emerge from a study of appropriately chosen model systems.
The natural goal of such a study will be to understand the sequence of
events necessary for DNA replication in terms of the secondary and ter-
tiary structure of the template, the enzymes required, and the structural
components necessary to maintain order in the system; and ultimately,
to reproduce the reaction in vitro.

To attain these goals the system chosen should fulfill four criteria:
1. The structure of the non-replicating DNA should be known; 2. Complete
genetic analysis should be possible; 3. The environment in which repli-
cation occurs should be amenable to study (in the case of a virus this
means the host should be well-characterized); and 4. To achieve the
final goal, the DNA chosen must possess biological activity. Only bacterial
viruses presently satisfy these criteria. However, the criteria are
rather stringent and there is no doubt that valuable information will
be gained from studying more complicated organisms. In fact, most of
the control principles acting in DNA replication have been elucidated
by studies of chromosomal replication in bacteria (see Lark (33) for a

review). Tritium autoradiography has demonstrated that E. coli DNA

can replicate as a circle with a single replication point (6), substan-

tiating earlier genetic indications that the chromosome is circular (26).

There is only one origin of DNA synthesis (after amino acid starvation

at least, 3) and normally one cycle of replication is completed before

the next one begins (30). Thymine starvation, however, induces premature

initiation of a new cycle of replication at the origin (33). Some CAM

resistant (at 30 μg/ml.) (structural?) protein is required for DNA rep-

lication(33). This might be involved in attaching the DNA to the mem-

brane (3, 16, 24).

The importance of circularity in DNA replication is also suggested

by the extensive analysis of the replication of phage ØX174 DNA. This

minute DNA bacteriophage fully satisfies the criteria discussed above.

As a result a detailed knowledge of its DNA replication is being obtained.

The single-stranded circular DNA of the virus is converted to a double-

stranded circular form which replicates semiconservatively. Later in

the infection viral single-strands are synthesized and immediately encap-

sulated (47, 48).

Another mode of DNA replication has been suggested by an analysis

of vegetative T4 DNA by Frankel (14). One interpretation of this data

is that T4 DNA is synthesized as a very long, linear concatenate (that

is, it contains many T4 genomes end-to-end). Phage DNA could be made

from the concatenate by cutting off lengths of the DNA which fit into

the head. This is an attractive hypothesis since it could explain

the occurrence of terminal redundancies (36). This interpre·

tation has been challenged, however, by Kozinski (personal communication), who maintains that the fragility and rapid sedimentation rate of the vegetative T4 DNA result from interruptions in one of the strands of the helix, and that the vegetative T4 DNA is the same length as T4 phage DNA. In any event, analysis of T4 DNA replication is certainly complicated by the large number (about 5 - 7) of mating events per genome. Recombining DNA might well have single-stranded regions (21) and be collapsed into a compact but shear-sensitive configuration which would give it a rapid sedimentation rate.

Analysis of λDNA replication is not hampered by frequent mating events (0.5 - 1 per genome). It, as ØX, satisfies the criteria described above and thus seems an appropriate system in which to study DNA replication. The discovery of circular (hydrogen-bonded) phage DNA (19) and later of circular (covalent) vegetative DNA (67, 2: vide ante) suggested that DNA replication involved circular forms in λ as well as in ØX (47), E. coli (6), Bacillus subtilis (Yoshikawa, personal communication) and polyoma virus (12, 20). However, the intracellular circular λ DNA is also found during infection of an immune host, in which no replication occurs (60). This prompted Bode and Kaiser(2) and more recently Hershey to claim that the circular DNA may be a form ".... to which replication is expressly forbidden"(18). That is, it is specifically a repressed form of the DNA. Hershey's statement was based partly on experiments by Smith and Skalka (52) who reported finding T4 - like concatenates of λ DNA which they claimed were precursors to phage DNA.

Circular λ DNA has been detected in vivo during three distinct

phases of the λ life cycle: 1. During infection of a sensitive host some

of both the parental and progeny DNA has sedimentation properties of

closed circular DNA (67); 2. After superinfection of an immune host in

which λ DNA replication does not occur (66), most of the parental DNA

is circularized (2); and 3. After either UV or thymine-less induction

of a λ-lysogen a small fraction of the newly synthesized DNA has been

reported to have the sedimentation properties of closed-circular DNA

(35). In the latter case no evidence was obtained that prophage material

(i.e. part of the bacterial genome) entered a circular form, although

it is doubtful whether the assay was sufficiently sensitive to detect

1% of the bacterial genome (the fraction which would be prophage mater-

ial).

The criteria used to identify a DNA molecule as a covalently-closed,

double-stranded ring are: 1. A very rapid sedimentation rate at a de-

naturing pH; 2. Easily reversible denaturation; 3. An increased buoyant

density in alkaline CsCl compared to non-circular DNA (59);and 4. An

enhanced buoyant density relative to linear DNA in a mixture of CsCl and

an intercalating dye such as ethidium bromide (42). Also necessary, but

not sufficient conditions for establishing covalent circularity, are an

increased sedimentation rate at neutral pH compared to linear DNA of the

same molecular weight, and observation of circular molecules in the

electron microscope. The rapid sedimentation rate at a denaturing pH

is caused by the conservation of turns in the helical molecule upon

denaturation, producing a collapsed structure with a mass twice as large

as the individual polynucleotide chains and a much smaller frictional

coefficient. The increased buoyant density at alkaline pH is not so well

understood. It might be caused by exclusion of H_2O from the denatured,

collapsed structure. The increased buoyant density of circular DNA relative

to linear DNA in CsCl-ethidium bromide is due to the smaller amount of

dye bound by the circular DNA (Bauer & Vinograd, personal communication).

The dye, being primarily made of carbon atoms, has a much lower density

than the DNA and thus the density of the dye-DNA complex is decreased

in proportion to the amount of dye bound by the DNA. Superficially, one

might imagine that the lesser extent of dye-binding by the circular DNA

is due to mechanical restraints caused by the change of the helix pitch

as the dye intercalates. On the other hand, theoretically at least, a

linear molecule might be completely unwound by the intercalating dye.

In addition to possessing the usual helical turns, most circular,

double-stranded ring DNA molecules are now believed to possess tertiary

twists or supercoiling (see reference 59 for a review). The initial evidence

was from electron microscopy which revealed tightly twisted forms of polyoma

DNA (61). Other evidence supports this interpretation; such as a rapid sedi-

mentation rate at neutral pH (up to 50% more rapid than open circular

forms of the DNA), and a decrease in the sedimentation rate or an increase

in the buoyant density at a pH just below that required to produce com-

plete denaturation. These changes probably represent local denaturation

(or a small change of pitch throughout the molecule) which removes the

tertiary turns and, in effect, produces a molecule with the properties

of an open ring. This conversion can also be effected by intercalation

of ethidium bromide (42). Another property of closed, twisted circular
DNA, as yet not understood, is the sensitivity of its sedimentation rate
at neutral pH to changes in the ionic strength of the medium (2; Part II).

Evidence that circular λ DNA is involved in the replication process
has been obtained by quantitative physical analysis of the λ DNA synthe-
sized after infection of a suseptible host by a cI mutant of λ. The time
at which the circular DNA is synthesized and the amount which accumulates
in the cell early in infection, provide an explanation for the eclipse
of DNA infectivity observed in the helper assay by Dove & Weigle (11).
The circular DNA replicates semi-conservatively, as demonstrated by
radioactive and density labeling experiments.

2. SUMMARY

The structure and replication of the fast-sedimenting form of λ
DNA found after infection have been examined. The sedimentation rate
at neutral and alkaline (strand-separating) pH, shear resistance, and
buoyant density in alkaline CsCl are consistent with the interpretation
that the rapidly-sedimenting DNA is a covalently-closed, circular duplex
wound in a superhelix.

Mitomycin C has been used to inhibit bacterial DNA synthesis in
E. coli hcr⁻ cells infected with λ. An analysis of the λDNA synthesized
under these conditions reveals that before intracellular phage are pro-
duced, synthesis of DNA with the sedimentation properties of the closed-
circular form predominates. At the end of the phage eclipse period,

net synthesis of circular DNA stops and synthesis of DNA with the sedi-
mentation properties of mature viral DNA begins. If bacteria are infected
with λ in the presence of 100 μg/ml. of chloramphenicol, the conversion
of the injected phage DNA to the fast-sedimenting form occurs, but no
replication ensues. In the presence of 30 μg/ml. of chloramphenicol
(added 15 minutes before infection) the circular DNA is able to replicate,
albeit more slowly than in the absence of chloramphenicol. The synthesis
of DNA with the sedimentation properties of mature viral DNA is com-
pletely inhibited in the presence of 30 μg/ml. of chloramphenicol.

The replication of the circular DNA has been studied using density
and radioactively labeled phage particles to initiate the infection.
The circular DNA of parental origin consists of both unreplicated and semi-
conservatively replicated molecules. If the infection is performed in
medium containing ^{3}H-thymine, the progeny circular DNA has either a
hybrid density or a fully light density. The ratio of progeny to parental
DNA rings rises to at least twenty by the end of the phage eclipse period.
Most closed-circular DNA molecules containing parental label replicate
more than once semi-conservatively. The DNA of hybrid density derived
from the fast-sedimenting component on a sucrose gradient is resistant
to strand separation in alkali. The formation of parental and progeny
circular DNA explains the eclipse of infectivity measured in the helper
assay (11).

3. MATERIALS AND METHODS

The bacterial and phage strains, media, solutions and techniques used, but not described below, are contained in the preceding paper (67).

(a) Bacterial strains

E. coli AB2497 is a thymine-requiring derivative of AB1157, and is uvr^+. E. coli AB2500 is a thymine-requiring derivative of the strain AB1886, and is uvr^- (uvr A-6) (22).

(b) Phage strains

The CII and CIII mutants, C68 and C67, respectively, were obtained from Dr. William Dove, and are from the original collection of Kaiser (29). CII (1000) was obtained by UV mutagenesis of λ^+.

λvir and λvir,h were obtained from Dr. Jean Weigle.

(c) Chemicals

CsCl, optical grade purity, Harshaw Chemical Company; chloramphenicol[1] was a gift of Parke, Davis Co.; mitomycin C[1], Nutritional Biochemicals; $^{15}NH_4Cl$, 99 atom % ^{15}N, Biorad Laboratories, Richmond, California; ^{13}C-glucose, D-glucose ^{13}C, 42 atom % ^{13}C (uniformly labeled), Merck, Sharp and Dohme, Ltd., Montreal, Canada.

[1]Abbreviations used: MC, mitomycin C; CAM, chloramphenicol; pfu, plaque-forming units; SDS, sodium dodecyl sulphate.

(d) Media

KG medium is K medium with glycerol replacing glucose at a final concentration of 0.1%

TG is the medium used to prepare phage containing $^{13}C^{15}N$ or $^{32}P^{13}C^{15}N$. It is similar to TPG medium (47) and contains per liter: 0.5 g NaCl, 8.0 g KCl, 0.2 g $MgCl_2 \cdot 6H_2O$, 12.1 g Trizma base, 0.046 g KH_2PO_4, 1.0 ml. of $0.1\underline{M}$ $CaCl_2$, and 0.02 g Na_2SO_4. The pH is adjusted to 7.4 before autoclaving. This is supplemented with ^{12}C-glucose and $^{14}NH_4CL$ or ^{13}C-glucose and $^{15}NH_4Cl$ to give a final concentration of 0.1% of each, to make either L(light)-TG or H(heavy)-TG, respectively. The generation time of E. coli K12 strain 3110 in either L-TG or H-TG is about 75 minutes.

(e) Preparation of λ phage containing radiophosphorus, ^{13}C-carbon and ^{15}N-nitrogen

E. coli 3110 is carried on an agar slant containing L-TG as the nutrient source. An inoculum from the slant is grown overnight to saturation (about 5×10^8 bacteria/ml.) in H-TG. This culture is diluted 100X into fresh H-TG and again grown to saturation. Typically, one-half ml. of this culture is diluted into 20 ml. of fresh H-TG and grown to an absorbance of 0.5 at 600 mμ (about 4×10^8 bacteria/ml.). The bacteria are centrifuged, resuspended in 2 ml. TM, and infected with λc26 at a multiplicity of about 5 pfu/bacterium. After 15 minutes adsorption at

37°, the infected bacteria are added to 18 ml. of fresh H-TG containing either one-half or one mc of carrier-free radiophosphorus and aerated vigorously at 37°. After lysis occurs the phage are purified as described previously. The density of the phage containing $^{13}C^{15}N$ is about 0.032 g/cm^3 greater than the density of phage containing ^{12}C and ^{14}N. More than 95% of the phage have this density increment; the DNA isolated from the phage has the same density increment. The density distribution of a typical preparation is presented in Figure 1.

(f) DNA extraction

Method A: This is the procedure described previously (68).

Method B: About 2×10^9 infected bacteria are pelleted gently, washed with TE, and resuspended in 0.40 ml. of 0.50 M sucrose dissolved in TE. 0.2 ml. of 1% grade 1 lysozyme (dissolved in 0.25 M tris, pH 8.1) and 0.2 ml. of 0.2 M EDTA are added and the suspension incubated for 15 minutes at 0°C. 1.2 ml. of SDS[1] (0.5-1.0%) is added with gentle mixing and then 0.1 ml. of Pronase, prepared as described below, is added to give a final concentration of 50 µg/ml. The resulting clear, extremely viscous solution is incubated at 37° for two hours and then frozen and kept at -20°C. This procedure is similar to that described by Massie & Zimm (37), but the lysate is not extracted with phenol. The procedure seems acceptable by the following criteria: 1. Phage DNA thus extracted has the same sedimentation properties (Figure 2), buoyant density, and infectivity in the helper assay as phenol-extracted phage DNA. 2. Component I is not converted to component II during the two hour incubation

in SDS and Pronase, although it is if the Pronase has not been self-digested and heated (Figure 3). Its sedimentation properties and buoyant density (at pH 10.5 and 8.1, respectively) are also not affected by omission of the phenol extraction (Figures 3 and 4). However, sedimentation at pH 12.5 is somewhat erratic. This might be due to co-precipitation with undigested protein in alkali. 3. The recovery of vegetative DNA from the washed cells is 100%. Pronase (Calbiochem, grade B) is dissolved at a final concentration of 1.0 mg/ml. in 0.01 $\underline{M}$ tris, 0.02 $\underline{M}$ EDTA, pH 8.1, and incubated ("self-digested") for two hours at $37°C$. It is then heated at $80°C$ for two minutes and quickly cooled.

This procedure has several advantages over Method A, or any other procedure involving extraction of the DNA by phenol. There is no possibility for the DNA to become trapped at the phenol-buffer interface, which occurs, to a variable extent, even if the lysate is treated with Pronase before phenol. Also the lysate can be immediately examined, e.g. by sucrose gradient sedimentation.

When cell extracts prepared by this technique are to be examined by sucrose gradient centrifugation, the sucrose is dissolved in 0.01 $\underline{M}$ tris, 0.001 $\underline{M}$ EDTA, pH 10.5. The high pH is used to help prevent aggregation. When the extracts are to be analyzed by CsCl density gradient centrifugation, it is preferable to lyse the cells with Sarcosyl rather than SDS in order to prevent the SDS (which is insoluble in concentrated CsCl) from trapping some of the DNA at the top of the gradient.

One disadvantage has been noticed with phage DNA extracted by this method. Such DNA is 2-3-fold less infective in the spheroplast assay

than phenol-extracted phage DNA, although the specific infectivities of

the two preparations in the helper assay are identical. This is shown

in Table 1. A preparation of ^{32}P-λb2b5c DNA was treated with SDS and

Pronase as usual and one-half of the sample was extracted with phenol

and dialyzed. The number of infective centers (helper assay) or phage

(spheroplast assay) produced per ^{32}P cpm by the two preparations is

recorded. The values are taken from the linear range of the assays,

and duplicate samples were assayed in both cases. The number of phage

produced was proportional to the DNA concentration in the range tested

in the spheroplast assay (from a 100-fold to a 10,000-fold dilution).

This indicates that some extraneous factor (SDS?) does not cause the

inhibition. Some tenacious protein or other constituent of the phage

particle not grossly affecting the physical properties of the DNA

is probably responsible.

Of the experiments reported here, all except those in Results g(ii)

were performed using DNA extracted by Method A.

(g) Shearing DNA solutions

DNA solutions are sheared with a Virtis homogenizer, essentially

as described by Kaiser (30).

(h) CsCl equilibrium density gradient centrifugation of DNA

1.15 g of the DNA solution to be analyzed are mixed with 1.95 g

of CsCl (final density = 1.73 g/cm^3) in a centrifuge tube. This solution

is overlayed with paraffin oil, and centrifuged at 30,000 rev./min at

20° for 36-48 hours in the SW39 rotor.

Alkaline CsCl equilibrium density gradient centrifugation (60)

is performed similarly, but in polyallomer centrifuge tubes. 2.88 g

of CsCl is dissolved in 2.00 g of a DNA solution in 0.05 $\underline{M}$ K_3PO_4 with

KOH added to bring the final pH to 12.5-12.8 as measured on a Beckman

Zeromatic pH meter with a general purpose glass electrode. The final

density of the solution should be 1.76 g/cm^3.

After centrifugation, a hole is pierced in the bottom of the tube

and drops are collected. If only radioactivity is to be assayed in the

fractions, the drops are collected directly into glass scintillation

vials containing Whatman GF/glass filters. After the filters have dried,

10 ml. of toluene containing Liquiflour (New England Nuclear Corp.) are

added and they are counted in a Beckman or Nuclear Chicago scintillation

counter. The efficiency of counting ^{3}H-DNA by this method is 23% (in

the Beckman counter), compared to a value of 30% obtained simply by drying

a small sample of ^{3}H-thymidine on the filter. The variation in CsCl

concentration from the top to the bottom of the gradient does not alter

the counting efficiency significantly. If bioassays are to be performed

on the fractions, drops are collected into the appropriate buffer, and

samples are spotted onto filters in scintillation vials and counted

as described above.

The density gradient is measured by collecting two drop fractions

into a vial containing 0.5 ml. of paraffin oil. After collecting all

of the gradients, the density of the CsCl in the vials is measured by

weighing 50 μl. of the solution in calibrated 50 μl. pipettes.

(i) <u>Lysogenization</u>

This procedure is adapted from that of Fry (15) and Sechaud (45).
E. coli 3110 is grown to 2 x 10^9 bacteria/ml. in K medium. The cells are
then collected on a filter, washed, and resuspended in TM and aerated
(starved) for an hour at 37°C. λc$^+$ is added to a multiplicity of 15
pfu/bacterium and allowed to adsorb for 15 minutes at 37°C. The infected
bacteria are then collected on a filter, washed with TM, and diluted
10X into prewarmed K medium and aerated for at least 15 minutes before
plating for lysogenic colony formers and infective centers. Lysogenic
colony formers, non-lysogenic colony formers, and infective centers are
assayed by a method developed by J. Weigle (personal communication).
An appropriate dilution is plated on UV-irradiated C600 (the UV dose being
about twice that required to induce a lysogen). Infective centers are
recognized as plaques, non-lysogenic bacteria as small colonies growing
faster than the lawn of C600, and lysogenic bacteria as a plaque centered
by a small colony of lysogenic bacteria. The plaque is produced by
spontaneous lysis and phage production by some of the lysogenic bacteria
in the central colony. This technique may be used whenever the lysogen-
ized bacteria are able to grow faster than the indicator strain.

90-95% lysogeny is routinely obtained. If a thymine-requiring
host is to be lysogenized, it is important to supply the culture with
thymine during the starvation in TM; otherwise very few cells are lyso-
genized. (They seemed to be "preinduced.") Even with this precaution,

we have never been able to lysogenize more than 70% of a thymine-requir-

ing strain of E. coli.

(j) Pulse labeling with ^{3}H-thymidine or ^{3}H-thymine

Pulse labeling of E. coli (a prototroph) with ^{3}H-thymidine is

done essentially as described by Smith and Levine (50).

E. coli AB2500 (hcr$^-$, thymine-requiring) has been used to study

the kinetics of DNA synthesis in sensitive cells infected with clear

mutants of λ after treatment with MC (Methods (k)) to inhibit host DNA

synthesis. The cells are grown in KG medium containing 5 μg/ml. each

of thymine and uracil (the uracil is present to prevent incorporation

of radioactivity into RNA which otherwise occurs with some of the

batches of ^{3}H-thymine obtained). They are treated with MC, washed and

resuspended in 1/10 volume TM, and infected with a multiplicity of 5

pfu/bacterium. After 5 minutes adsorption at 37°C they are diluted 10X

into prewarmed (37°C) KG medium supplemented as described above. At

the desired times 1.0 ml. is removed and added to an aeration tube con-

taining 10 μc of ^{3}H-thymine and aerated for 2.5 or 5 minutes. Then

0.5 ml. of cold 15% TCA containing 2.0 mg/ml. ^{1}H-thymidine is added and

30 minutes later the precipitate is collected on GF/A filters and counted

in the scintillation counter.

Artificially high rates of incorporation occur if thymine-requiring

bacteria, growing in ^{1}H-thymidine containing media, are pulsed with radio-

active thymidine. This does not occur if the same culture is pulsed with

^{3}H-thymine, or if the bacteria are grown in thymine-containing media and

pulsed with ^{3}H-thymine. The bacteria may degrade the thymidine in the

medium and then take up the radioactive thymidine preferentially when

it is added during the pulse.

(k) Mitomycin C treatment of bacteria

The amount of MC used in each experiment is based on the mg

equivalents contained in the vial, as indicated by the manufacturer,

not on the actual weight of material in the vial. In practice, the

contents, two mg equivalents of MC, are dissolved in two ml. of TM just

prior to the experiment and kept in the dark. This solution is then added

to the culture to give a final concentration of 20-50 μg equivalents of

MC/ml. of culture. The culture is kept in the dark without aeration

for ten minutes at 37°C and then the bacteria are collected on a DA

millipore filter and washed with several volumes of TM. The washed

bacteria are resuspended in the desired medium. Solutions of MC are

used only once (34).

4. RESULTS

(a) Sedimentation properties of injected λDNA

It has been shown that λDNA may be converted to a fast-sedimenting

form after infection of sensitive or homoimmune lysogenic cells(67, 2, 65).

The evidence presented in this section supports the idea that the fast-

sedimenting form, component I, is a twisted, closed-circular DNA duplex, analogous to polyoma viral DNA component I (61) or $\emptyset$X replicative form component I (5, 27, 43). The sedimentation properties of component I, whether extracted from a homoimmune lysogen or a susceptible cell, are the same.

In order to examine the intracellular DNA of both types (i.e. both repressed and non-repressed) extracted from the same cell, E. coli 3110 (λ) was infected as described in the legend to Figure 5 with (a) ^{3}H-λvir, (b) ^{32}P-λc26, or (c) a mixture of ^{3}H-λvir and ^{32}P-λc26.

The infection with λvir, both alone and in the mixed infection, produced a burst size of 50-100 λvir pfu/cell. In the mixed infection, less than 2 pfu/cell were λc26, in agreement with the observation of Thomas & Bertani (57) that in a mixed infection with a heteroimmune phage, λvir in this experiment, and a homoimmune phage, λc26, the homoimmune phage is still repressed despite the replication of the heteroimmune phage. (When λvir infects a λ-lysogen as here, it, too, is apparently initially repressed, as evidenced by a lower initial rate of λ-mRNA synthesis (49).)

The sedimentation of the ^{3}H-λvir DNA, extracted 35 minutes after the single infection, is shown in Figures 5a and 5b. To obtain Figure 5a the DNA was sedimented through a sucrose density gradient at pH 8.1. After sedimentation each fraction was assayed for radioactivity and infectivity in the helper assay. Figure 5b shows a sedimentation profile obtained with the same DNA, mixed with a marker ^{32}P-λDNA, after centrifugation through a sucrose gradient at about pH 12.6. Sedimentation proceeds from right to left in this and in all succeeding sedimentation

diagrams. The slow sedimenting peak of radioactivity at pH 8 sediments

at the same rate as the helper infectivity and by this criteria would

be component III, mature phage DNA (2). The fast sedimenting radioactivity

has sedimented 1.85 times as far as the helper infectivity at pH 8 and

3.5 times as far as the ^{32}P-λDNA marker at pH 12.6. These properties

are identical to those of component I (2) isolated from homoimmune in-

fected cells. This is also shown in Figure 2c which illustrates the

sedimentation pattern at pH 8 obtained with the repressed DNA extracted

35 minutes after infection of E. coli 3110(λ) with ^{32}P-λc26. Two

major radioactive components are again observed, only one of which is

infective in the helper assay. By sedimentation and biological properties

these correspond to components I and III.

The DNA extracted 35 minutes after mixedly infecting E. coli

3110(λ) with ^{3}H-λvir and ^{32}P-λc26 was sedimented on a sucrose density

gradient at pH 8. The results are shown in Figure 5d. The distributions

of ^{3}H and ^{32}P are remarkably similar, in that the same two components

are present, presumably components I and III, and they are present in

almost exactly the same proportions.

Some of the DNA (Figure 5a) sediments to the bottom of the centri-

fige tube. However, if the bottom fraction is recentrifuged, it yields

approximately the same pattern as the original extract, and hence is

probably an artifact caused by the non-radial walls of the centrifuge

tube. Also, less material is found in the pellet after centrifugation

if the DNA is diluted several-fold before centrifugation.

A summary of some of the sedimentation properties of component I

obtained from infected, susceptible or homoimmune lysogenic cells is shown

in Table 2. The ratic of the distance sedimented by component I to the

distance sedimented by a marker phage DNA is recorded. The alkaline suc-

rose gradients contained 0.10 $\underline{M}$ NaOH plus NaCl to provide the desired

final ionic strength. A gradient of pH, as well as sucrose, was present,

varying from pH 12.6 (in 5% sucrose) to pH 12.0 (in 20% sucrose). The

pH values are corrected by determining the Na^+ correction in the res-

pective sucrose solutions. At low ionic strength in alkali a minor peak

moving about twice as fast as the marker is sometimes observed. This

may be a partially renatured form of component I (5) and it is likely

that this is the middle component we first reported (67).

(b) <u>Shear</u> <u>resistance</u> <u>of</u> <u>component</u> <u>I</u>[2]

If the relatively rapid sedimentation rate of component I is a

consequence of a decrease in the frictional coefficient of the molecule

rather than an increase in its mass, then the DNA should be more compact,

and hence less sensitive to hydrodynamic shear than is a molecule of the

same sedimentation rate but a more extended configuration. This expec-

tation is at least partially realized.

Intracellular ^{32}P-λ DNA was obtained from E. coli 3110 (λ) superin-

fected with ^{32}P-λc26. High molecular weight E. coli DNA containing ^{3}H-

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[2]Similar experiments have recently been reported by Ogawa and

Tomizawa (41).

thymine was obtained by growing E. coli CR34 in the presence of ^{3}H-thymidine for several generations and then gently extracting the nucleic acids as described in Methods (using Method A). The ^{32}P- and ^{3}H-DNA samples were mixed and a portion was sheared in the Virtis homogenizer at 0° for twenty minutes at a setting which should break linear λDNA molecules into halves (30). Samples of the sheared and unsheared DNA were then layered on separate sucrose density gradients (pH 8) and centrifuged. After centrifugation samples from each fraction were assayed for ^{3}H, ^{32}P, and infectivity in the helper assay.

The results are shown in Figures 6a and 6b. In the unsheared sample the E. coli DNA sediments at about the same rate as component I, about 1.9 times faster than the helper infectivity. If the helper infectivity is assumed to correspond to the position of linear λDNA, and the molecular weight relation, $D_1/D_2 = (M_1/M_2)^{0.35}$ (4), holds for such high molecular weight DNA, then the weight average molecular weight of the E. coli DNA in this preparation is about 200×10^6 daltons. The sheared E. coli DNA sediments only 0.38 as far as before shearing, indicating a reduction in the molecular weight to about 12×10^6 daltons.

The peak of infectivity measured in the helper assay sediments only 0.75 as far after shearing as before. This reduction of the sedimentation rate corresponds approximately to a halving of the molecular weight of λDNA. The relative specific infectivity of the slow peak decreases to about 30% after shearing, which is also consistent with a conversion to half-molecules (30).

However, component I sediments at approximately the same rate after shearing as before. The recoveries after the shearing procedure, of both the ^{32}P and ^{3}H were only 25 and 33%, respectively. For this reason the only conclusion possible is that some of component I is resistant to a shear force which breaks intact λDNA molecules into half-length fragments. It also appears from the distribution of ^{32}P in Figure 6 that component I was either preferentially lost (compared to the smaller fragments) during the shearing or that some of it was converted to slower sedimenting DNA.

The resistance to shear of component I makes it appear unlikely that it contains regions of single-stranded DNA, since these would be quite easily broken by shear stress (9).

(c) <u>Buoyant density of component I in alkaline CsCl</u>

· Another characteristic of covalently-closed circular DNA is its increased buoyant density in alkaline CsCl compared to that of a linear or "nicked" circular form (64, 5). <u>E. coli</u> CR34 was infected with ^{32}P-λ c26 and the extracted DNA was sedimented on a neutral sucrose gradient. Fractions containing component I were pooled and mixed with ^{3}H-<u>E. coli</u> DNA and the density adjusted to about 1.76 g/cm^{3} with CsCl. The buffer was 0.05 $\underline{M}$ K$_{3}$PO$_{4}$-KOH, pH 12.8 (measured in CsCl). The solution was centrifuged for 60 hours at 17° C and 30,000 rev./min. After centrifugation, fractions were collected from a hole punched in the bottom of the tube.

As shown in Figure 7a the majority of the ^{32}P-DNA has a buoyant density in alkaline CsCl about 0.02-0.03 g/cm^3 heavier than the ^{3}H-E. coli DNA. Because of the hydrolysis of DNA in alkaline CsCl (60, 58) this component cannot be estimated quantitatively by this means. About 20% of the ^{32}P-DNA has the same density as the marker. If component I is centrifuged in alkaline CsCl at 36°, in order to prevent crystallization of CsCl at the bottom of the gradient, only a small fraction of component I has a density greater than that of phage DNA. This is shown in Figure 7b.

(d) Sedimentation of intracellular λDNA extracted without deproteinization

Assuming component I is a twisted, circular duplex, as the evidence indicates, it would be of interest to know the cause of the tertiary twisting. Since all of the twisted, circular forms of DNA thus far studied have been examined after deproteinization, usually by extraction with phenol, it seemed possible that the tertiary coiling resulted from the removal of protein.

E. coli 3110(λ) was grown and infected with ^{32}P-λ c26. Thirty minutes after infection the bacteria were harvested, converted to spheroplasts, and lysed by dilution into distilled H$_2$O. A ^{3}H-λDNA sedimentation marker was added and the solution was centrifuged through a sucrose gradient at pH 8. The results are shown in Figure 8. The majority of the intracellular DNA has the sedimentation properties of component I at this pH and ionic strength (0.1 M). Apparently removal of a protein moiety from the DNA(unless removed by dilution in this experiment) is not related to the high sedimentation rate of component I at neutral pH.

(e) Radioactive labeling of vegetative λDNA

In order to study the replication of λ DNA, and specifically to determine whether or not the circular form of λDNA replicates, it is important to be able to distinguish bacterial DNA, which might be synthesized by the host after infection, from the phage-specific DNA. Such a distinction can be made by studying the production of infectious λDNA in the spheroplast assay described previously (68). It would also be advantageous to be able to label vegetative λ DNA with radioisotopes without the problem of concurrent host DNA synthesis. The observation that mitomycin C treated hcr⁻ bacteria are able to support ØX174 growth normally without appreciable host DNA synthesis (34) prompted us to apply this technique to λ-infected cells.

(i) Effect of MC pretreatment on phage development and ^{3}H-thymidine incorporation in λ-infected E. coli AB2500(hcr⁻)

E. coli AB2500 (T⁻, hcr⁻:uvr A-6, 22) was grown in KG medium plus thymidine and uracil and the culture was divided into two portions. One portion was treated with 50 μg/ml. of MC for 10 minutes prior to infection (see Methods for details), washed and resuspended in KG medium plus 5 μg/ml. each of thymidine and uracil. The other portion was washed and resuspended identically but not treated with MC. One-half of each portion was then infected with 4.3 c26/colony former, ^{3}H-thymidine was added to all four cultures, and they were aerated at 37°C. At the times indicated in Figure 9a samples were withdrawn to measure the amount of cold TCA-insoluble radioactivity incorporated and to assay for intra-

cellular phage. The zero-minute sample was taken within 30 seconds
after adding the ^{3}H-thymidine to the culture.

The data on the infection itself, colony formers before and after MC
treatment and infection, and infective centers plus and minus MC are shown
in Table 3a. The data on the isotope incorporation and phage growth are
shown in Figure 9a. The effect of MC in this experiment is to reduce the
final phage yield by about 50% and to slow somewhat the rate of production
of intracellular phage. In other essentially identical experiments, no ef-
fect of MC on the final phage yield has been noted. The amount of ^{3}H-thymidine
which is incorporated by the two non-MC treated cultures (plus and minus phage)
is very similar, and is about three times the amount which is incorporated in
the MC-treated, infected culture. The large number of viable uninfected
bacteria (Table 3a) in the non-MC-treated, infected culture make it seem
likely that a large fraction of the ^{3}H-thymidine incorporation in this cul-
ture is due to bacterial DNA synthesis. The amount of ^{3}H-thymidine incorpor-
ated by the MC-treated, uninfected culture is reduced to about 3% of the in-
corporation in the MC-treated, infected culture. The initially high level of
incorporation by the former is not usually observed (see Results (f) for
example).

(ii) <u>Effect of MC pretreatment on phage development and ^{3}H-thymidine
incorporation in λ-infected E. coli AB2497(hcr$^+$)</u>

The data from an experiment identical to the one described above,
but using an hcr$^+$ host (AB2497), are shown in Table 3b and Figure 9b.
The treatment with MC reduced the phage yield to 1-2% of the yield in

the non-MC-treated, infected control. As is evident from Table 3b,

the phage are not produced by 2% of the cells releasing a normal burst

since the capacity to become an infective center is only reduced to 20%.

The average burst size in this population of infective centers is only

2.5 pfu/cell, compared to 35 in the control. The time these few phage

are made is delayed by about 20 minutes compared to the control.

The total incorporation of ^{3}H-thymidine in the MC-treated cultures,

infected or not, is only 1-2% of the incorporation measured in the un-

treated cultures.

As may be noted in Tables 3a and 3b, a large fraction of bacteria

survived λ infection at a multiplicity of about 4 pfu/cell, even when

85% of the pfu had adsorbed (as measured by $CHCl_3$ treatment of the

infective centers). For example, in the experiment shown in Table 3b,

85% of the pfu had adsorbed by ten minutes but only 48% of the bacteria

were converted to infective centers, i.e. the other 52% were still able

to form colonies. Since a cI mutant of λ was used, the surviving bacteria

could not be lysogenic. This is not an uncommon result in our exper-

ience. Washing and resuspending the bacteria at a higher concentration

in TM and then allowing adsorption and injection to occur in TM at 37°C

yields better killing of the bacteria than does infection in the growth

medium. Phenotypic resistance to λ-infection is shown by some strains

of E. coli when grown prior to infection in glucose- but not glycerol-

containing medium (23). However, the bacteria used to obtain the data

in Table 3 had been grown in glycerol-containing K medium.

MC pretreatment (of an hcr$^-$ strain) is thus especially useful when it is more convenient to infect the bacteria in the growth medium (or if the infection is done at a low multiplicity of phage per bacterium) since any uninfected bacteria present will synthesize little, if any, DNA.

(iii) <u>Inhibition of DNA synthesis in uninfected hcr$^+$ and hcr$^-$ cells and the capacity to support λ growth as a function of MC concentration</u>

To determine the concentration of MC needed to reduce bacterial DNA synthesis to a low level, AB2500 and AB2497 were grown as usual, the cultures divided into 6 portions, and then treated with 0, 0.2, 1.0, 5.0, 25 or 50 μg/ml. of MC. They were then washed once by centrifugation and resuspended in medium containing ^{3}H-thymidine. After 45 minutes aeration at 37$^°$ the amount of acid insoluble ^{3}H-thymidine incorporated was determined both before and after hydrolysis with 0.5$\underline{M}$ NaOH (14 hours at 37$^°$C).

A similar experiment using the same concentrations of MC was performed withλc26-infected AB2497(hcr$^+$) to determine at which concentration of MC the capacity for λ growth was retarded. As noted before, MC at a concentration of 50 μg/ml reduces the burst size of λ in an <u>hcr</u>$^-$ host by at most a factor of two, and probably not at all. Capacity was measured by determining the mean burst size 90 minutes after infection.

The results of the experiments are shown in Figure 10. In all cases the residual fraction of the quantity measured, compared to the value obtained without MC pre-treatment, is recorded.

The loss of the ability to synthesize DNA occurs at about the same concentration of MC in both hcr$^+$ and hcr$^-$ cells, although the ability to form colonies is much more sensitive in the latter. In both cases, however, the surviving DNA synthesis is greater than the survival of colony-forming ability, indicating that dead cells are able to synthesize DNA, as expected. If this is mainly repair synthesis, it occurs to the same extent in cells able or not to excise thymine dimers.

The capacity to produce λ is drastically reduced between 1.0 and 5.0 µg/ml of MC. Bacterial DNA synthesis is reduced to 20 and 5%, respectively, at these concentrations. Depending on the level of labeling of bacterial DNA which one could tolerate in an experiment, it is thus possible to MC-treat hcr$^+$ bacteria before infection to suppress host DNA synthesis without too drastically curtailing the phage capacity. However, it seems much more advantageous to infect an hcr$^-$ host after treatment with, say, 20 µg equivalents per ml. of MC, which reduces host DNA synthesis in uninfected bacteria to about 1-2% without affecting the phage capacity. This is especially true since the potency of different lots of MC seems to vary[3] and the tolerance, in terms of phage capacity, of the hcr$^-$ host drops very abruptly as a function of MC concentration (Figure 10).

[3]As noted in Methods, the MC is sent by the manufacturer in mg equivalents of the drug. The potency of the drug is measured in terms of LD_{50} for mice, thus "equivalent" preparations might behave quite differently toward bacteria.

(f) Kinetics of DNA synthesis in λ-infected cells

(i) DNA synthesis after infection with c+, cI, cII, and cIII

The rate, at various times after infection, of incorporation of ^{3}H-thymidine in a culture in which 90% of the cells, E. coli 3110, were lysogenized (as described in Methods) by λc+ is shown in Figure 11a. An uninfected control is shown in the same figure. The rate of incorporation is determined as described in Methods, following the procedure Smith & Levine (50) used to measure the rate of DNA synthesis in P22-infected S. typhimurium. As they pointed out, this is a much more sensitive method to detect subtle changes in the rate of DNA synthesis caused by phage infection than to measure the total uptake over longer periods of time. Of course, the rate of incorporation is a valid measure of the rate of DNA synthesis only if uptake of the isotopically labeled component and its entry into the endogenous pool are not rate-limiting. In our experiments using E. coli 3110, the ^{3}H-thymidine incorporation is linear between 15 and 120 seconds after adding the isotope, indicating that uptake and entry into the pool are not rate limiting.

During lysogenization by λ, as with P22, there is a maximum in the rate of incorporation of ^{3}H-thymidine which occurs 6 minutes after infection (Figure 11a). (In our experiments, 6 minutes after infection means 6 minutes after dilution from TM into growth medium at 37°C. In the experiments of Smith & Levine (50) the maximum occurred 6 minutes after phage had been added to the growing bacteria.) Between 12 and 35 minutes after infection the rate of DNA synthesis is reduced to the back-

ground level, indicating a cessation of both phage and host DNA synthesis
at this time. After 35 minutes the rate begins to increase and there-
after parallels the uninfected control. The analogy with P22 is striking,
both in the sequence of events and the timing.

Similar pulse-labeling experiments with previously-starved E. coli
(as were used for the lysogenization experiment described above) were
performed after infections with a cII (c1000) and with a cI(c71) mutant
of λ. The results are shown in Figures 11b and 11c. The cII mutant
clearly shows the same peak of ^{3}H-thymidine incorporation at 6 minutes
as does the wild-type during lysogenization. However, no subsequent
repression of incorporation is evident, and lysis occurs at 40 minutes
after infection, as judged by a loss of turbidity and a decrease in the
rate of isotope incorporation. The cI mutant (c71) shows a slight maximum
in the rate of incorporation at 8-10 minutes after infection and DNA
synthesis appears to be somewhat repressed (compared to the control or
the cII infected culture) between 12 and 18 minutes. The culture does
not lyse until about 60 minutes. Neither the maximum in the rate of
incorporation nor the repression are as marked as during lysogenization
with wild-type λ. The interpretation of the experiment done with λc71
is somewhat complicated because it is a temperature-sensitive clear
mutant (M. Lieb, personal communication), being c+ at $30°$ and cI at
$40°$C. The temperature used in these experiments ($37°$) may have allowed
a partial wild-type phenotype even though a normal yield of phage was
produced.

In order to establish further that the early-synthesized DNA was
phage-specific, and to confirm these results in a thymine-requiring host,
MC-treated AB2500 was used as a host for infections with cI(c26),
cII(c68), and cIII(c67).   (No infection with λc+ is included because
we have been unable to achieve a high enough frequency of lysogeny in
a thymine-requiring host to permit an unambiguous evaluation of the
results.) The rates of ^{3}H-thymine incorporation in the infected and con-
trol cultures, together with phage growth curves are shown in Figures
12a, b, and c.

The rates of isotope incorporation and phage production are very
similar in the three infected cultures. The only clear difference is
the slower rate of phage production and lysis in the cI infected culture.
There is no evidence that DNA synthesis is repressed at any time in any
of the infected cultures (except after lysis). It seems unlikely that
the lack of repression is due to the MC pretreatment since the P22-
induced repression in the rate of ^{3}H-thymidine incorporation occurred
even in UV-irradiated cells. In general, the kinetics of DNA synthesis
in MC-treated AB2500 are similar to those observed after infection
of starved E. coli 3110 with λcII(c1000).

DNA synthesis starts within minutes after dilution into growth
medium. The rate of ^{3}H-thymine incorporation continues to increase until
about 20-25 minutes after infection when it becomes nearly constant.
Since the pulses in this experiment were relatively long, 2.5 minutes,
it is difficult to discern a maximum in the rate of incorporation between
5 and 10 minutes as was seen in infected E. coli 3110. The time at which

the rate of incorporation reaches its maximum level, which it then maintains, is the same time that mature phage begin to appear in the cell (Figures 12a, b, c), and could represent the onset of steady-state conditions of phage DNA synthesis and withdrawal for maturation.

(ii) DNA synthesis in MC-treated E. coli AB2500 infected with λ in the presence of CAM

After treating the bacteria with MC they were incubated in the presence of 0, 30, and 100 μg/ml. of CAM for 15 minutes in TM at 37°C. The cells were then infected with λc26 and after dilution into medium containing the appropriate amount of CAM the rate of DNA synthesis was determined by measuring the amount of TCA-insoluble ^{3}H-thymine incorporated in sequential five minute intervals during the infection. The results are shown, together with those from an uninfected control (without CAM) in Figure 13a and b. Figure 13b shows the integrated form of the rate curve obtained in Figure 9a at 0 and 30 μg/ml. of CAM.

Less than 0.1 phage/cell was produced in the cultures infected and maintained in the presence of CAM. The burst size was 26 phage/cell in the absence of CAM. The number of infective centers was the same at 0, 30, and 100 μg/ml. of CAM and did not decline during the course of the experiment in the cultures containing CAM.

Cells infected in the presence of 100 μg/ml. of CAM do not incorporate a detectable amount of ^{3}H-thymine during the course of the experiment. However, the culture infected and incubated in the presence of 30 μg/ml. of CAM synthesizes DNA at an approximately constant rate for

the duration of the experiment. Sixty minutes after infection the culture

incubated in the presence of 30 μg/ml. of CAM has incorporated 25% as

much ^{3}H-thymine as the infected culture without CAM. The culture without

CAM incorporates ^{3}H-thymine with kinetics similar to those shown in

Figure 12.

(g) Fractionation by sucrose density gradient centrifugation

of the DNA synthesized during infection with λcI

(i) In the absence of CAM

A culture of MC-treated E. coli AB2500 was infected with ^{32}P-λc26

in TM. After allowing 10 minutes at 0°C for adsorption to occur without

injection (2), a sample was taken for DNA extraction (= 0 minute sample

or phage DNA); the remainder of the culture was brought to 37°C, incu-

bated for ten minutes, and blended in a Waring Blendor (to remove un-

injected DNA) for two minutes. The blended cells were centrifuged and

resuspended in KG medium containing ^{3}H-thymidine and ^{1}H-thymidine at

final concentrations of 5 μc/ml. and 10 μg/ml., respectively, and aerated

at 30°C. Samples were withdrawn at various times to measure the amount

of incorporated, TCA-insoluble ^{3}H-thymidine, to assay for intracellular

phage, and for DNA extraction (by Method A). Figure 14 shows the incor-

poration of ^{3}H-thymine, intracellular phage growth, and the appearance

of helper-infective DNA.

Only 30-60% of the ^{3}H-thymine incorporated between 0 and 55 minutes

was recovered after phenol extraction; the remainder could not be re-

moved from the phenol-buffer interface. At 70 and 85 minutes about 90%

of the total incorporated ^{3}H was recovered, suggesting that the DNA syn-

thesized in the early part of the latent period is bound more firmly

to a structure which remains at the interface after phenol extraction.

After 55 minutes most of the incorporated ^{3}H was presumably in phage

particles and more readily extracted (similar results have been found

by others working with phage-infected bacteria, for example, Smith &

Burton (51) and Frankel (14)). More recent experiments using the pronase

extraction procedure described in Methods have verified these results

as is shown in the next section (Figure 18). This method of extrac-

ting DNA from the infected cells avoids the losses which occur as a

consequence of phenol extraction.

The fractionations, by sedimentation analysis through a sucrose

gradient at pH 8, of the ^{32}P, ^{3}H, and helper-infectivity present in the

cell extracts made at 20, 30, 55, and 70 minutes after resuspension in

^{3}H-thymidine-containing medium are shown in Figure 15b-e (sedimentation

analysis of the zero minute (phage DNA) sample is shown in Figure 15a).

Figure 15f illustrates the sedimentation properties of the DNA extracted

at 55 minutes after infection and sedimented through an alkaline sucrose

gradient containing 0.30 $\underline{M}$ NaOH and 0.30 $\underline{M}$ NaCl.

The distribution of ^{32}P-parental DNA on the sucrose gradients does

not change significantly with time. However, the synthesis of vegetative

λDNA containing ^{3}H-thymine shows two phases, early and late, distinguish-

able by the sedimentation properties of the DNA synthesized. From 20

to 40 minutes (at $30°$ C) (the sedimentation patterns of the lysates pre-

pared at 30 and 40 minutes were qualitatively identical) the principal

DNA component synthesized is component I. After 40 minutes, net syn-

thesis of component I ceases and synthesis of component III predominates

thereafter. Before 55 minutes it is also observed that component II

(recognized by its sedimentation 15% faster than the helper-infectious

DNA, component II) is synthesized in significant quantities.

An analysis of the distribution of the ^{32}P-DNA injected by the

infecting phage particles reveals that a considerable fraction has been

degraded. Since only about 20% of the phage in the preparation were

active (able to form a plaque), this may represent degradation of the

injected but non-viable phage DNA. The degradation probably did not

occur during extraction since the ^{32}P-DNA in the 0 minute sample (phage

DNA) appears to be intact. The undegraded ^{32}P-DNA sediments partly at

the position of component I and partly at what appears to be component

III, judging from its cosedimentation with the peak of infectivity in

the helper assay.

The helper infectivity is included on the graphs only as a sedi-

mentation marker (to indicate the position of linear phage DNA molecules)

and cannot be analyzed quantitatively since the assays on the different

gradients were performed with different preparations of competent cells.

The fraction of the ^{3}H-containing progeny DNA, synthesized by 55

minutes after infection, which sediments as component I is 37% as judged

by centrifugation at pH 8, but only 16% as determined by sedimentation

at pH 13. This discrepancy may result from alkaline degradation or storage before the alkaline analysis. The distribution of ^{3}H on the alkaline sucrose gradient (Figure 15) does not resemble that of pulse-labeled DNA from λ-infected cells obtained by Smith & Skalka (52) after a similar analysis.

The results described above indicate that late in an infection with λ about 10-15% of the total (extractable) DNA in the cell has the sedi-mentation properties of component I. The distribution of spheroplast-infectious DNA after sedimentation through a sucrose gradient (pH 8) confirms this observation. The experiment was done similarly to the one described above except the infected bacteria were incubated for 60 minutes at 37°C before the nucleic acids were extracted. The dis-tribution of ^{3}H-containing vegetative DNA and spheroplast-infectious DNA is shown in Figure 16. Before centrifugation λb5 DNA was added as a sedimentation marker. The marker apparently contains a mixture of linear and hydrogen-bonded circular molecules. The hydrogen-bonded circles are infective in the spheroplast assay (68) but not in the helper assay (32).

If extracts made before the end of the phage eclipse are analyzed similarly, the majority of the spheroplast-infectious DNA sediments as component I.

(ii) In the presence of CAM

The uptake of ^{3}H-thymine in MC-treated cells infected with λ in the presence of 30 μg/ml. of CAM suggested that this concentration of

CAM allowed some λ DNA synthesis to occur. This synthesis was inhibited

by 100 μg/ml. of CAM (Figure 9). In the presence of 30 μg/ml. of CAM

neither whole phage nor helper-infectious DNA is produced (11). The

sedimentation analysis, at pH 8, of the DNA synthesized in 0 and 30

μg/ml. of CAM is shown in Figure 17a and b. The infection was carried

out essentially as described in the legend to Figure 13 but phage

λb2b5cI containing radiophosphorus was used to initiate the infection

in the presence of 0, 30 and 100 μg/ml. of CAM. The cultures containing

0 and 30 μg/ml of CAM also contained ^{3}H-thymine during the infection,

but the culture containing 100 μg/ml. of CAM contained only ^{1}H-thymine

since no DNA synthesis occurs at this concentration of CAM (Figure 13).

The nucleic acids were extracted 35 minutes after infection in the

control without CAM and at 45 minutes after infection in the cultures

containing 30 and 100 μg/ml. of CAM. The cell lysates were prepared by

Method B (see Methods) which avoids phenol extraction. Fractions from

the sucrose gradients containing DNA synthesized in the presence of

0 and 30 μg/ml. of CAM were analyzed for ^{32}P (contributed by the infec-

ting phage), ^{3}H-thymine-containing vegetative DNA, DNA infectious in

the helper assay, and DNA infectious in the spheroplast assay. Before

centrifugation of a sample of the DNA from the culture infected in 100

μg/ml. of CAM, ^{3}H-containing λc26 DNA was added as a sedimentation marker

(no ^{3}H was present during the infection in the presence of 100 μg/ml. of

CAM). The results are shown in Figures 17a, b, and c.

The conversion of linear phage DNA to component I can occur in 100

μg/ml. of CAM as shown in Figure 17c. A minority of the intracellular

DNA in this extract sediments at the same rate as the marker. However, since the intracellular DNA is from a delection mutant of λ (b2b5c), it is uncertain whether the slower-sedimenting ^{32}P-DNA is component II or III.

In 30 µg/ml. of CAM only components I and II have been synthesized by 45 minutes, as indicated by an analysis of the sedimentation properties of both the incorporated ^{3}H-thymine and the spheroplast-infectious DNA.[4]

There is a suggestion of a fourth component (which is not infectious) containing ^{3}H-thymine which sediments faster than component I on these sucrose gradients (Figures 13a and b). This component has been observed irreproducibly in several experiments.

THe extracts prepared at 45 minutes from the culture kept in 30 µg/ml. of CAM and the extracts made at 15 and 35 minutes in the absence of CAM were phenol extracted and sedimented as above. Qualitatively, the results were the same as for the non-phenol extracted samples.

[4]As pointed out in Methods, phage DNA extracted (from virus) without phenol has a 2- to 3-fold lower infectivity per DNA molecule in the spheroplast assay than phage DNA extracted with phenol. In the helper assay, however, they have the same specific infectivity. The reason for this is unknown but it may explain the lower infectivity toward spheroplasts per ^{3}H disintegration of component III compared to component I observed in Figure 7a. Phenol-extracted component I and phage DNA have the same specific infectivity in the spheroplast assay (68).

The sedimentation patterns of the ^{3}H-vegetative DNA extracted with

or without phenol from the culture infected in the absence of CAM are

presented in Figure 18. These results are shown to indicate that losses

occurring during phenol extraction introduce little, if any, bias in the

recovery of λ-specific components. The relative amounts of components

I and II (or III) are nearly the same with or without phenol extraction,

even though the total recovery of ^{3}H-containing vegetative DNA was only

17% (at 15 minutes) and 73% (at 35 minutes). Of course, since the re-

covery at early times is much lower than at late, a distorted idea of the

relative amounts of I and III will be obtained if the recovery is not

measured.

A larger fraction of the samples centrifuged without phenol extrac-

tion pellets on the bottom of the tube (Figure 18a, b, c) than is observed

after phenol extraction (Figure 183). This does not occur with phage DNA

or with non-replicating λDNA extracted from an immune lysogen (Figures

2 and 3). The pelleted DNA might represent replicating DNA. A larger

fraction pellets at early times than late, just as the recovery of veg-

etative DNA is usually lower at early than late times. For these reasons

DNA actually in the process of replicating might not be resolved as a

unique component on these gradients. This point will be unearthed again

in Part III in which the structure of pulse-labeled λ DNA is investigated.

(h) Replication of component I studied by

density and radioactive labeling

The replication of component I has been studied by the use of heavy isotopes in a manner similar to that used by Meselson & Stahl (38) in their pioneering study of the replication of the E. coli chromosome. Phage were prepared (as described in Methods) which contained radio-phosphorus and heavy isotopes of carbon and nitrogen (^{13}C and ^{15}N). The DNA of such phage is more dense than is DNA which has been syn-thesized in medium containing ^{12}C and ^{14}N and hence such DNA may be isolated and purified on the basis of its unusual density in a CsCl equilibrium density gradient. The density distribution of the pfu and radiophosphorus in a typical "heavy" phage preparation is shown in Figure 1.

E. coli CR34 was infected with ^{32}P^{13}C^{15}N-λc26 in light (^{12}C^{14}N), ^{1}H-thymine medium, so that the progeny DNA (except those molecules con-taining contributions from the parent) would be "light" and nonradio-active. The nucleic acids were extracted after 30 minutes of infection at 30°C and fractionated by sedimentation through a neutral sucrose gradient. Fractions containing component I were pooled, as were fractions contain-ing the unresolved components II and III. A control culture was infec-ted with ^{32}P ^{12}C^{14}N-λc26 and treated similarly. CsCl was added to the pooled fractions to raise the density to 1.70 g/cm^3, a density reference ^{3}H-E. coli DNA was added, and the solutions were centrifuged at 30,000 rev./min in the SW39 rotor at 20°C for 42 hours.

The results from two similar experiments are shown in Figures 19 and 20. Figure 19 shows the separation of $^{32}P^{13}C^{15}N$-λc26 DNA (extracted from mature virus particles) and ^{3}H-E. coli DNA. ($^{32}P^{12}C^{14}N$-λc26 DNA has the same density as E. coli DNA (Figure 20b)). Figures 19b and 19c demonstrate the density distribution of component I, containing parental ^{32}P and $^{13}C^{15}N$, obtained in two similar experiments. The results shown in Figure 19b suggest that about one-half of the parental label which sedimented as component I in this experiment was in molecules which replicated semi-conservatively. In the next experiment about 90% of component I had a hybrid density (Figure 19c). In no experiment have we observed any indication of a continuous shift in density associated with component I. The parental ^{32}P is found in DNA which has either the density of $^{13}C^{15}N$ phage DNA (fully conserved) or a density exactly (within experimental error) intermediate between fully conserved (HH) DNA and the marker (equivalent to light phage DNA, designated LL). The position of the hybrid DNA, designated HL, indicates that it is composed of approximately equal amounts of H($^{13}C^{15}N$) and L($^{12}C^{14}N$) DNA.

The density distribution of those components II and III which contain ^{32}P is shown in Figure 20a. Only about 10% of the parental radioactivity which sedimented as components II and III has shifted in density from the HH position, whereas the component I material from the same sucrose gradient was at least 90% hybrid (HL) (Figure 19c).

The extent of replication of component I has been measured in cells infected with "heavy"λ in the presence of ^{3}H-thymine. E. coli AB2500 was grown and treated with MC as described previously to inhibit host

DNA synthesis. After thoroughly washing the bacteria with TM, $^{32}P^{13}C^{15}N$-

λc26 was added at a multiplicity of 0.8 pfu/colony former. KG medium

lacking thymine was added and the infected culture starved of thymine for

thirty minutes (the number of infective centers did not decrease). The

thymine starvation was intended to allow component I to accumulate in the

absence of DNA synthesis, if possible, and synchronize the subsequent

replication. After 30 minutes in the absence of thymine a sample was

removed for DNA extraction. ^{3}H-thymine at a concentration of 2.5 μg/ml.

and a specific activity of 0.62 c/mmole was then added. Samples were

removed for DNA extraction after an additional 10 and 15 minutes incu-

bation at 30°C. Samples of the extracts were sedimented on neutral

sucrose gradients as usual. The distributions of ^{32}P, ^{3}H-thymine, and

helper infectivity after sedimentation of the extracts are shown in

Figures 21a and b. Components I, II, and III can be distinguished by

virtue of their sedimentation rate and infectivity, or lack thereof, in

the helper assay. The sedimentation pattern is similar to that observed

in the early part of the latent period as exemplified in Figure 11.

The fractions containing component I were pooled and centrifuged

to equilibrium in a CsCl density gradient as before. A density reference,

^{3}H-E. coli DNA, was added before centrifugation to the sample of the

extract made just prior to thymine addition. The results are shown in

Figures 22a, b, and c.

It is apparent from Figure 22a, which shows the density of DNA from

cells infected and deprived of thymine for 30 minutes, that the washing

and thymine starvation were not sufficient to prevent component I from

replicating at least once. About 70% of the ^{32}P is in the HL region of the

CsCl gradient; the remainder has not replicated (HH). Ten minutes after

^{3}H-thymine is added the density distribution of ^{32}P remains unchanged

(Figure 22b). Newly synthesized component I, evidenced by the position

of the ^{3}H-thymine, is present in two peaks, in the HL and LL regions of

the CsCl gradient. At 15 minutes after addition of ^{3}H-thymine the amount

of ^{3}H-DNA in the LL position has increased about three times relative to

the amount present in the extract made five minutes earlier. The amount

of ^{3}H-DNA in the HL region does not change significantly in this period.

We presume these three peaks of radioactivity correspond, in order of

decreasing density, to: 1. fully conserved component I, (HH) containing

two parental strands; 2. semi-conservatively-replicated component I (HL)

consisting of one parental strand and one newly synthesized strand (the

newly synthesized strand will contain either ^{1}H-thymine or ^{3}H-thymine,

depending on whether it was synthesized before or after addition of ^{3}H-

thymine); and 3. progeny component I made of newly synthesized DNA (LL).

The amount of component I synthesized by 15 minutes after the ad-

dition of ^{3}H-thymine can be estimated from the relative amount of ^{3}H in

the LL and HL peaks of Figures 22b and c. Assuming there are two HL

component I molecules per cell (the multiplicity was less than one and

most of the sedimenting ^{3}H cts/min were in component I (Fig. 21)), the

total number of component I DNA molecules per infective center is 6 and

20 at 40 and 45 minutes after infection, respectively (including the

30 minutes of thymine starvation). This is probably a minimum estimate

since 70% of component I had replicated at least once before ^{3}H-thymine

was added. In an experiment similar to the one just described, but with the period of thymine starvation omitted, about 25 component I molecules, estimated as outlined above, were synthesized by 30 minutes after infection.

In this experiment, component II isolated from a neutral sucrose gradient was also subjected to CsCl equilibrium density gradient analysis. The results were essentially identical to those shown in Figure 22b except that a smaller fraction of the ^{32}P -parental DNA was in the HL region of the gradient.

The ability of the HL DNA to maintain its structure after alkaline denaturation has been studied. If HL component I is a closed circular molecule containing one strand of ^{3}H^{12}C^{14}N-DNA and one strand of ^{32}P-^{13}C^{15}N-DNA, alkaline denaturation should not allow the strands to separate since they are topologically linked. Denaturation of component II, however, should allow the two strands of differing density to separate and be resolved on a CsCl density gradient, assuming that it is a circular duplex with a scission in at least one strand.

Fractions containing HL component I or component II were pooled and dialyzed to remove CsCl and NaOH was added to raise the pH to about 12.8. After ten minutes at 37°C, HCl was added to lower the pH to 8.1. CsCl was added to raise the density to 1.71 g/cm^3 and the solutions were centrifuged for 42 hours at 30,000 rev./min at 20° C. The results are shown in Figures 23a and b.

The hybrid DNA which had originally sedimented as component II on a neutral sucrose gradient shows distinct strand separation after treat-

ment with alkali. The hybrid component I, on the other hand, does not. Most of the ^{32}P is still associated with ^{3}H after the treatment with alkali. The peak of ^{3}H in the LL region of this gradient is probably due to the presence of LL DNA which was originally pooled with the HL DNA, since the separation of HL and LL DNA is incomplete (Figure 22c). These results are consonant with the hypothesis that component I is a form of DNA whose two strands are prevented from separation in denaturing conditions. In contrast, the strands of component II are separated by treatment with alkali.

(i) Synthesis of spheroplast-infectious supercoils

If the increase in infectious DNA measured during the early part of the latent period is due to accumulation of component I as postulated earlier, it should be possible to observe this increase after fractionating the DNA on a neutral sucrose gradient. Spheroplast-infectious DNA sedimenting at the position of component I (after infecting a sensitive host) was demonstrated in Figure 16 and 17 but an increase in the amount of infectious component I during the latent period was not shown since extracts made at only one time were examined. The experiments in this section demonstrate net synthesis of spheroplast infectious component I and indicate that the increased infectivity is due to progeny DNA, not an increased specific infectivity of parental DNA.

E. coli CR34 (T$^-$) was grown and starved of thymidine for 40 minutes and then infected with an adsorbed multiplicity of 1.0 λc26 per bacterium and aerated an additional 30 minutes in the absence of thymidine. The

phage used in the infection contained ^{32}P and also ^{13}C and ^{15}N so the

parental DNA could be separated from the progeny duplexes by CsCl density

gradient centrifugation. The intended purpose of the thymidine starva-

tion was two-fold: 1. To demonstrate that component I can be formed

in the absence of DNA synthesis; and 2. To synchronize the infection with

respect to DNA synthesis. As will become evident, the period of thymidine

starvation served only to confuse the results of the experiment.

DNA was extracted from the infected complexes just before restitution

of thymidine and 10 and 20 minutes thereafter. Biological assays of this

DNA and also DNA extracted from the phage used in the infection are shown

in Table 4. As expected, a loss of infectivity after infection is ob-

served if the infectivity is measured in the helper assay. However,

DNA infective in the helper assay appears to be synthesized sooner than

is observed if the infected cells have not been deprived of thymidine.

Analogous to the results presented in Part I, the synthesis of sphero-

plast- infectious DNA precedes that of DNA infective in the helper assay.

Samples of the infected cell extracts were mixed with a DNA sedi-

mentation marker (b2b5c DNA) and centrifuged through sucrose density

gradients. The distribution of infectious DNA is shown in Figure 24.

The results from the three gradients are shown on one graph, the position

of the sedimentation marker is indicated by an arrow. There is obviously

an increase in the amount of infectious DNA which sediments as component I

when the extracts prepared 0, 10, and 20 minutes after adding thymidine

are compared. Contrary to the results obtained previously (studying

the labelled progeny DNA), there is also an increase in the amount of

slowly-sedimenting DNA at this "early" time. Since the fractions were
not assayed in the helper assay after sedimentation, it is not possible
to determine whether this DNA corresponds to mature phage DNA. Its co-
sedimentation with the marker, however, lends credence to that notion.
The early synthesis of phage DNA is probably caused by the previous
thymine starvation.

Sedimentation of the extracts prepared at 10 and 20 minutes reveals
three components. In the latter extract their sedimentation properties
clearly correspond to components I, II, and III.

Component I was isolated after sucrose gradient centrifugation of
a sample of the extract prepared 10 minutes after addition of thymidine,
mixed with b2b5c phage DNA and ^{3}H-E. coli DNA, and centrifuged to near
equilibrium in a CsCl equilibrium density gradient. A sample of ^{32}P^{13}C^{15}N-
phage DNA was treated similarly. After centrifugation fractions were
collected and assayed for radioactivity and then dialyzed to remove
CsCl (which is inhibitory in the spheroplast assay). Fractions of the
gradient bracketing the region containing radioactivity were assayed in
the spheroplast assay and the fractions containing either radioactivity
or infectivity are shown in Figure 25. Figures 25a and 25b are from the
same tube. The density markers centrifuged in the tube with component I
were distributed similarly to that shown in a and are therefore not
included.

The heavy (^{13}C^{15}N) ^{32}P-containing phage DNA (λc26) is well resolved
from the ^{3}H-E. coli DNA and the b2b5c infectivity. The λc26 infectivity
peaks at the same position as the ^{32}P radioactivity (Figure 25c). Most

of the infectivity associated with component I (Figure 25c), however,

is in the position expected for light (LL) progeny DNA. The distribution

of ^{32}P component I reveals a small peak of non-replicated DNA (desig-

nated HH), a large peak in the position of semi-conservatively replicated

DNA (HL), and, surprisingly, some of the ^{32}P is in a lighter position.

In experiments in which there has been no thymidine starvation, the

radioactivity in the light region is absent. Since it is known that

thymine starvation facilitates recombination in λ (J. Weigle and R. D'Ari,

personal communication), this might be the explanation for this observa-

tion. Alternatively, the light radioactivity could be due to breakdown

of some of the phage DNA involved in abortive infections and re-incor-

poration into the bacterial chromosome (which might be very efficient

during thymine starvation).

The truncation of the peak of infectivity in the LL region is

probably due to inhibition by E. coli DNA.

A quantitative interpretation of these results is difficult to

make because of the inhibition mentioned above. However, it is clear

that most of the infective supercoils have a LL density. Thus the

increase in infectivity observed in extracts early in the latent period

cannot be due to an increased specific infectivity of the parental

supercoils.

An estimate of the extent of replication of the supercoils can be

made by comparing the amount of infective DNA in the HL and LL region of

the gradient shown in Figure 25c. There are about 4 LL supercoils per

HL supercoil. This is a minimum since the infectivity in the LL region

is probably inhibited by the E. coli DNA.

5. DISCUSSION

The physical and biological properties of component I summarized

in Table 5 are consistent with the interpretation that it is a covalently

closed, twisted form of DNA. Its sedimentation properties, whether

extracted after infection of an immune lysogen or a sensitive cell,

are identical. Removal of a protein moiety (by phenol extraction) is

probably not involved in producing the tertiary coiling since extraction

of infected cells simply by osmotic lysis also reveals a fast sedimenting

component. An increased pitch at the time of final closure, perhaps

caused by a different ionic environment, would result in a twisted form

of the circular DNA after purification. Such twists would have the

same sense as the Watson-Crick helix, right-handed, as was also pointed

out by Vinograd & Lebowitz (59).

Attempts to measure, specifically, DNA synthesis after infection

or induction have been hampered by the similarity in base composition

between λ and E. coli DNA (31). Also, no chemical modifications of the

phage DNA composition which would allow it to be distinguished from

E. coli DNA have been found. Initial efforts to study synthesis of λDNA

utilized a marker rescue technique (25, 56, 45) . This method requires

that the DNA whose "genome multiplication" is being measured be able to

recombine with the superinfecting phage. In one case, however, it has

been shown that DNA was synthesized but was unable to recombine (28).

More recently, Joyner et al. (28) have used DNA-DNA hybridization

to measure λ and host DNA synthesis after induction of λ-lysogens. They

concluded that host DNA synthesis continued approximately unabated after

temperature or MC induction (using hcr$^+$ bacteria).

However, the levels of λ and host DNA synthesis after infection

might be different than after induction. Smith & Skalka (52), also

using DNA-DNA hybridization, estimated that 85% of the DNA synthesized

in a one minute pulse at 25 minutes after infection with a clear-plaque

mutant was λDNA. The value of 15% E. coli DNA synthesized during the

pulse is about one-half the value obtained by Joyner et al. (28) who

measured the total DNA synthesized from 0 to 30 minutes after induction

and might reflect a decrease in the relative rate of host DNA synthesis

during the latent period.

Lambda infection can inhibit host DNA synthesis, as is shown in

Figure 11 (assuming that the rate of incorporation of ^{3}H-thymidine into

TCA-insoluble material in a non-thymidine requiring bacteria can be

equated with the rate of DNA synthesis). The effect of lysogenization

by λ on total DNA synthesis is essentially identical to that observed

during lysogenization of S. typhimurium by P22 (50).

MC treatment of hcr$^-$ bacteria has enabled us to incorporate ^{3}H-

thymine specifically into the λDNA synthesized after infection. The

hcr$^-$ allele is necessary in order not to damage the capacity of the

bacteria for phage growth and to reduce the host DNA synthesis to a very

low level. No direct evidence has been obtained that the rate of host

DNA synthesis is reduced to the same level in the infected culture as

in the uninfected culture. However, the qualitative agreement between
the kinetics of synthesis of infectious (in spheroplasts) λDNA and ^{3}H-
thymine incorporation, and the identification in extracts of infected
cells of ^{3}H-thymine in only λ-specific components, recognized by their
infectivity (Figures 16 and 17) and sedimentation properties (Figure 15)
after sucrose density gradient fractionation, support this conclusion.

The kinetics of ^{3}H-thymine incorporation in cI, cII and cIII
infected cells, whether hcr$^-$ and treated with MC, or hcr$^+$ and non-MC
treated, do not reveal a marked repression of DNA synthesis between 12
and 30 minutes post-infection as was observed during lysogenization.
Infection with λcI does result in a slightly slower rate of phage pro-
duction and lysis, and with one cI mutant (c71) a slight depression of
DNA synthesis may have been observed (Figure 11). These results agree
qualitatively with those obtained in the S. typhimurium-P22 system in
which P22 cII (analogous to cI) caused an early repression of ^{3}H-
thymidine incorporation, delayed phage synthesis, and delayed lysis,
whereas P22 cI (analogous to λcII) did not. The magnitude of the re-
pression caused by the λcI mutants tested, however, was much smaller
than that observed after P22 infection. Conceivably, part of the dif-
ference could be a gene dosage effect since Smith & Levine (50) used
higher multiplicities than were used in these experiments.

Characterization of the DNA synthesized by λc26 (cI) after infection
of MC-treated bacteria reveals three components which are synthesized
after λ infection. Component I sediments 1.9X as fast as phage DNA at
pH 8 at low ionic strength, and 3.5X as fast as denatured phage DNA in

alkali (2). It is infectious in the spheroplast assay but not in the helper assay. Net synthesis of component I begins shortly after infection and continues until synthesis of intracellular phage begins. Component II sediments about 1.15X as fast as phage DNA at neutral pH. It is also non-infectious in the helper assay but is able to infect spheroplasts (68). Component III is presumed to be identical to phage DNA for the following reasons: 1. It sediments at the rate of added marker phage DNA (Figure 16); 2. It is infective in the helper assay (Figure 17); 3. Its synthesis begins at the same time and in parallel with the synthesis of both intracellular phage and helper-infective DNA (Figures 14 and 15).

It cannot be excluded that some of the DNA sedimenting in the position of component III might have a lower specific infectivity than DNA extracted from purified phage. However, the specific infectivity of the ^{3}H-DNA synthesized after the synthesis of helper-infectious DNA begins is approximately constant, as can be calculated from Figures 14 and 15. This would suggest that there is at most a short lag between the production of any linear DNA not infectious in the helper assay and linear DNA containing cohesive ends which is infectious in the helper assay.

Some indication that genome multiplication can occur in the presence of 25 μg/ml. of CAM was presented by Sechaud (45). (It should be noted, however, that Thomas (56), using the same technique, concluded that no increase in recombination-proficient DNA occurred in the presence of 25 μg/ml. of CAM.) The uptake of ^{3}H-thymine in MC-treated bacteria

infected with λc26 in the presence of 30 μg/ml. of CAM confirms the

synthesis of λDNA at this concentration of CAM (Figure 13). At 100 μg/ml.

of. CAM no λDNA is sythesized. However, if 100 μg/ml. of CAM is added

15 minutes prior to infection, conversion of phage DNA to component I

still occurs (Figure 17). The conversion also occurs in an amino acid

auxotroph which is starved of an essential amino acid before and during

infection (Young, unpublished observations). The DNA synthesized in

30 μg/ml. of CAM is primarily component I; the remainder is component II

(Figure 17). Since genome multiplication, as indicated by recombination,

apparently occurs, albeit more slowly, in this concentration of CAM

(45), this would suggest that component I may be able to recombine.

Alternatively, the accumulation of component I could increase the gene

pool of the initial infecting phage and allow more rapid synthesis of

DNA which could recombine after CAM was removed.

The experiments using density- and radioactivity-labeled phage

demonstrate that the component I molecules containing parental label

are, in part, unreplicated and, in part, semiconservatively replicated.

Meselson & Weigle (40) also showed that injected DNA replicates at

least once semiconservatively. The incorporation of ^{3}H-thymine into

hybrid component I which was synthesized in ^{1}H-thymine light medium

demonstrates that component I which has replicated once semiconservatively

is able to replicate again semiconservatively (Figures 22a and b). Of

course, only the end product of the replication is observed in these

experiments. It seems most likely, since a closed circular duplex must

open in at least one strand in order to replicate semiconservatively,

that a stage similar to component II is directly involved in the synthesis of component I.

Some gratifying parallels between the processes of infection with ØX174 and λ may be noted in these experiments. In summary these include: 1. Synthesis of (ØX174) or conversion to (λ) a double-stranded, circular replicative form (ØXRF or λRF). The conversion is resistant to 100 μg/ml. of CAM and to amino acid starvation, and apparently does not require a phage function, since with no phage mutants examined has this conversion been inhibited, either of ØX (48) or λ (65, 44). In the case of λ, Gellert (17) has found an activity in E. coli extracts which converts hydrogen-bonded, circular phage DNA (formed by annealing viral DNA) into alkali-resistant circular duplexes. 2. Net synthesis of the ØXRF and λRF occurs specifically in the early part of the latent period, before synthesis of mature phage begins. In both instances, RF replication occurs in the presence of 30 μg/ml., but not 100 μg/ml., of CAM. Apparently, at least one protein involved in the replication of RF is phage-specified since both ØX and S13 mutants exist which do not show RF replication (48, 53). Several early mutants of λ, sus N, O and P, are also blocked completely in DNA synthesis (3, 10, 28). Since this protein is resistant to 30 μg/ml. of CAM it might be analogous to the CAM-resistant protein proposed by Lark (33) to be involved in the replication of the E. coli chromosome. A host function is probably also involved in the replication of ØXRF (Denhardt, personal communication). 3. Synthesis of mature viral DNA occurs after net synthesis of ØXRF or λRF ceases (47; Figures 15 and 16). CAM at 30 μg/ml. inhibits synthesis of viral DNA

(47, 11; Figure 17) as do some mutations of ØX (12) and in λ (10). All

of the λ mutants which do not synthesize mature viral DNA are, of course,

also defective in the synthesis of functional heads. The mutants which

cannot act as head donors in Weigle's in vitro phage assembly assay

(sus A-E) (63) have been implicated in the maturation (conversion to

helper-infective) of DNA, since these mutants do synthesize λDNA but

it is non-infective in the helper assay (10, 28). The maturation step

involved might be the conversion from synthesis of circular DNA to syn-

thesis of mature phage DNA. The defects which prevent formation of

normal λ heads could act in a manner perhaps analogous to CAM to prevent

synthesis of viral DNA.

Similarly, in ØX infection a mutant which is specifically unable

to synthesize the serum-blocking power protein does not synthesize single-

stranded viral DNA (12, 48). Thus it seems that synthesis of mature

viral DNA may depend on several functions not directly involved in DNA

synthesis.

Early and late functions in λ infection appear clearly exemplified

by the early synthesis of λRF and the late synthesis of mature viral

DNA. The multiplication of λRF presumably enhances the probability of

lysogenization during infection with λc^+ by providing more circular DNA

which may pair and recombine with the host chromosome (7). The increased

frequency of lysogenization which results from interference with protein

synthesis in P1 and P2 infections (8, 1), if applicable to λ, may be

explained by accumulation of λRF without initiation of late functions

which lead to a lytic rather than a lysogenic response.

TABLE 1

Infectivity of Phage DNA Extracted with SDS and

Pronase with or without Phenol

Extraction procedure	cpm/ml	Helper Infectivity		Spheroplast Infectivity	
		$IC/ml \times 10^{-7}$	$IC/^{32}Pdpm$	$pfu/ml \times 10^{-7}$	$pfu/^{32}Pdpm$
SDS-Pronase	3.0×10^5	5.3, 5.0	180, 170	1.1, 1.3	37, 43
SDS-Pronase - Phenol	1.9×10^5	3.5, 3.2	180, 170	1.8, 2.9	95, 150

^{32}P-λb2b5c phage were extracted with SDS and Pronase and then half of the sample was extracted with phenol. The two infectivity values represent duplicate assays of the DNA. The values are from the range of the assay in which the response was proportional to the DNA concentration.

TABLE 2

Relative Sedimentation Rate of Component I Extracted from

Lysogenic and Non-lysogenic Cells

Ionic Strength	pH = 8.1		pH = 12.8	
	K12S	K12(λ)	K12S	K12(λ)
0.01	1.9	1.9, 1.9[*]		
0.10	1.6		4.5	
0.20			3.4	
0.60			3.1	
1.0	1.6	1.5-1.6[*]	3.5	3.5[*]

[*]Bode & Kaiser, (2)

TABLE 3

Effect of Mitomycin C (MC) Pretreatment on the Production of Infective

Centers and Phage

(a) E. coli AB2500, hcr$^-$

Infected culture	Colony formers per ml.		Infective centers per ml.	Unadsorbed pfu per ml.	% pfu adsorbed	Phage yield per ml.
	Before infection	After MC and infection				
Minus MC	2.4×10^8	1.0×10^8	2.2×10^8	6.0×10^8	40%	1.3×10^{10}
Plus MC	2.4×10^8	5.0×10^3	1.5×10^8	5.4×10^8	46%	6.0×10^9

The bacteria were treated with 50 μg equivalents/ml. of MC for ten minutes at 37°C in the dark without aeration, filtered, washed, and resuspended in prewarmed KG medium containing 5 μg/ml. of thymidine. Phage and ^{3}H-thymidine were added to the cultures and samples taken to measure TCA-insoluble ^{3}H-thymidine incorporation and to assay for intracellular phage. 4.3 pfu/colony former were added; the multiplicity of adsorbed phage was 1.7. Colony formers, infective centers, and unadsorbed phage were measured ten minutes after infection. The infective centers were measured after inactivating the unadsorbed phage with λ antiserum.

(b) E. coli AB2497, hcr$^+$

Infected culture	Colony formers per ml.		Infective centers per ml.	Unadsorbed pfu per ml.	% pfu adsorbed	Phage yield per ml.
	Before infection	After MC and infection				
Minus MC	2.6×10^8	1.4×10^8	1.3×10^8	1.6×10^8	84%	4.6×10^9
Plus MC	2.6×10^8	1.7×10^5	2.4×10^7	1.8×10^8	82%	6.0×10^7

The infection and biological assays were performed as described in the legend to Table 3a. 4.0 phage were added per colony former and the multiplicity of infection of adsorbed phage was 3.6.

TABLE 4

Synthesis of Helper and Spheroplast Infectious DNA after Infection of a

Thymine Starved Bacterium

	Normalized Specific Infectivity	
Source of DNA	Helper Assay	Spheroplast Assay [1]
Phage	1.00	1.00
Thymine starved cells infected and deprived of thymine for 30 min	0.079	0.1-1
Same, 10 minutes after adding thymidine	0.13	11-25
Same, 20 minutes after adding thymidine	3.00	60-140

[1] The efficiency of this assay was quite low, thus the values

obtained varied within the range shown.

TABLE 5

COMPARISON OF PROPERTIES OF VIRAL λ DNA AND COMPONENT I

	RELATIVE DISTANCE SEDIMENTED		BUOYANT DENSITY IN ALKALINE CsCl	TWISTED MOLECULES IN ELECTRON MICROGRAPHS[+]	SHEAR RESISTANCE RELATIVE TO VIRAL DNA	SEPARABILITY OF STRANDS	INFECTIOUS AFTER DENATURATION[+]	INCREASED BUOYANT DENSITY IN ETHIDIUM BROMIDE-CsCl[+,++]
	pH 8*	pH 12.5**						
VIRAL DNA	1.0	1.0	~1.770	NO	SAME	YES	NO	NO
COMPONENT I	1.9	3.5	~1.790	YES	MORE RESISTANT	NO	YES	YES

* 0.01 M Tris
   0.001 M EDTA
** 0.10 M NaOH
   0.9 M NaCl

+ Kiger & Young, in preparation
++ Relative to Viral DNA. Radloff, Bauer & Vinograd (1967).

Figure 1. <u>CsCl equilibrium density gradient centrifugation of</u>
<u>$^{32}P^{13}C^{15}N$-λc26</u>.   A small portion of purified heavy phage were mixed

with vir, h and λb2b5c (which have densities of 1.508 and 1.483 g/cm^3,

respectively) and CsCl was added to raise the density to about 1.51 g/cm^3.

After 40 hours of centrifugation at 30,000 rev./min at 20°C in the SW39

rotor drops were collected into TM from a hole pierced in the bottom of

the tube and each fraction was assayed for phage and ^{32}P. The positions

of the phage density markers are designated by arrows in the figure.

The regions of the gradient not shown did not contain significant amounts

of either radioactivity or phage.
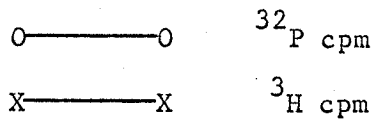
o————o   iλ pfu

x————x ^{32}P cts/min
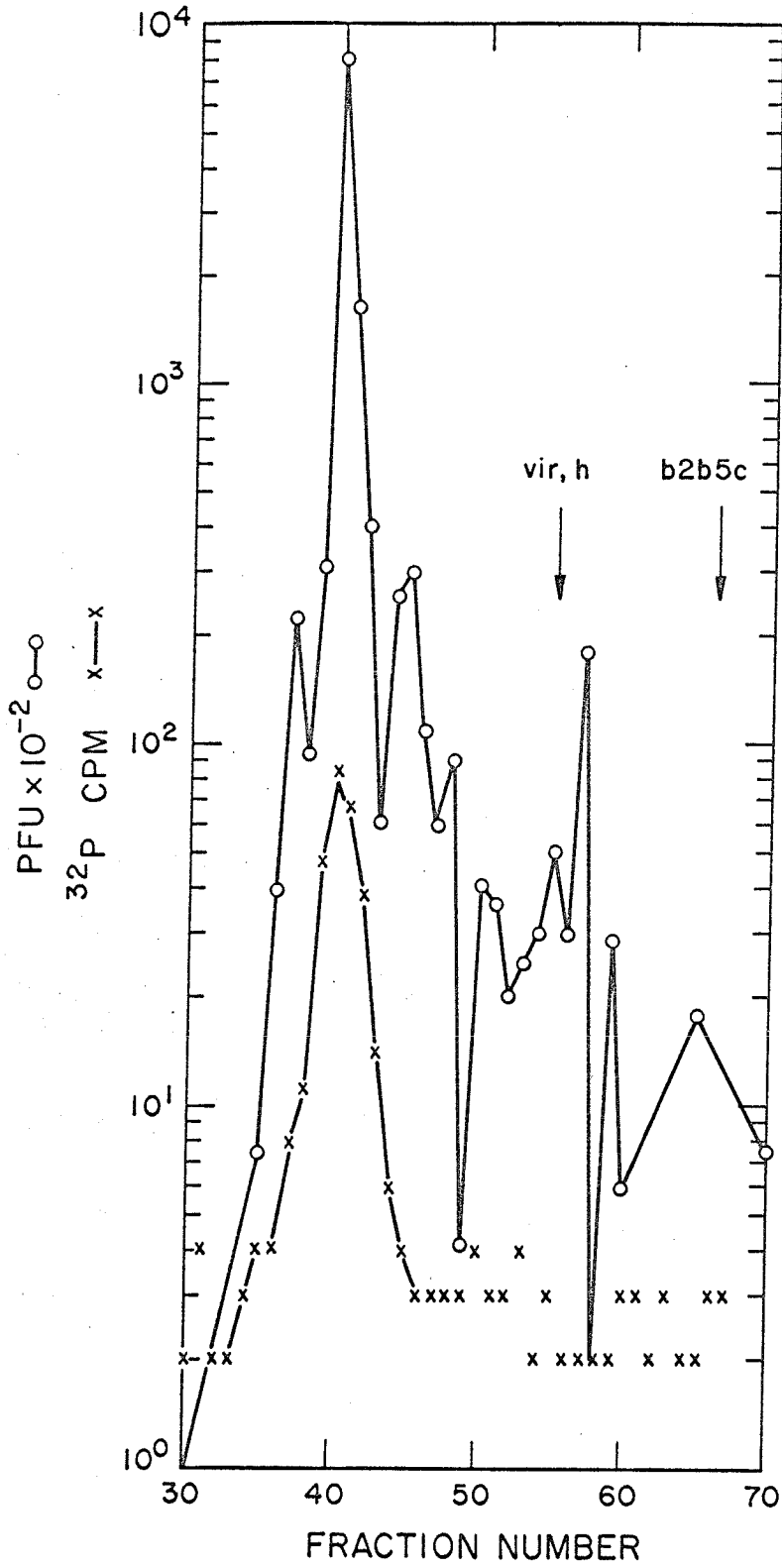
Figure 1

Figure 2. Sedimentation of phage DNA extracted with or without phenol.
^{32}P- b2b5c DNA extracted with SDS and pronase was mixed with phenol

extracted ^{3}H-λDNA and centrifuged for 6 hours at 25000 rpm and 6°C.in

the SW 25.1 rotor. The sucrose gradients contained 0.01 $\underline{M}$ tris 0.001 $\underline{M}$

EDTA , pH 10.5. The DNA extracted from wild type phage (^{3}H) sediments

about 9% faster than the DNA from the deletion mutant (^{32}P), as expected

from its higher DNA content.

        O————O ^{32}P cpm
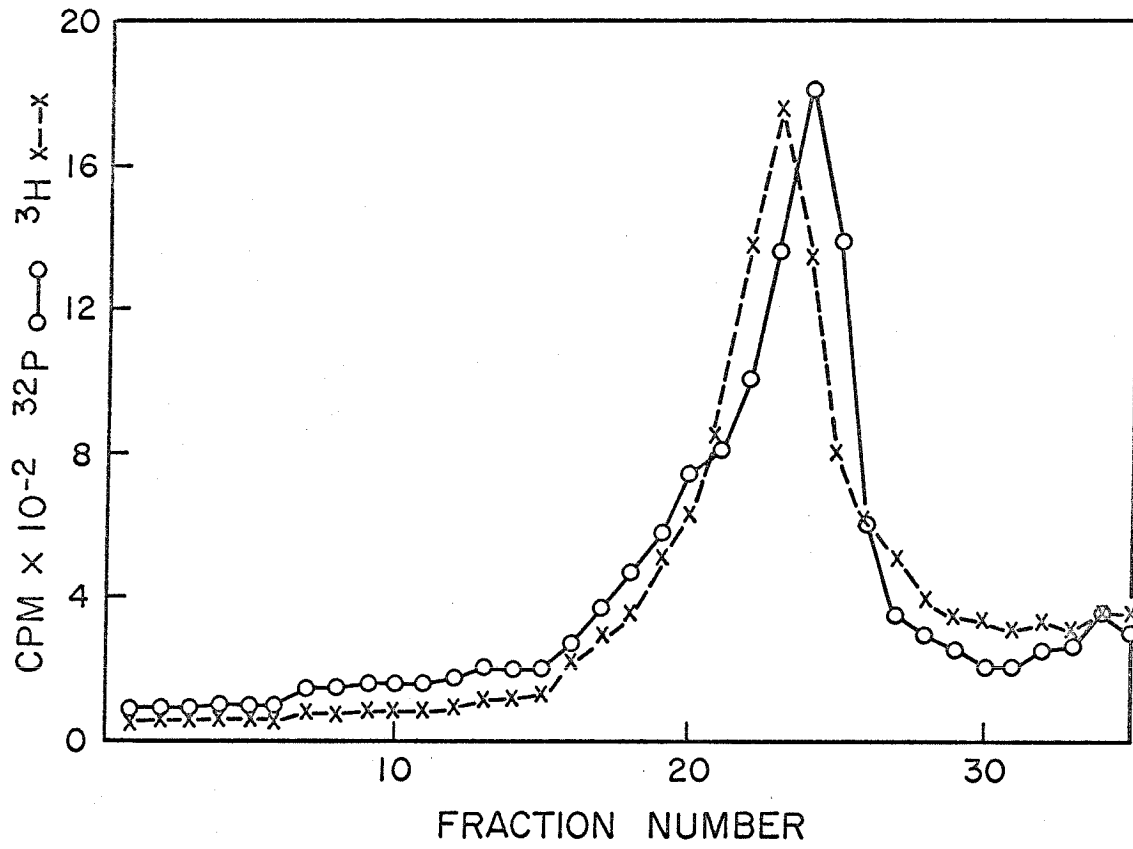
        X————X ^{3}H cpm

Figure 2

Figure 3. Sedimentation of intracellular λDNA extracted with or without

phenol. C600(b5) was infected with ^{32}P- b2b5c and cultured for 40

minutes at 37° and then the cells were collected and the nucleic acids

extracted by:

    (a) lysozyme, SDS, pronase (2 hours) and phenol. (Recovery = 47%).

    (b) lysozyme, SDS, and pronase (2 hours). (Recovery = 100%).

    (c) lysozyme, SDS, and phenol. (Recovery = 45%).

The lysates were layered with a ^{3}H-λDNA sedimentation marker onto pH 10.5

sucrose gradients and centrifuged for 6 hours at 25000 rpm in the SW 25.1

rotor.

    0———0 ^{32}P cpm

    X———X ^{3}H cpm
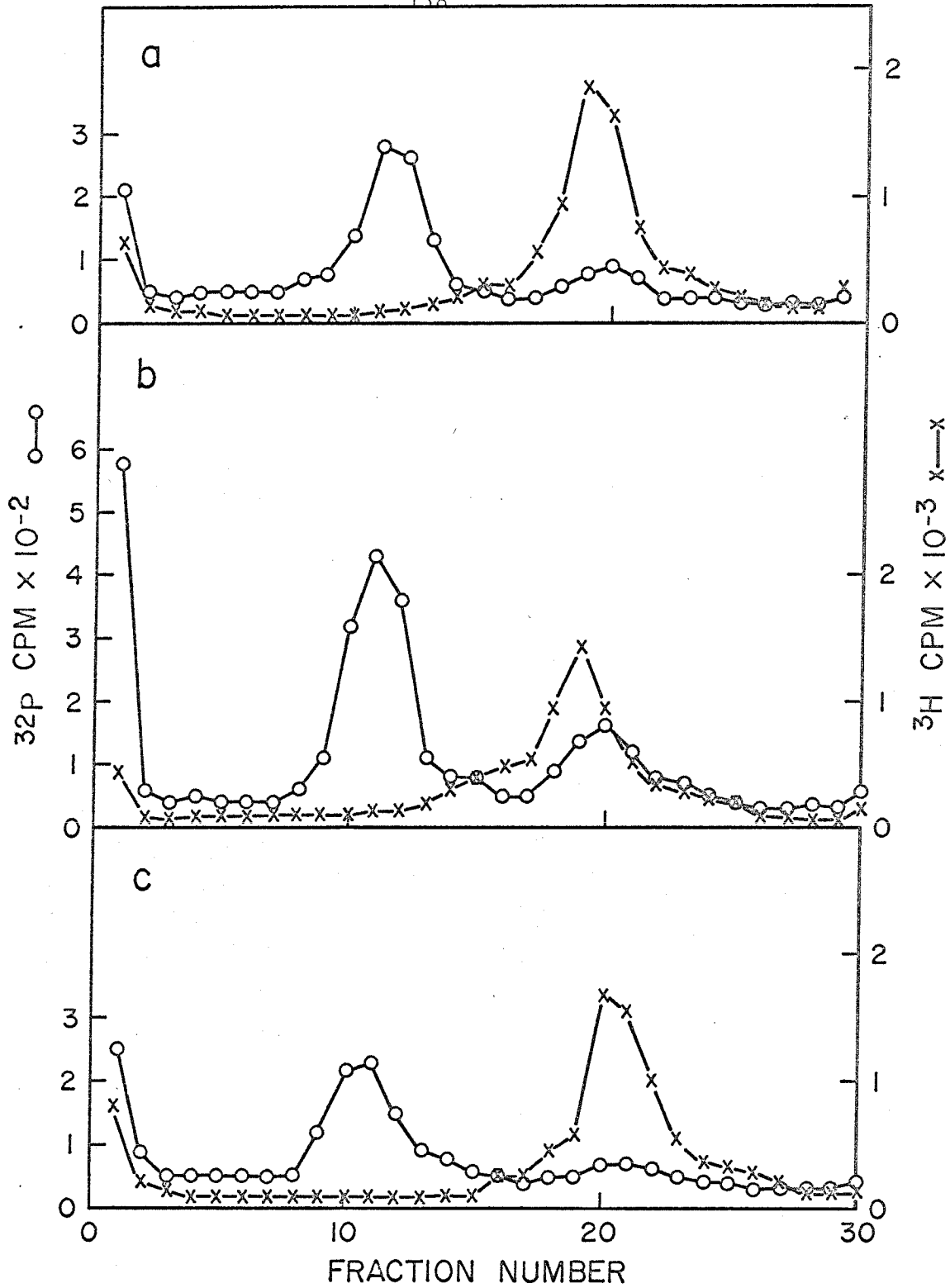
Figure 3

Figure 4. <u>Buoyant</u> <u>density</u> <u>of</u> <u>intracellular</u> λDNA <u>extracted</u> <u>without</u>

<u>phenol</u>. The DNA extracted as described in the legend to Figure 3b

was mixed with phenol extracted phage DNA and centrifuged to near equi-

librium in a CsCl density gradient. Upon addition of CsCl to the DNA

solution a flocculent precipitate formed and floated to the top of the

tube. This was probably precipitated SDS and might explain the radio-

activity found at the top of the gradient (Fraction 25) after centri-

fugation as due to DNA trapped in the precipitate. Density increases

from right to left.

$$O\text{———}O \qquad ^{32}P \text{ cpm}$$

$$X\text{———}X \qquad ^{3}H \text{ cpm}$$

Figure 4

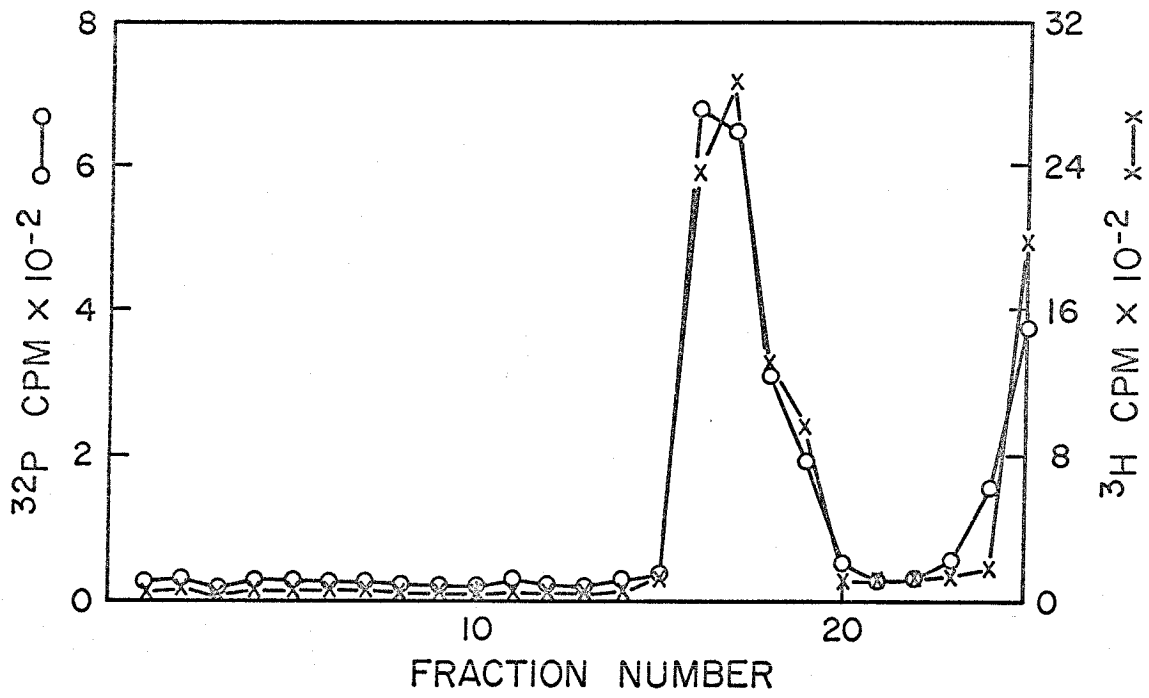Figure 5. <u>Sedimentation</u> <u>of</u> <u>intracellular</u> λDNA <u>through</u> <u>sucrose</u> <u>gradients</u>

<u>at</u> <u>neutral</u> <u>and</u> <u>alkaline</u> <u>pH</u>. <u>E. coli</u> 3110(λ) was grown in KG medium, cen-

trifuged, resuspended in three portions of TM at $0°C$, and infected with

(a,b) ^{3}H-λvir, (c) ^{32}P-λc26, or (d) a mixture of ^{3}H-λvir and ^{32}P-λc26.

The multiplicity of each genotype was about 8. After 15 minutes at

37°C KG medium was added, the suspensions were blended in a Waring

Blendor for two minutes, and then aerated for 35 minutes at $30°C$. The

DNA was extracted by Method A. The recovery of radioactivity in the

dialyzed phenol extracts was between 85 and 115%. The nucleic acids

from about 3 x 10^8 cells were layered on 5-20% sucrose gradients in

TE (a,c,d), and centrifuged for 5 hours at 25,000 rev./min and $6°C$ in the

SW 25.1 rotor. Fractions were collected as described in Methods and

analyzed for radioactivity and infectivity in the helper assay. The

recovery of the radioactivity applied to the gradient was between 75

and 90%. The alkaline sucrose gradient (b) contained 0.10 $\underline{M}$ NaOH,

0.90 $\underline{M}$ NaCl. ^{32}P-phage DNA was added before centrifugation for 2 hours

at 25,000 rev./min at $6°C$. 0.8 ml. of glycerol was layered on the bottom

of the neutral sucrose gradients before centrifugation. a: Intracellular

^{3}H-λvir DNA radioactivity and helper infectivity. b: Intracellular

^{3}H-λvir DNA and ^{32}P-λ phage DNA, pH 12.8; c: Intracellular ^{32}P-λc26

DNA radioactivity and helper infectivity. d: Intracellular ^{3}H-λvir

and ^{32}P-λc26 DNA from the mixed infection.

x———x ^{3}H cts/min

0———0 ^{32}P cts/min
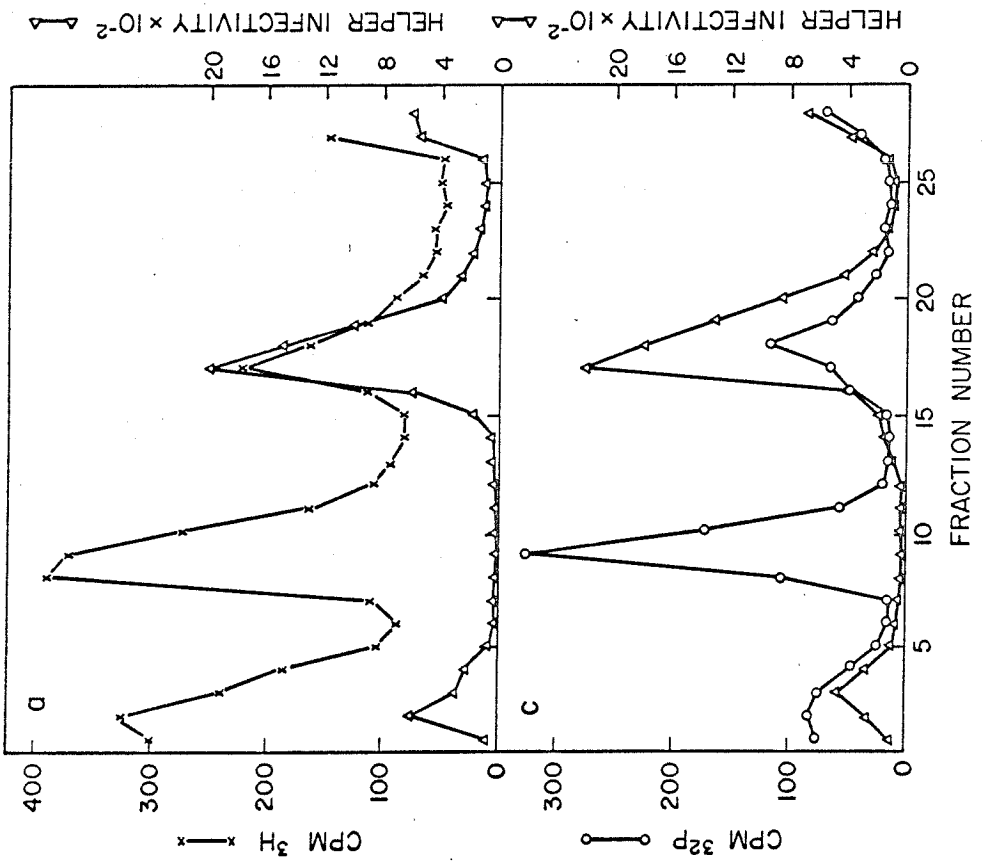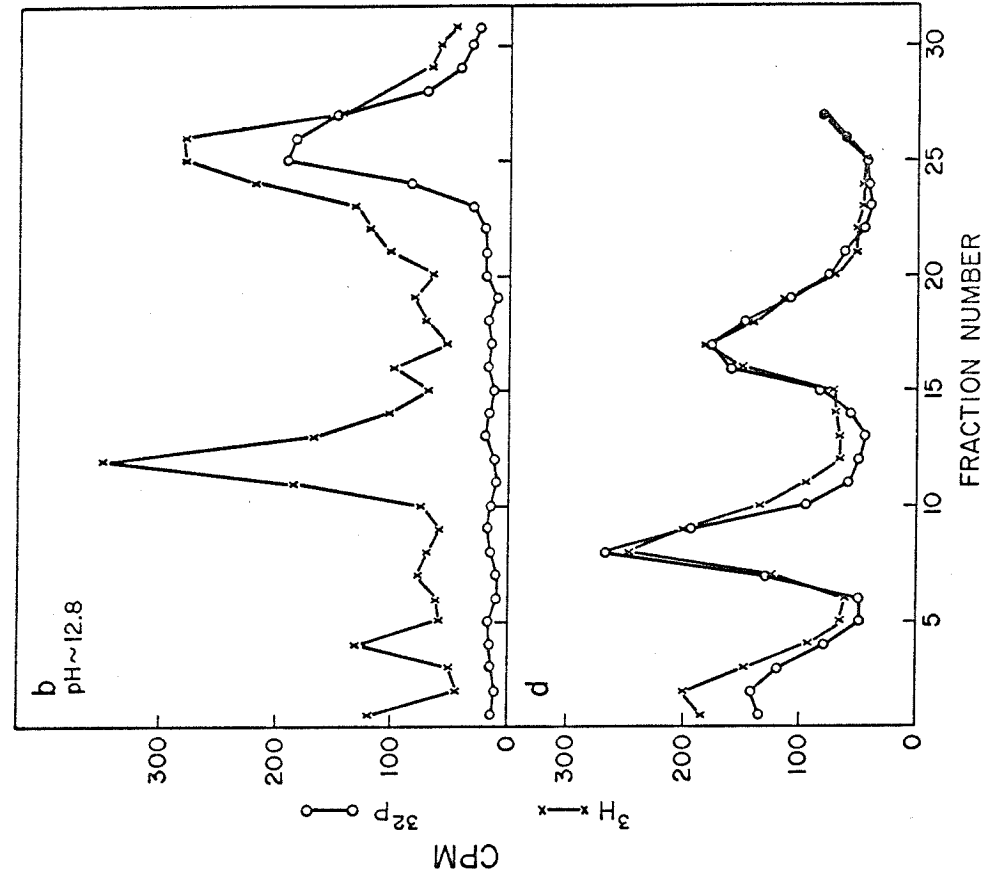
△———△ Infectivity in the helper assay

Figure 5

Figure 6. The effect of shear on the sedimentation rate of intracellular λDNA and E. coli DNA. Intracellular ^{32}P-λc26 DNA from the experiment described in the legend to Figure 2 was mixed with high molecular weight ^{3}H-E. coli DNA and one portion was sheared at $0°$C (in 0.1 M NaCl) in a Virtis homogenizer at a setting which breaks λ phage DNA approximately in half. Each portion was centrifuged through a neutral sucrose gradient as described before and the fractions were assayed for radioactivity and infectivity in the helper assay. a: Unsheared, recovery: ^{3}H 140%; ^{32}P 80%. b: Sheared, recovery ^{3}H 50%; ^{32}P 20%.

0———0 ^{32}P cts/min

X———X ^{3}H cts/min

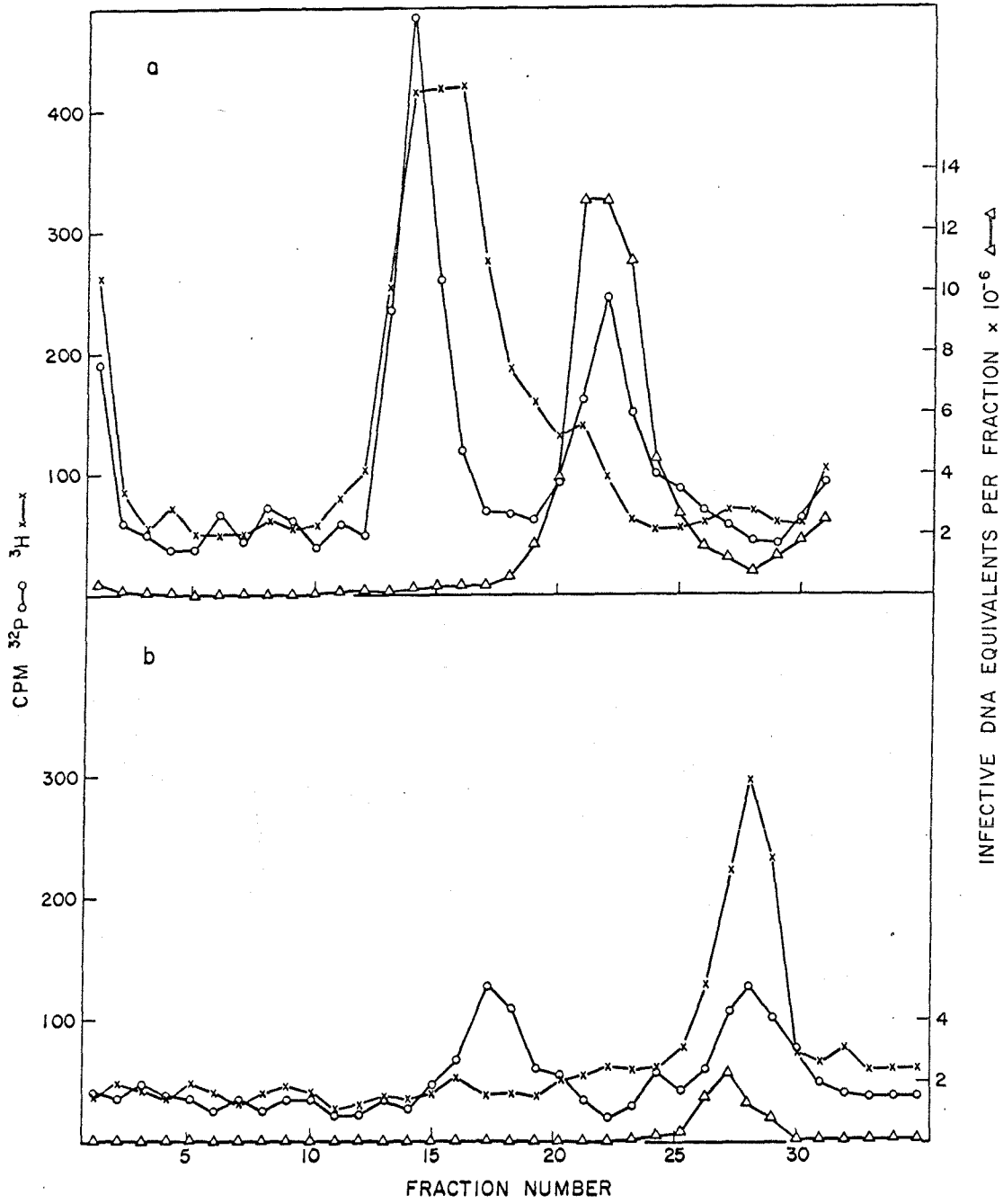$\triangle$———$\triangle$ Helper infectivity

Figure 6

Figure 7. <u>Buoyant density of component I in alkaline CsCl</u>. Component

I was isolated from neutral sucrose gradients as described in the text

and mixed with CsCl to raise the density to about 1.76 g/cm^3. The DNA

was dissolved in 0.05 $\underline{M}$ K$_3$PO$_4$ with KOH added to bring the pH to 12.8.

    a: ^{32}P component I, and ^{3}H-$\underline{E.\ coli}$ DNA were centrifuged for 60

hours at 17°C at 30,000 rev./min.

        0———0 ^{32}P cts/min

        X———X ^{3}H ctx/min

    b: ^{32}P-λc26 and ^{3}H-λvir components I from the mixed infection

experiment presented in Figure 2 were mixed with 1.7 A$_{260}$ units of

λb2b5c DNA and centrifuged for 48 hours at 30,000 rev./min and 36°C.

        0———0 ^{32}P cts/min

        X———X ^{3}H cts/min

        0- - -0   A$_{260}$

        □———□   density

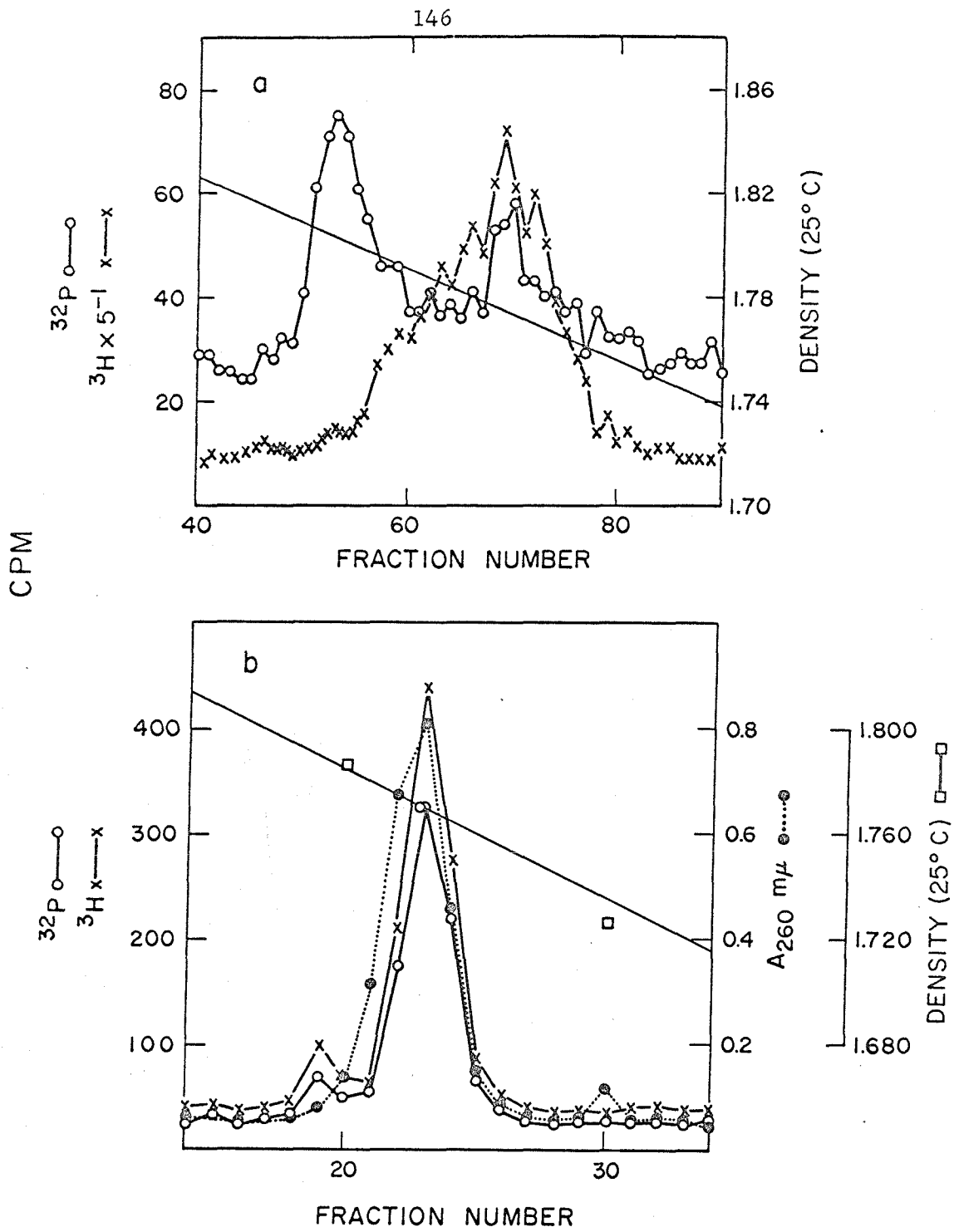Figure 7

Figure 8. Sedimentation of intracellular λDNA extracted by osmotic
lysis. ^{32}P-λc26 was used to infect E. coli 3110(λ). 30 minutes after
infection the infected bacteria were converted to spheroplasts and lysed
by dilution into distilled H_2O. The solution was immediately mixed
with ^{3}H-phage DNA, layered on a neutral sucrose gradient, and centri-
fuged for 7 hours at 25,000 rev./min at 3°C.

        0———0 ^{32}P cts/min

        X———X ^{3}H cts/min

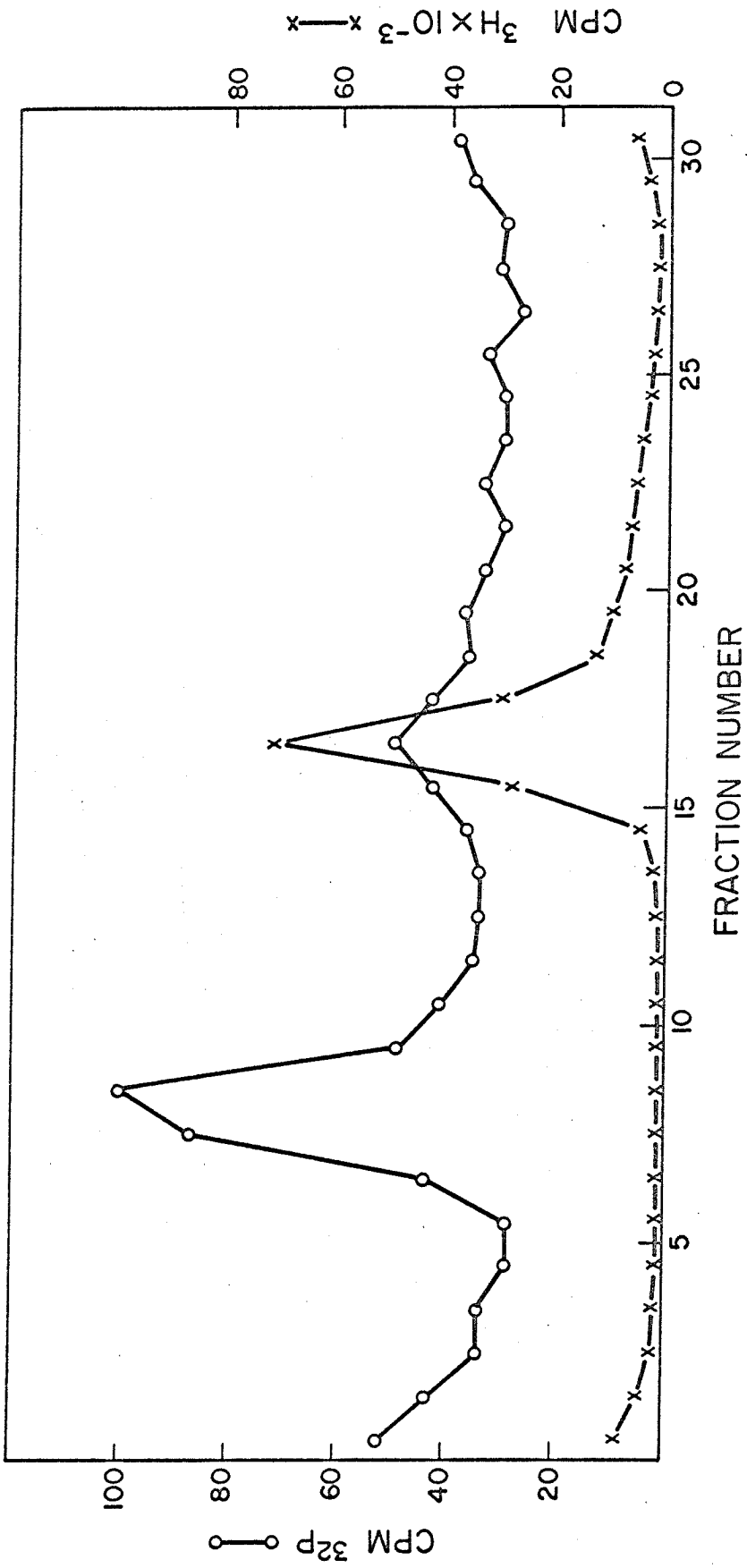Figure 8

Figure 9. <u>Effect</u> <u>of</u> <u>mitomycin</u> <u>C</u> <u>pretreatment</u> <u>on</u> ^{3}H-thymidine incor-

poration <u>and</u> <u>phage</u> <u>growth</u>. Bacteria were grown in KG medium plus uracil

and thymidine and then treated with 50 μg equivalents/ml. of MC in the

dark without aeration for 10 minutes. They were washed and resuspended

in four portions in the medium described above with ^{3}H-thymidine (0.5

μc/ml. final concentration) added and then λc26 was added to two portions.

TCA-insoluble radioactivity incorporated was measured by pipetting 1.0 ml.

from the culture into 2 ml. of cold 10% TCA containing 2 mg/ml. ^{1}H-

thymidine. After 30-60 minutes at 0°C the samples were filtered through

GF/A filters and washed with 3-5 ml. aliquots of 5% TCA containing

^{1}H-thymidine.

a. <u>E. coli</u> AB2500 hcr$^-$ b. <u>E. coli</u> AB2497 hcr$^+$

X————X ^{3}H cts/min-uninfected, minus MC

X- - -X ^{3}H cts/min-uninfected, plus MC

0————0 ^{3}H cts/min-infected, minus MC

0- - -0 ^{3}H cts/min-infected, plus MC

□————□   intracellular phage, minus MC

□- - -□   intracellular phage, plus MC
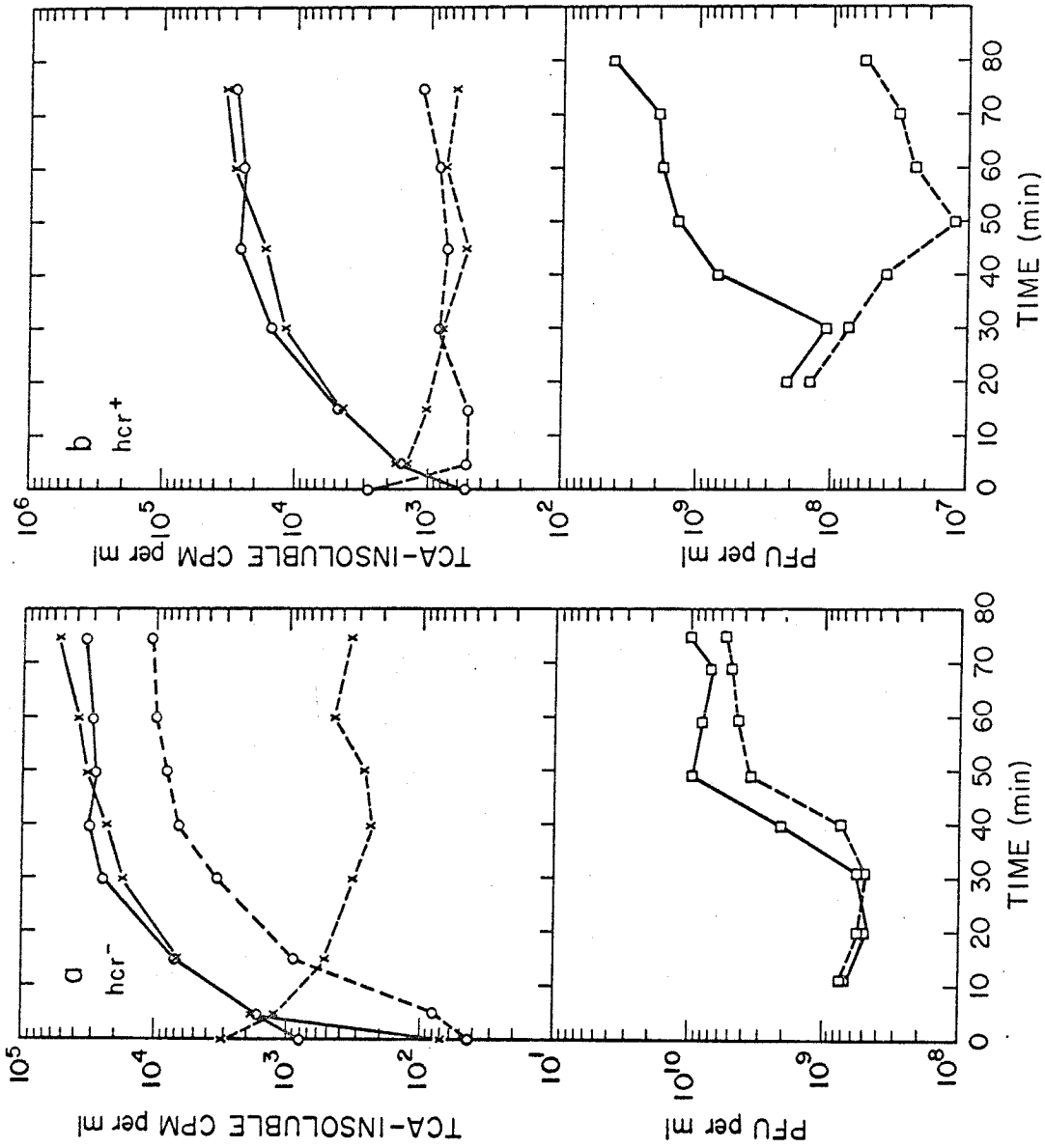
Figure 9

Figure 10. <u>DNA</u> <u>and</u> <u>phage</u> <u>synthesizing</u> <u>capacity</u> <u>after</u> <u>MC</u> <u>treatment of</u>

<u>hcr^{+}</u> <u>and</u> <u>hcr^{-}</u> <u>cells</u>. <u>E. coli</u> AB2497 or AB2500 were treated with MC as

described in Methods. After washing by filtration the cultures were

resuspended in KG medium containing ^{3}H-thymidine and aerated at 37° for

45 minutes. Acid insoluble ^{3}H-thymidine incorporation in each sample

was measured by TCA precipitation before and after hydrolysis with

0.5$\underline{N}$ NaOH (14 hours at 37°). The capacity for λ growth was measured

in a separate experiment. The results are plotted as the ratio of the

quantity obtained after treatment with x·μg/ml of MC to the quantity

obtained without MC pre-treatment.

      0‒ ‒ ‒0   colony-forming ability, hcr^{-}

      ●───────●   colony-forming ability, hcr^{+}

      · · · · · · · ^{3}H-thymidine incorporation, hcr^{-}

      _·_·_·_ ^{3}H-thymidine incorporation, hcr^{+}

$\square$, before alkaline hydrolysis; ◼, after alkaline hydrolysis, hcr^{-}.

$\triangle$, before alkaline hydrolysis; ▲, after alkaline hydrolysis, hcr^{+}.
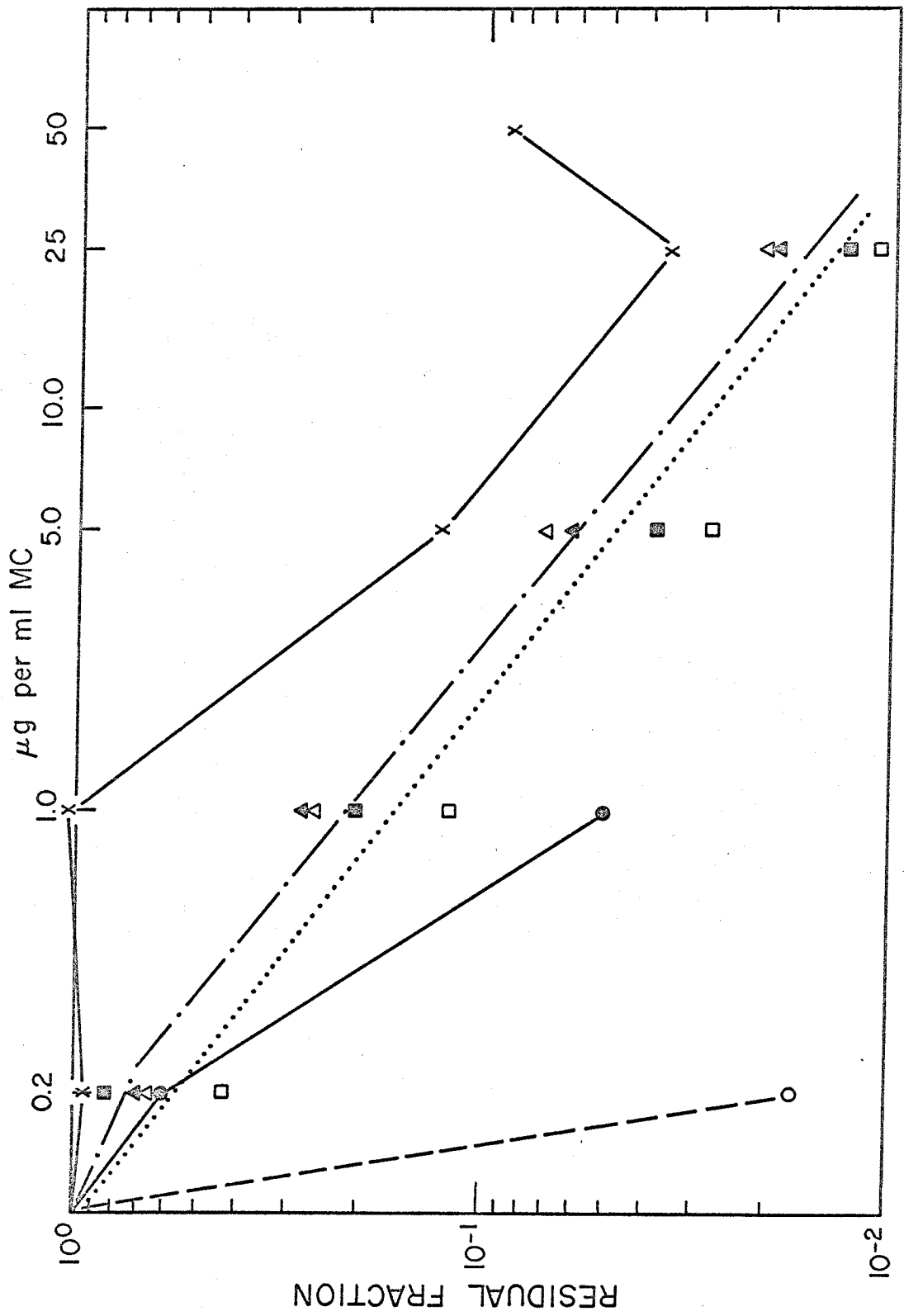
X───────X   phage capacity, hcr^{+}

Figure 10

Figure 11. ^{3}H-thymidine incorporation into E. coli 3110 infected with
λ^+, cII, and cI. E. coli 3110 was grown in K medium to 2×10^9 bacteria/
ml., filtered, washed with TM, and resuspended at the same concentration
in TM and aerated for one hour at 37°C. Phage were added at a multi-
plicity of 15 pfu/cell and adsorbed for 15 minutes. They were then
diluted into K medium and aerated at 37°C. Every two minutes (in the
infected cultures) 1.0 ml. was removed, aerated in the presence of 0.5 μc
of ^{3}H-thymidine for one minute, and then the incorporated, TCA-insoluble
radioactivity was determined as in Figure 6. About 90-95% of the cells
were lysogenized by λc^+ (section a). 95% of the cells became infective
centers after infection with cII or cI (sections b and c).

        0———0   TCA-insoluble cts/min, infected

        X———X   TCA-insoluble cts/min, uninfected

Figure 11

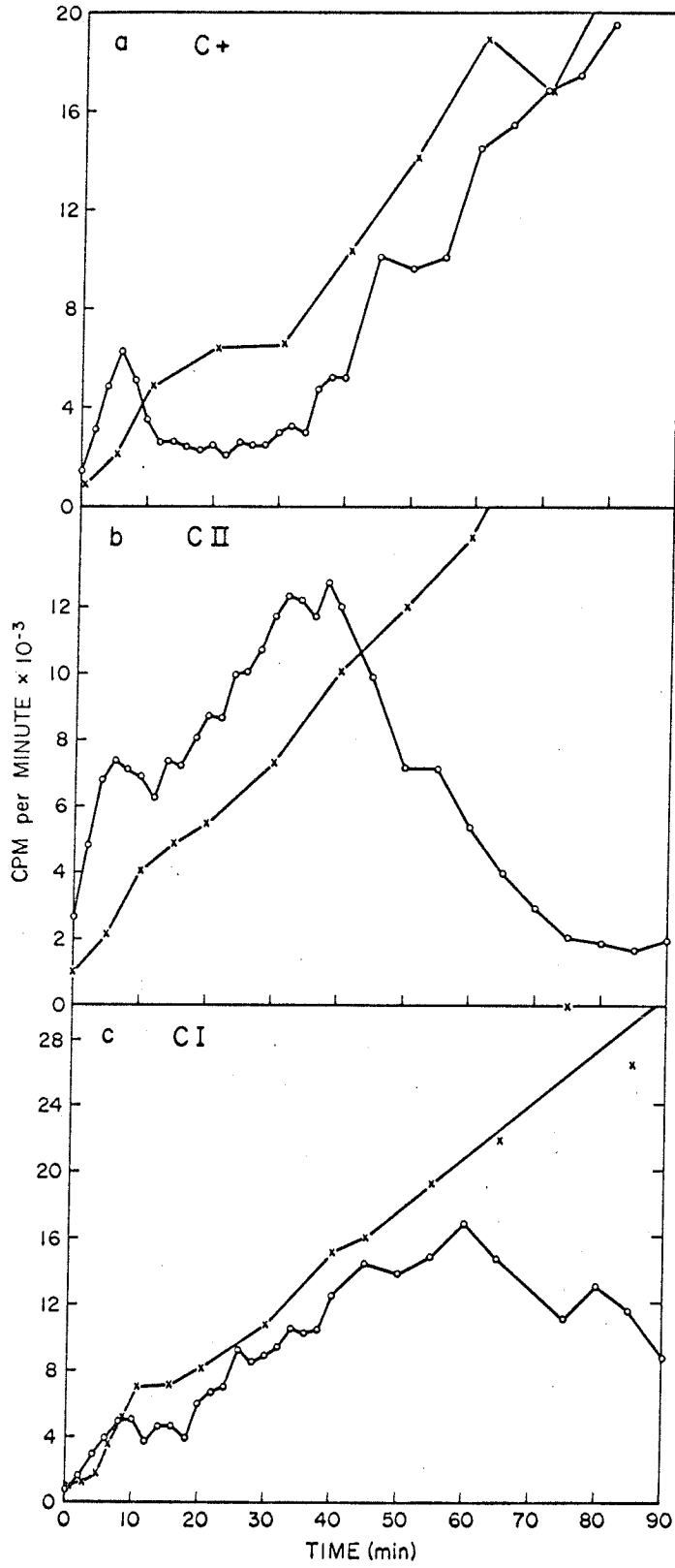Figure 12. ^{3}H-thymine <u>incorporation</u> <u>and</u> <u>phage</u> <u>growth</u> <u>in</u> <u>MC-treated</u>

<u>E. coli AB2500</u> <u>infected</u> <u>with</u> <u>λcI</u>, <u>λcII</u>, <u>or</u> <u>λcIII</u>. Cells were grown to

2×10^8 bacteria/ml. in KG medium plus thymine and uracil. The culture

was treated with MC as described in Methods, filtered, washed, and the

bacteria were resuspended in 1/10 volume TM. The appropriate phage

stocks were added to a final multiplicity of 5 pfu/cell to three samples

of the bacteria at 37°C. A fourth portion was used for an uninfected

control. Ten minutes after adding phage the cultures were diluted

with 9 volumes of prewarmed medium containing 5 μg/ml. each of thymine

and uracil, and aerated at 37°C. At 2.5 minute intervals, 1.0 ml. of

each culture was exposed to 10 μc of ^{3}H-thymine for 2.5 minutes and

then the TCA-insoluble radioactivity determined as in Figure 6. The

growth of intracellular phage is also presented.

    a.   cI (c26) and uninfected bacteria. b.   cII(c68) c.   cIII(c67).

        0———0   TCA-insoluble cts/min infected cultures

        X———X   TCA-insoluble cts/min uninfected
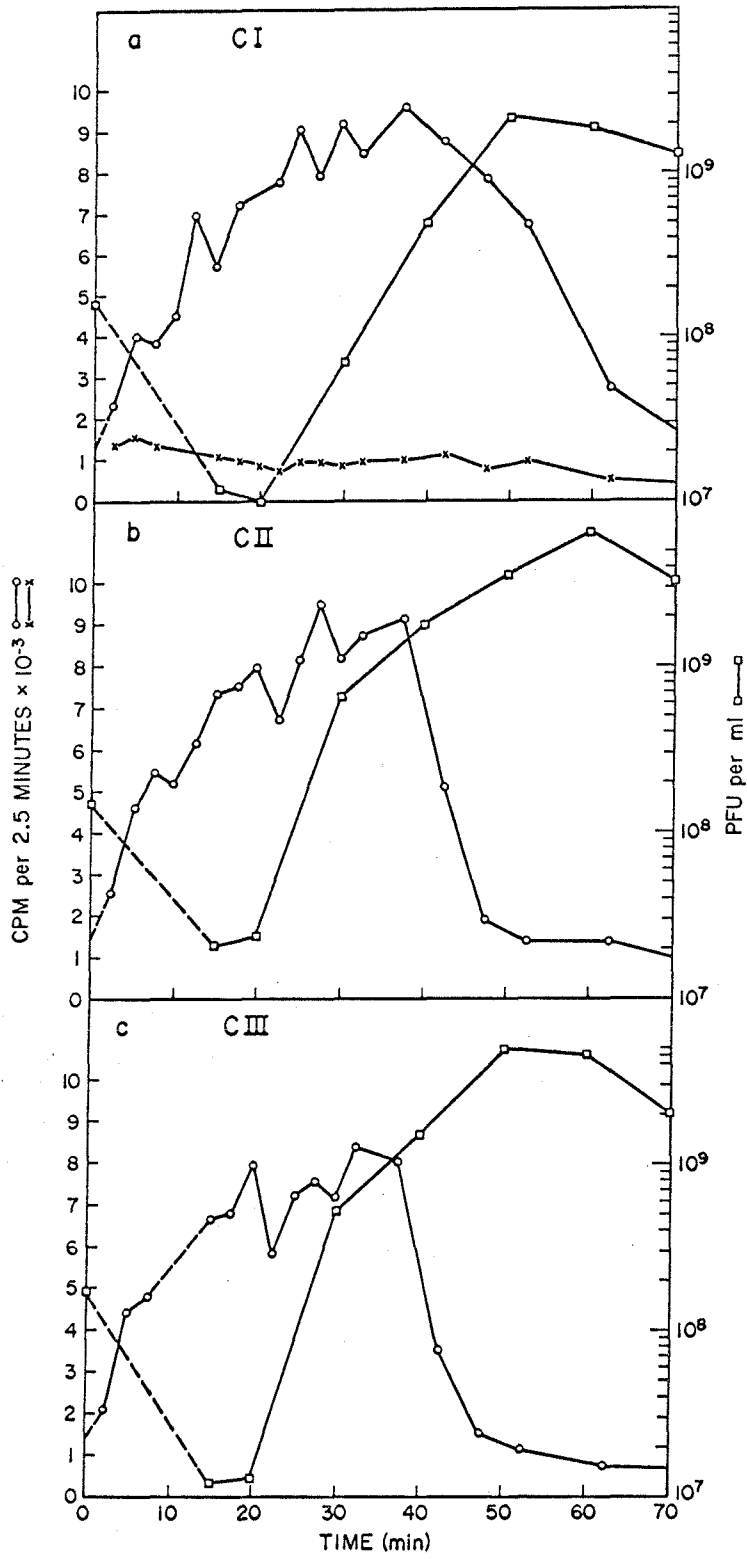
        □———□   intracellular phage

Figure 12

Figure 13. <u>DNA synthesis in MC-treated E. coli AB2500 infected with</u> <u>λ in the presence of 0, 30 and 100 μg/ml. of chloramphenicol (CAM)</u>. Bacteria were treated with MC as described in Methods and then incubated in TM containing 0, 30, or 100 μg/ml. of CAM for 15 minutes at 37°. They were then infected with 5 λc26/cell and diluted into KG medium containing the appropriate amount of CAM and 5 μg/ml. each of thymine and uracil. At 5 minute intervals, 1.0 ml. of each culture was aerated with 5.0 μc of ^{3}H-thymine for 5 minutes. The total cts/min incorporated, measured as in Figure 6, at the end of each pulse is shown in (a). The cumulative cts/min incorporated is shown in (b).

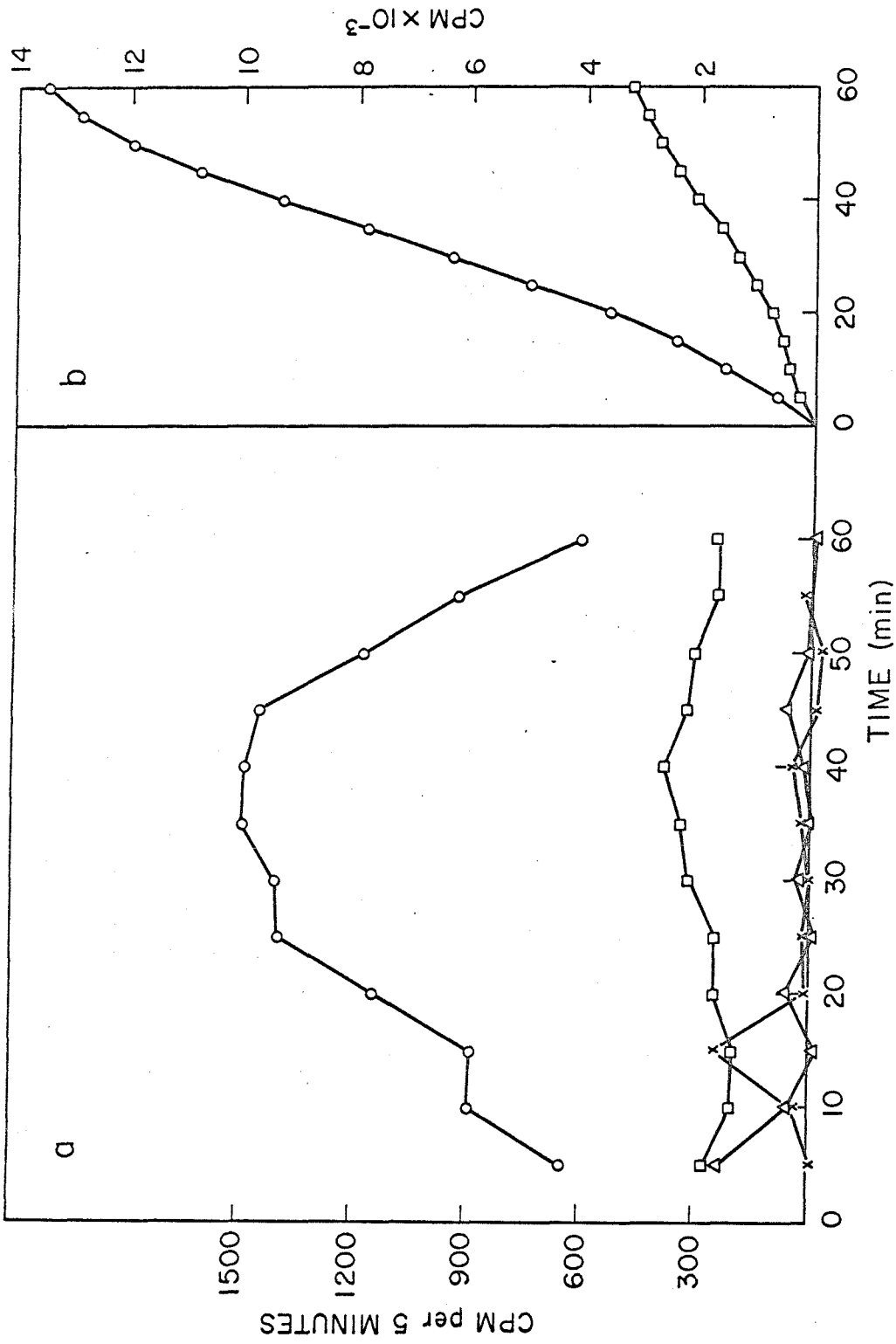|	
O——O	0 μg/ml. CAM
□——□	30 μg/ml. CAM
△——△	100 μg/ml. CAM
X——X	uninfected, 0 μg/ml. CAM

Figure 13

Figure 14. Synthesis of DNA, helper-infective DNA, and phage in MC-
treated E. coli AB2500 infected with λ. Cells were grown and treated
with MC as described in Figure 6. They were then infected and cultured
at 30°C as presented in the text. The recovery of DNA after phenol
extraction was only 30-60%, for both ^{3}H and ^{32}P. The incorporation of
^{3}H was measured by directly precipitating a sample from the culture
with TCA. The amount of infective DNA (in the helper assay) has been
normalized to ^{32}P dpm in the dialyzed extract to reduce some of the
uncertainty introduced by the variable recovery of DNA.

X———X ^{3}H cts/min

$\triangle$———$\triangle$ helper-infective DNA

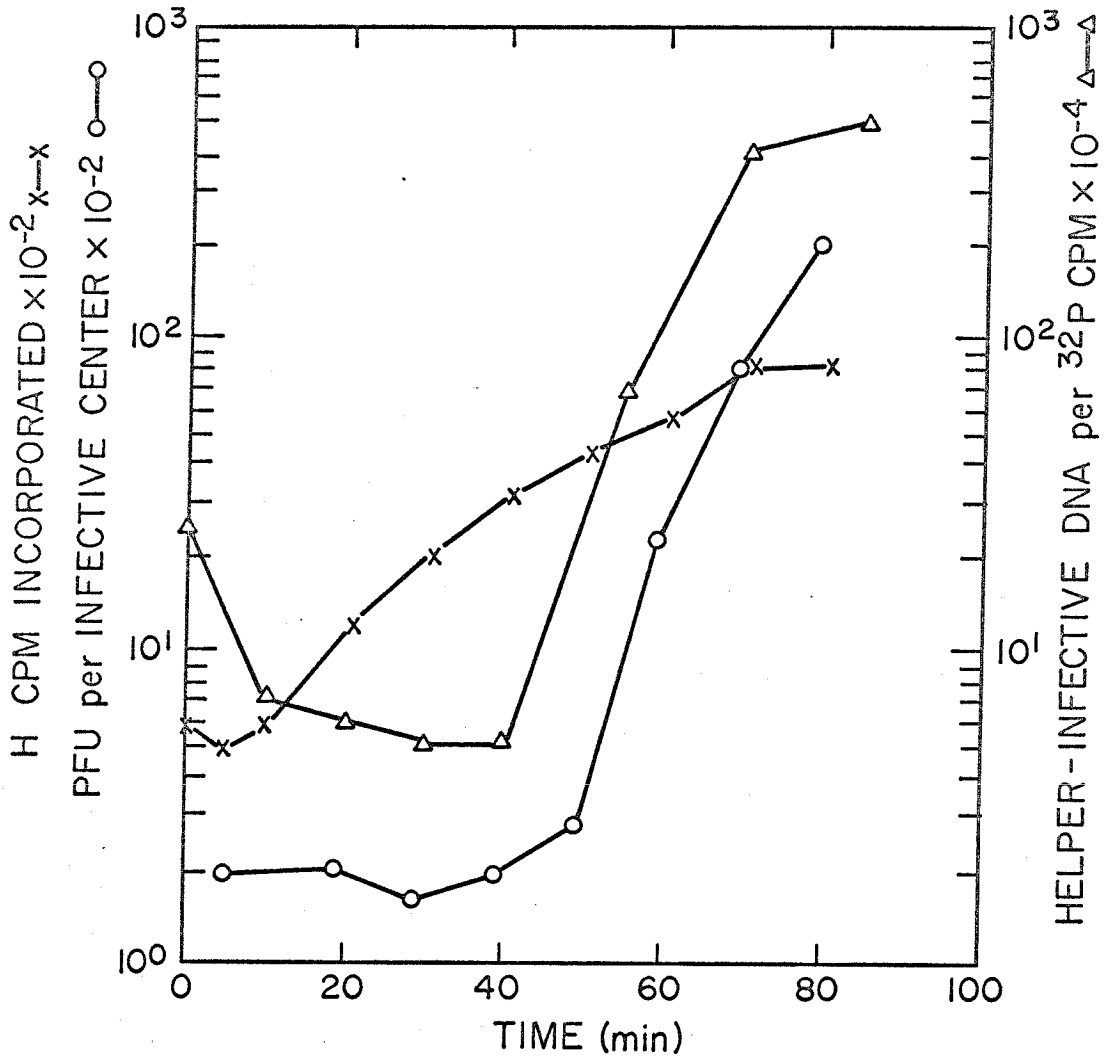0———0.   intracellular phage

Figure 14

Figure 15. <u>Sedimentation analysis of vegetative</u> λDNA <u>at various stages</u>

<u>of infection</u>. Extracts of cells (containing the nucleic acids from

about 4×10^8 cells) prepared as described in Figure 10 were layered on

5-20% sucrose gradients containing (a-e) TE, pH 8, or (f) 0.3 $\underline{M}$ NaOH,

0.3 $\underline{M}$ NaCl, and centrifuged for 5 hours (a-e) or 3 hours (f) at 25,000

rev./min and 6°C in the SW 25.1 rotor. Fractions were collected and

analyzed for radioactivity and infectivity in the helper assay. 80-

100% of the radioactivity layered on the gradients was recovered. The

efficiency of the helper assays varied between gradients so the specific

infectivity cannot be compared precisely.

0————0 ^{32}P parental DNA cts/min

X————X ^{3}H-vegetative DNA cts/min
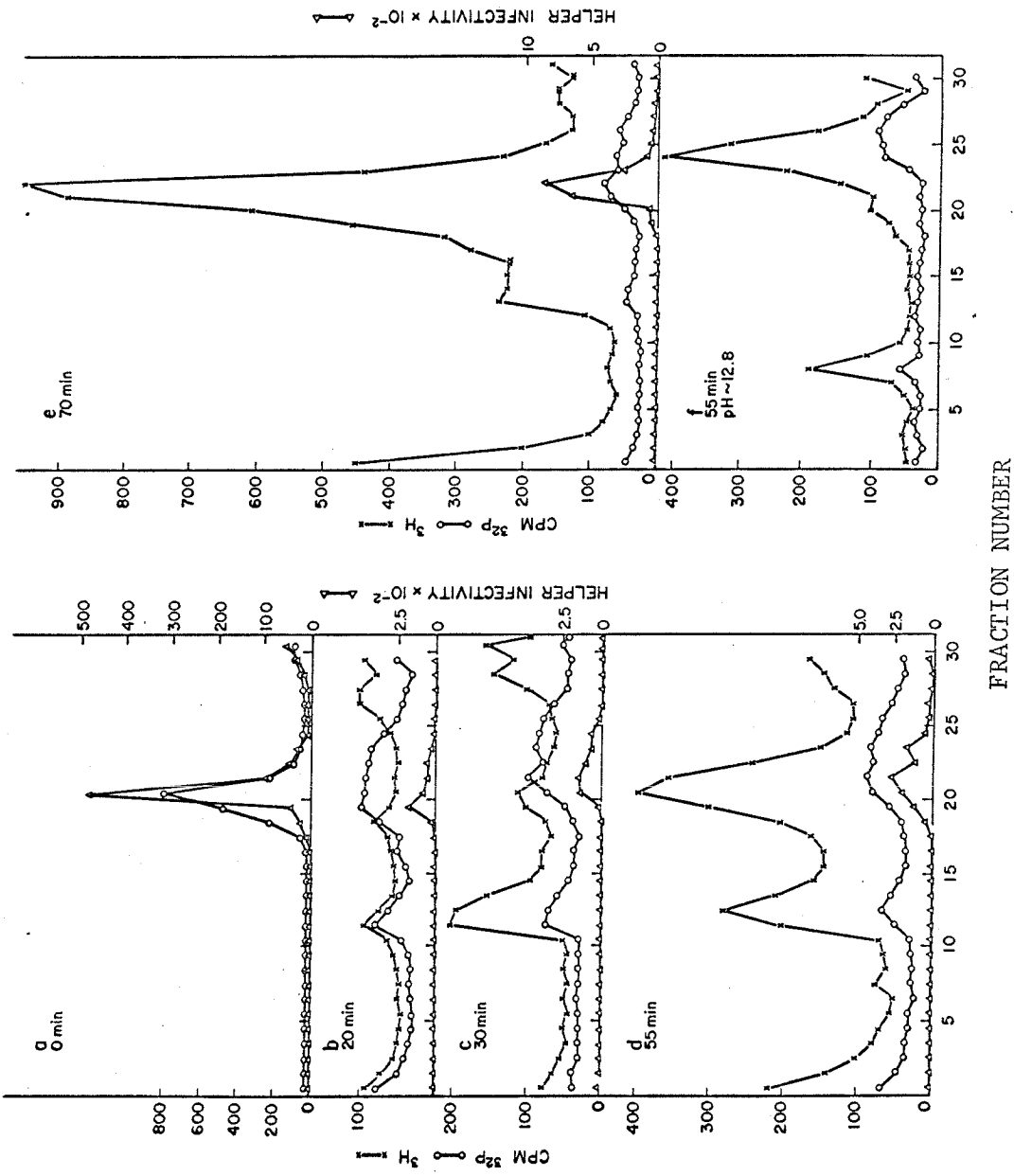
Δ————Δ   helper infectivity

Figure 15

Figure 16. Sedimentation analysis of vegetative λDNA extracted 60

minutes after infection.   The experiment was performed essentially as

described in Figure 6 but the infected bacteria were incubated at 37°C

for 60 minutes in the presence of ^{3}H-thymine before lysis and extraction

with phenol. The recovery of ^{3}H in the dialyzed phenol extract was

about 60%. A sample of the extract was layered on a neutral sucrose

gradient with a b5 DNA sedimentation marker and centrifuged for 5 hours

at 25,000 rev./min and 6°C in the SW 25.1 rotor. 0.5 ml. of each frac-

tion was TCA-precipitated and counted. 0.9 ml. of 0.05 M tris, pH 8.1,

was added to the remaining approximately 0.3 ml. and 0.3 ml. of sphero-

plasts was added. The remainder of the bioassay was performed as des-

cribed in Methods.

□————□   marker i^{b5}DNA spheroplast infectivity

X————X ^{3}H cts/min

△————△   vegetative iλDNA spheroplast infectivity
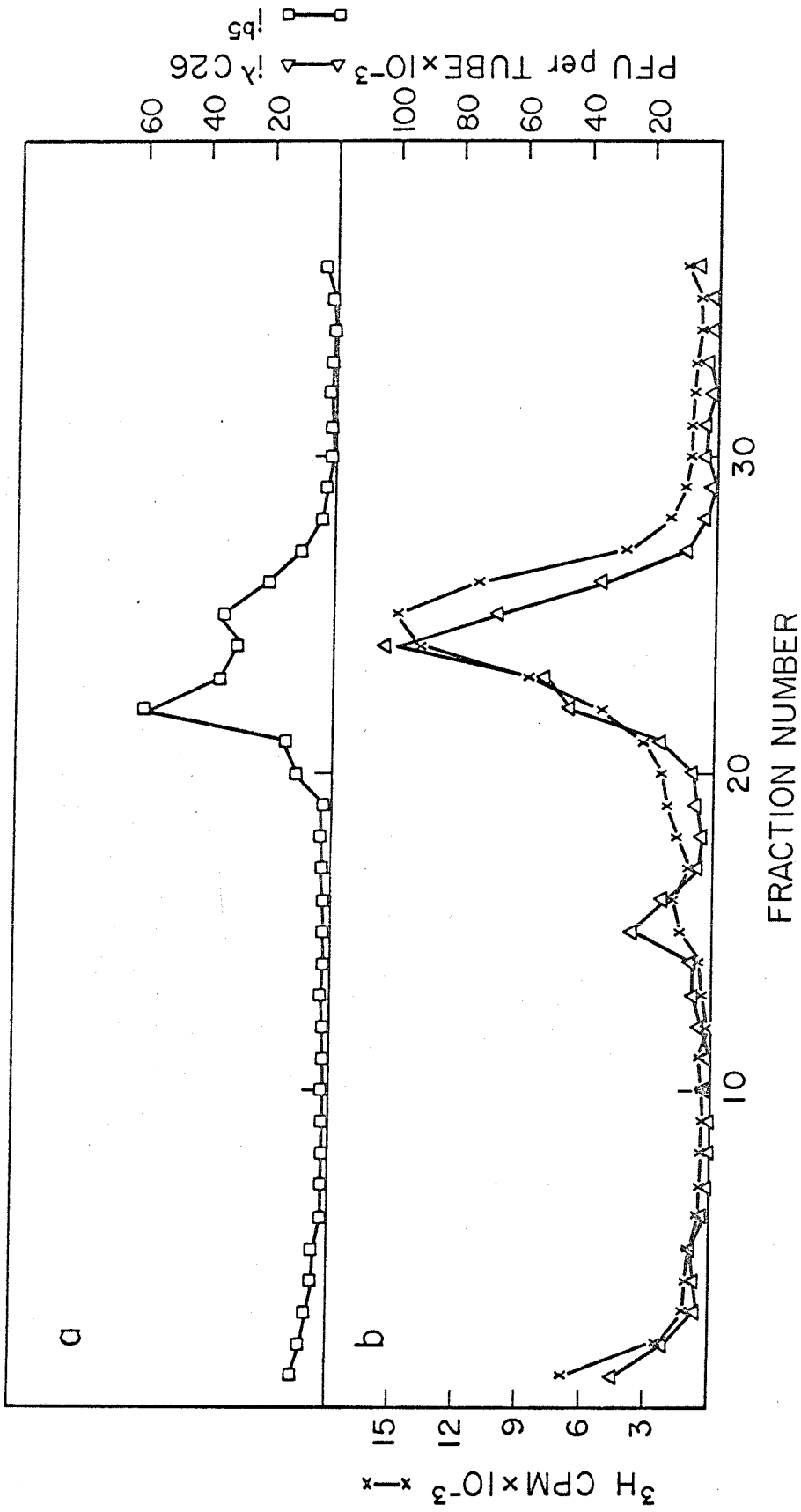
Figure 16

Figure 17. <u>Sedimentation analysis of vegetative</u> λ <u>DNA synthesized in</u>

<u>the presence of CAM.</u> E. coli AB2500 was infected as described in

Figure 9 and in the text. The infections in the presence of 30 μg/ml.

of CAM and in the absence of CAM were done in medium containing ^{3}H-

thymine in order to label the progeny DNA. The infection performed

in 100 μg/ml. of CAM was done in medium containing only ^{1}H-thymine since

no DNA synthesis occurs in this concentration of CAM (Figure 9). Lysates

of the infected cultures were made by method B (without phenol extraction)

at 35 minutes (0 μg/ml. CAM) or 45 minutes after infection (30 and

100 μg/ml. of CAM). Samples of the lysates were layered on 5-20%

sucrose gradients containing 0.01 M tris, 0.001 M EDTA, pH 10.5, and

centrifuged for 5 hours at 25,000 rev./min and 6 C. ^{3}H- DNA was

added as a sedimentation marker before centrifugation of the DNA ex-

tracted from the culture containing 100 μg/ml. CAM.

   a. No CAM, ^{32}P parental DNA, ^{3}H progeny DNA. b. 30 μg/ml. CAM,

^{32}P parental DNA, ^{3}H progeny DNA. c. 100 μg/ml. CAM, ^{32}P parental DNA,

^{3}H phage DNA sedimentation marker added before centrifugation.

0——0	parental ^{32}P cts/min
X——X	^{3}H cts/min
△——△	helper infectivity
□——□	spheroplast infectivity
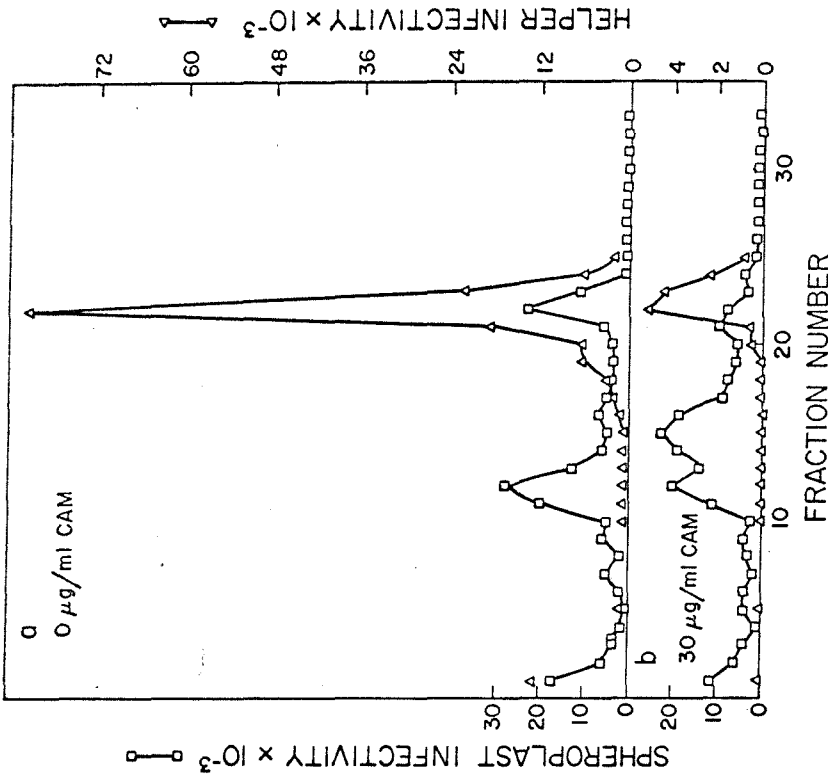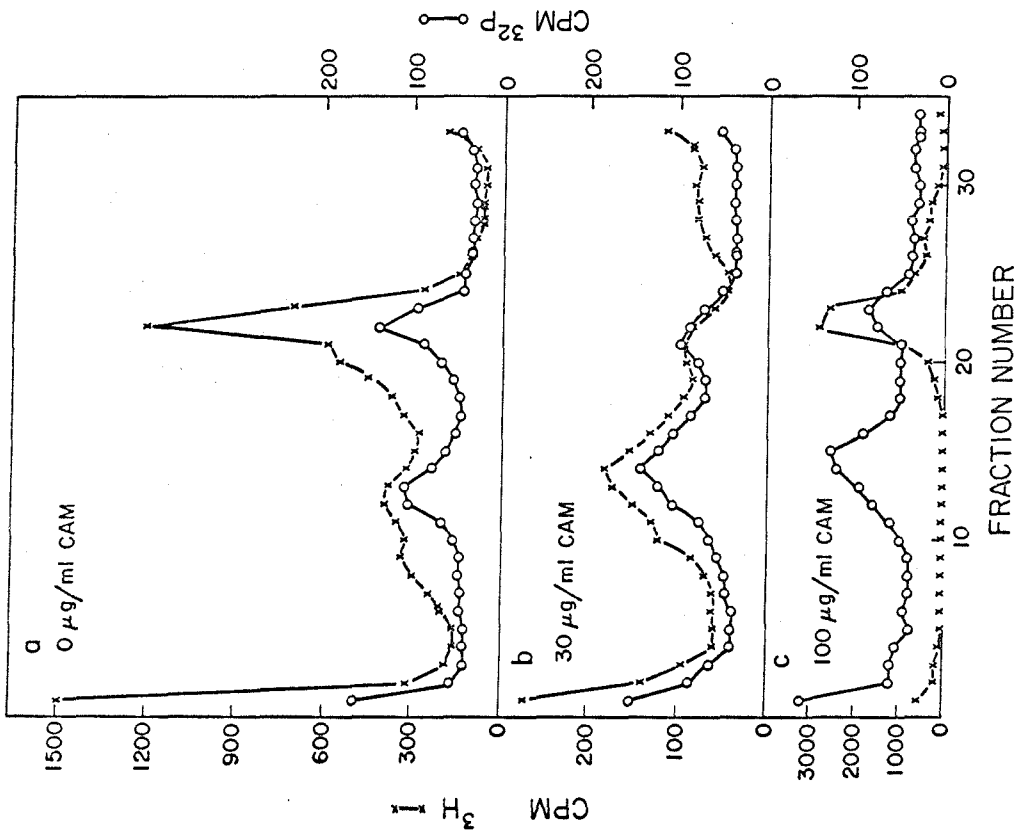
Figure 17

Figure 18. <u>Sedimentation analysis of vegetative DNA extracted at</u> <u>various times with or without phenol</u>. The cells were infected, lysed, and sedimented as described in the legend to Figure 17. All the lysates were first treated with SDS and pronase. The extracts are from the culture infected in the absence of CAM. Sedimentation shown in <u>e</u> was for only 4-5 hours compared to 6 hours for the others.

168
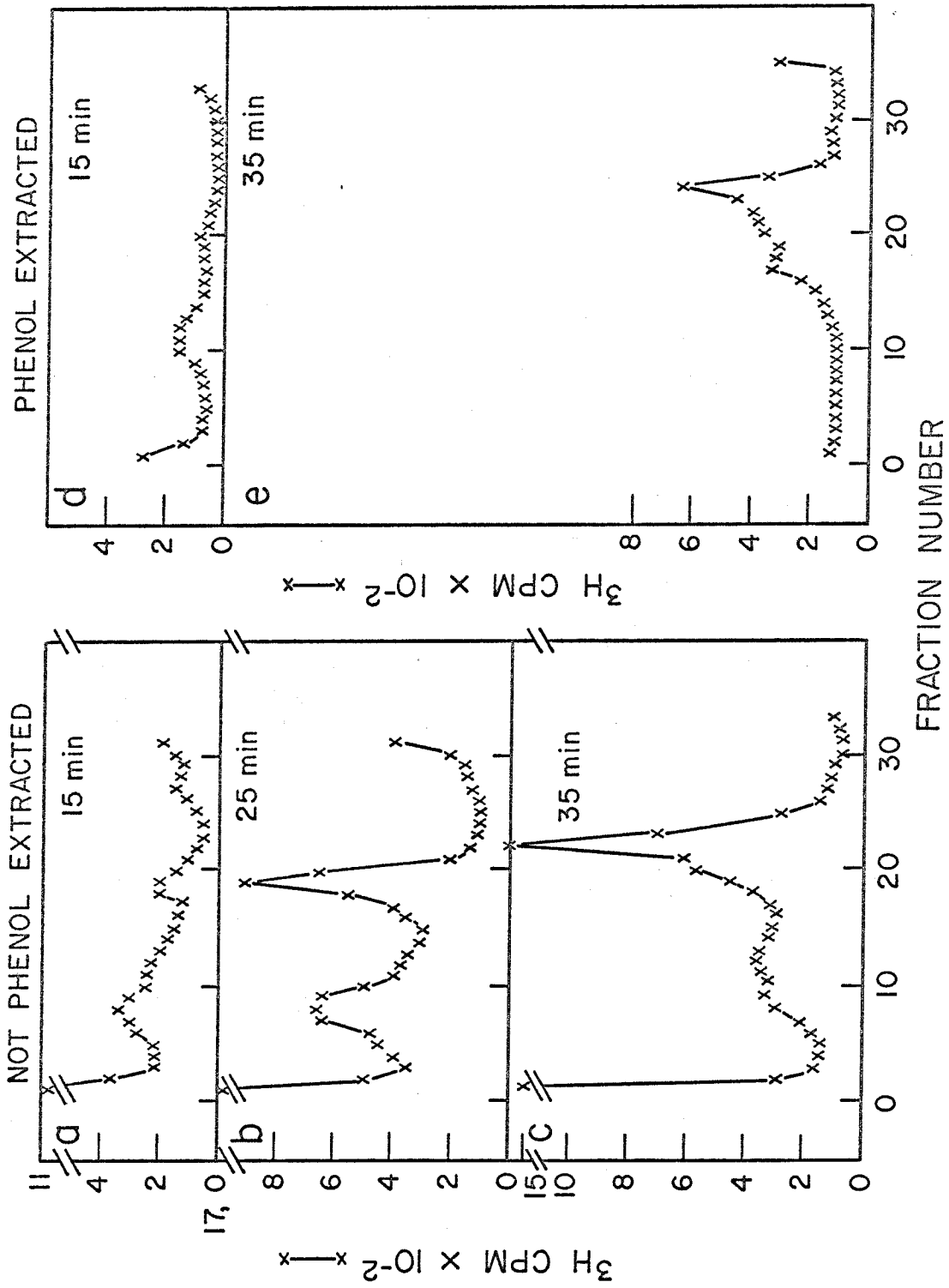
Figure 18

Figure 19. <u>CsCl equilibrium density gradient centrifugation of</u> $^{32}P^{13}C^{15}N$-

λ <u>phage DNA and component I extracted 30 minutes after infection</u>.

    a. Phenol extracted DNA from purified heavy phage was mixed with

<u>E. coli</u> ^{3}H-DNA in 1.71 g/cm^3 CsCl at pH 8 and centrifuged for 42 hours

at 30,000 rev./min and 20° C. The density gradient was measured pycno-

metrically as described in Methods.

    b. and c. Component I was isolated after sucrose gradient sedi-

mentation of extracts made 30 minutes after infection of CR34 with

^{32}P $^{13}C^{15}N$-λc26 in light, nonradioactive medium. A density marker,

^{3}H-<u>E. coli</u> DNA, was added before centrifugation. The results in (b)

and (c) are from two similar experiments.

        0———0 ^{32}P cts/min

        X———X ^{3}H cts/min

        □———□   density

Figure 19

171
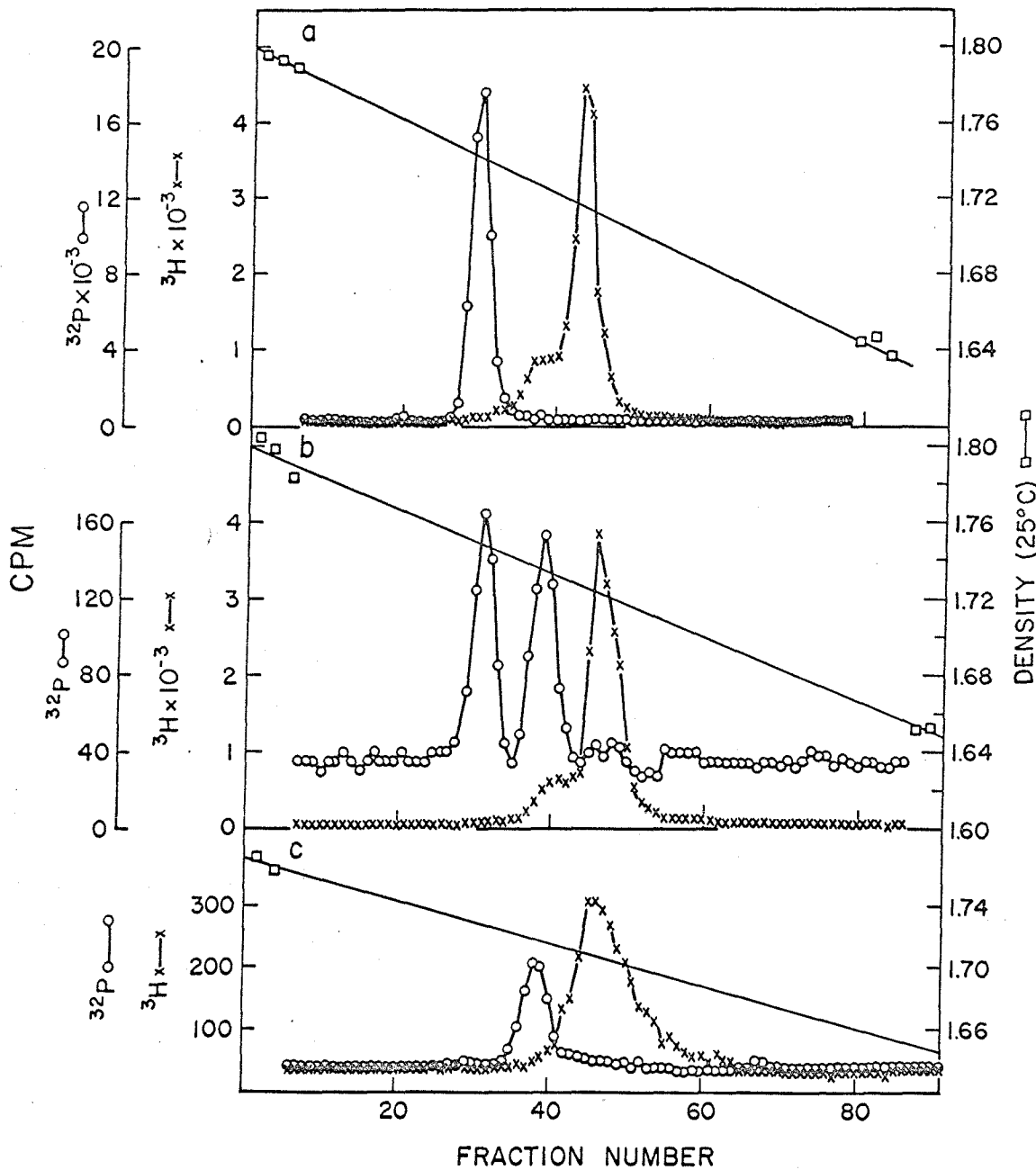
Figure 20. <u>CsCl equilibrium density gradient centrifugation of</u> $^{32}P^{13}C^{15}N-$ <u>components</u> <u>II</u> <u>and</u> <u>III</u>, $^{32}P^{12}C^{14}N$<u>-component</u> <u>I</u>.

    a. Components II and III were isolated from the same sucrose gradient from which component I, Figure 14c, had been obtained and centrifuged in the same way after ^{3}H-<u>E. coli</u> DNA had been added.

    b. Component I obtained as described in Figure 14 but radioactive, <u>light</u> phage were used in the infection. It was mixed with CsCl and ^{3}H-<u>E. coli</u> DNA before centrifugation.

        0———0 ^{32}P cts/min

        X———X ^{3}H cts/min
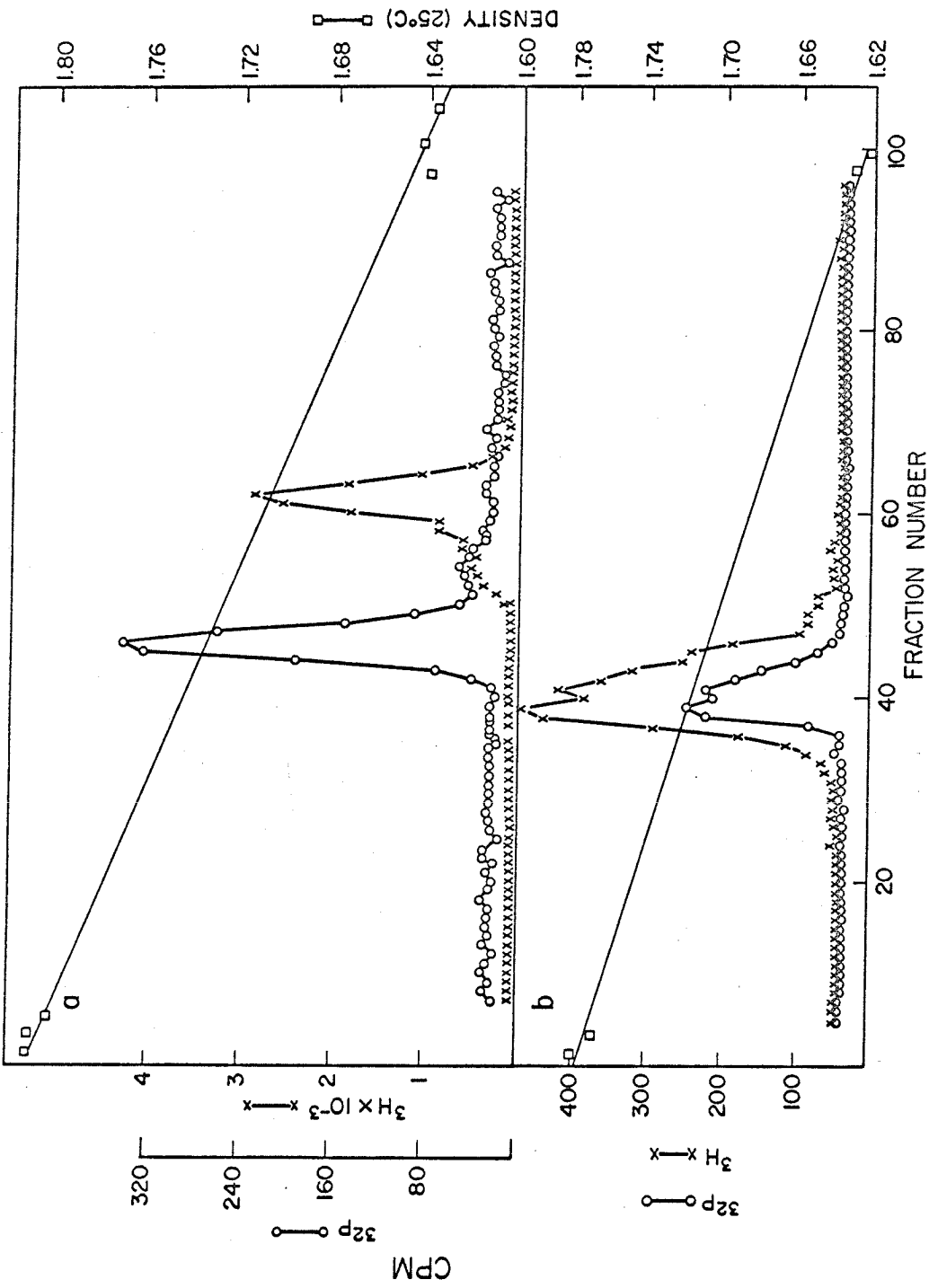
Figure 20

Figure 21. <u>Sedimentation</u> <u>analysis</u> <u>of</u> <u>vegetative</u> λDNA. <u>E.</u> <u>coli</u> AB2500 was infected with $^{32}P^{13}C^{15}N$-λc26 as described in the text and starved of thymine for 30 minutes. A portion was taken for DNA extraction (using method A) at the end of the thymine starvation. The sedimentation analysis of this extract is not shown but the distribution of parental ^{32}P was similar to that shown in (a) and (b). ^{3}H-thymine was added to a final concentration of 2.5 μg/ml. and 0.62 c/mmole. Ten and 15 minutes later, portions were taken for DNA extraction. The recoveries of label in DNA in the dialyzed phenol extracts made 0, 10 and 15 minutes after adding ^{3}H-thymine were: ^{32}P: 54, 38, and 61%; ^{3}H: (label not added to zero-minute sample) 50 and 73%. Samples from the extracts were layered on sucrose gradients as usual at pH 8 and centrifuged for 5 hours at 9°C at 25,000 rev./min in the SW 25.1 rotor. The recovery of radioactivity from the gradients was greater than 90%.

a. Sedimentation of the extract prepared ten minutes after addition of ^{3}H-thymine. b. Same, but 15 minutes after ^{3}H-thymine addition.

0———0 ^{32}P cts/min

X———X ^{3}H cts/min

△———△   helper infectivity
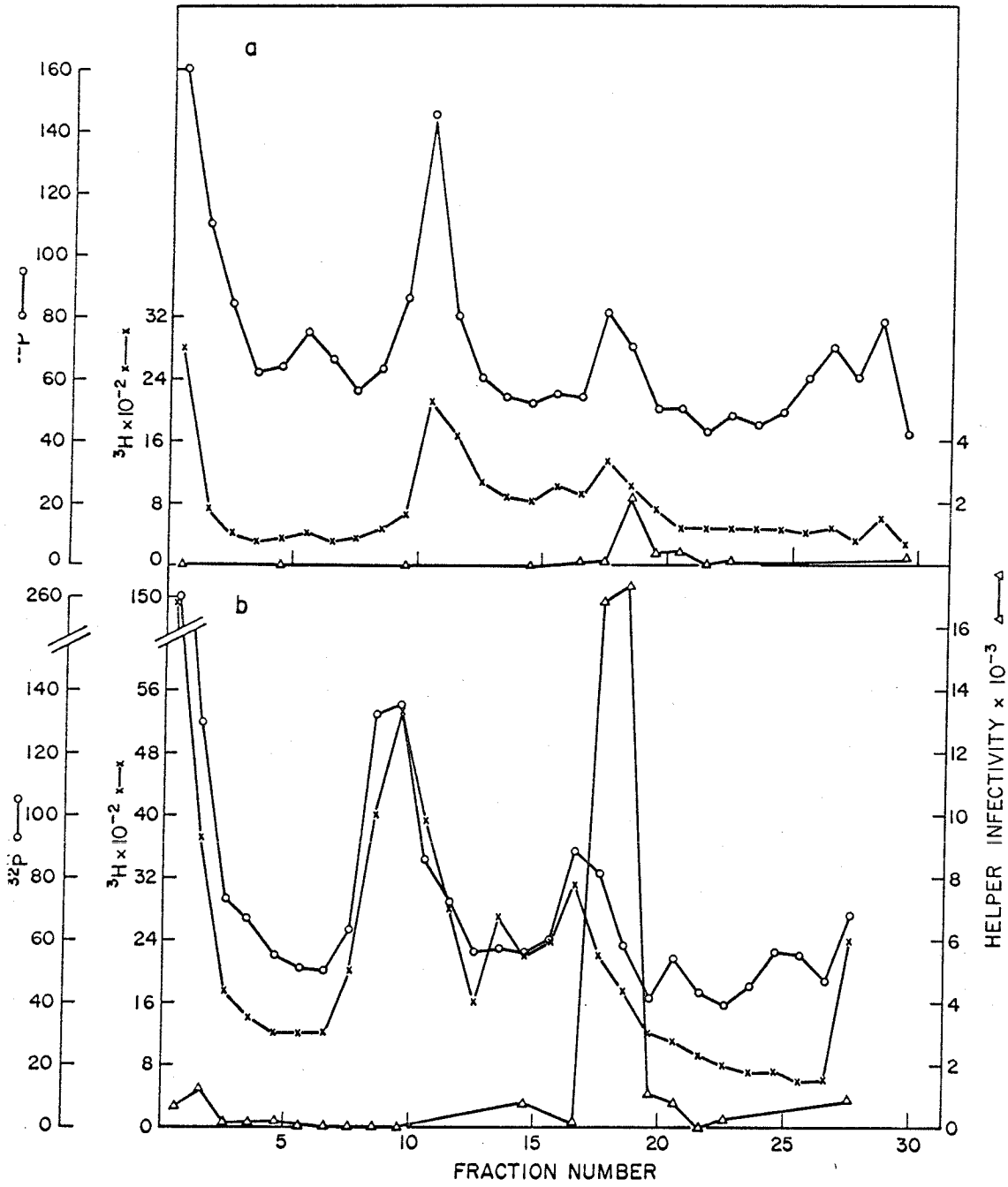
Figure 21

Figure 22. <u>CsCl equilibrium density gradient centrifugation of isolated</u>

<u>component I</u>. Fractions containing component I were pooled from the

sucrose gradients shown in Figures 16a and b and also from the sucrose

gradient of the extract made before the addition of thymine (not shown).

^{3}H-<u>E. coli</u> DNA was added as a density marker to the latter sample before

centrifugation since in this case the progeny DNA was not labeled with

^{3}H. CsCl was added to bring the final density to 1.71 g/cm^3 (pH 8)

and the solutions were centrifuged to equilibrium (36 hours at 30,000

rev./min at 20°C). Drops were collected onto GF/A filters in vials,

dried, scintillation fluid added, and the vials counted in a scintil-

lation counter.

    a. Component I from "thymine starved" cells; ^{32}P^{13}C^{15}N-parental

DNA; <u>E. coli</u> DNA density marker. b. Component I, 10 minutes after ad-

dition of ^{3}H-thymine. c. Component I, 15 minutes after addition of

^{3}H-thymine.

        0————0 ^{32}P

        X————X ^{3}H

        □————□   density
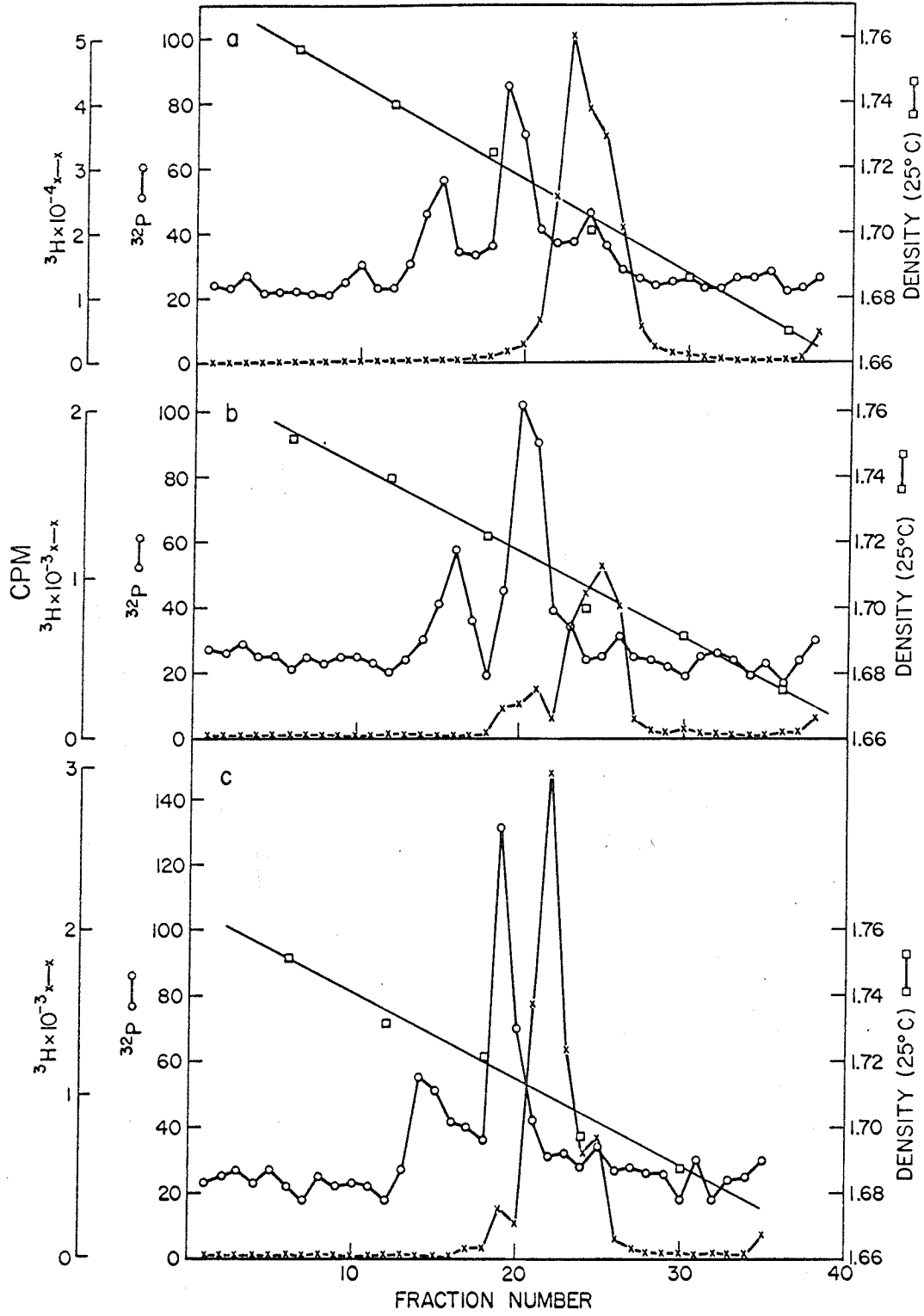
Figure 22

Figure 23. <u>Stability</u> <u>of</u> <u>HL</u> <u>DNA</u> <u>after</u> <u>alkaline</u> <u>denaturation</u>. Components

I and II were isolated from neutral sucrose gradients and centrifuged

(separately) to equilibrium in neutral CsCl density gradients. The

fractions containing HL_I were pooled and dialyzed to remove CsCl, and

then denatured with alkali and reneutralized as described in the text.

·Pooled HL_{II} was treated identically. The reneutralized solutions were

mixed with CsCl to bring the final density to approximately 1.71 g/cm^3,

and the solutions were centrifuged to equilibrium (42 hours at 30,000

rev./min at 20°C). Fractions were collected and counted as described

in Methods. The samples of component I and component II are from dif-

ferent experiments in which the vegetative DNA was labeled with ^{3}H-

thymine at different specific activities, hence the ratio $^{32}P/^3H$ is

different for HL_I and HL_{II}. The parental DNA contained ^{32}P, ^{13}C, and

^{15}N.

      a. Component II.   b. Component I.

          X——X ^{3}H cts/min

          0——0 ^{32}P cts/min

          □——□   density
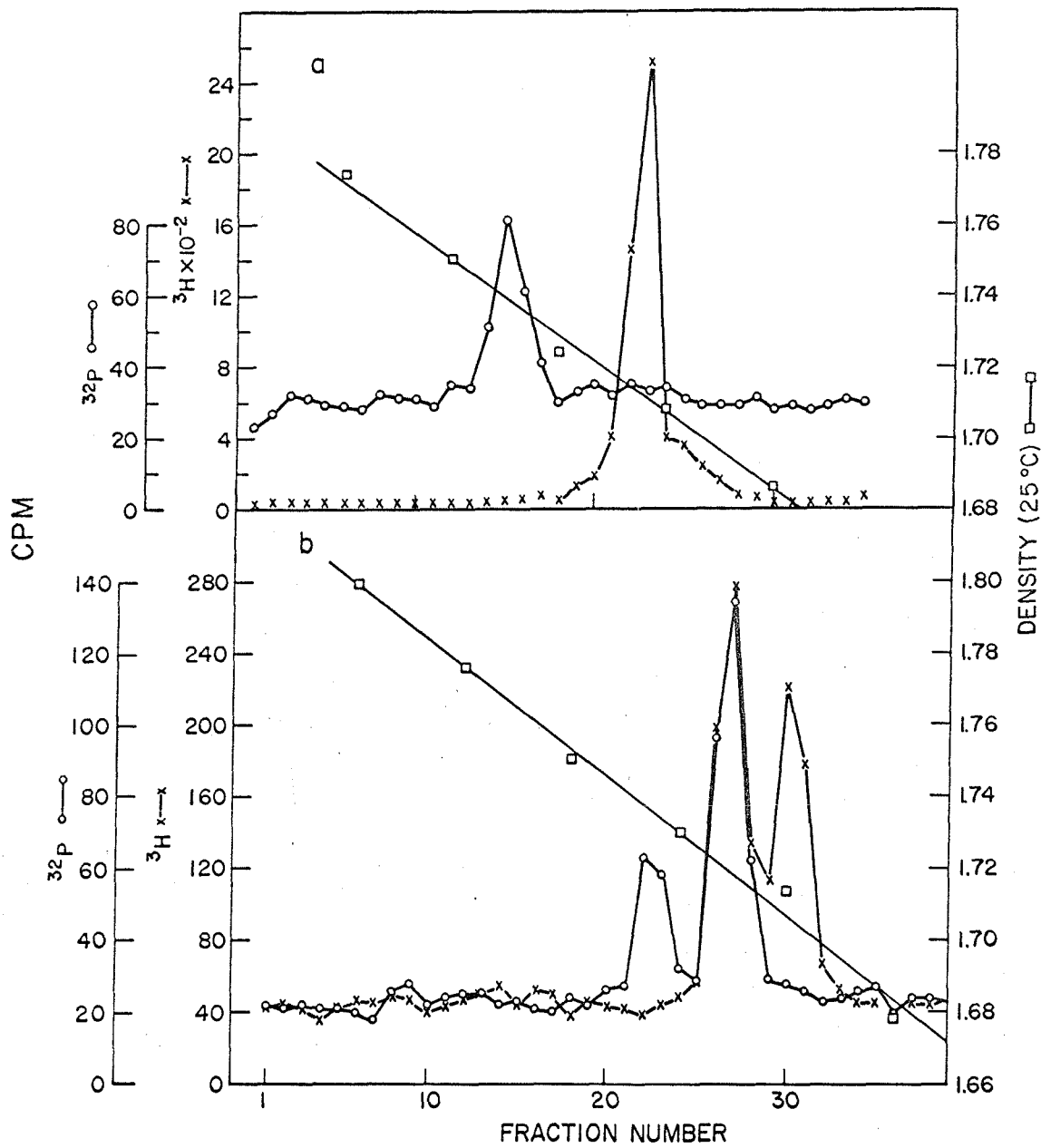
Figure 23

Figure 24. <u>Spheroplast</u> <u>infectivity</u> <u>associated</u> <u>with</u> <u>component</u> <u>I</u> <u>at</u>

<u>various</u> <u>stages</u> <u>of</u> <u>infection</u>. Details of the infection and analysis

are presented in the text. The results from three separate sucrose

gradient sedimentation runs are presented.

       0————0   "0" minutes (40 minutes after infection in

                          the absence of thymidine).

       △------△   10 minutes after adding thymidine.

       □·····□   20 minutes after adding thymidine.

The large arrow (↓) indicates the position of the sedimentation marker

(λb2b5c DNA) on the gradients.
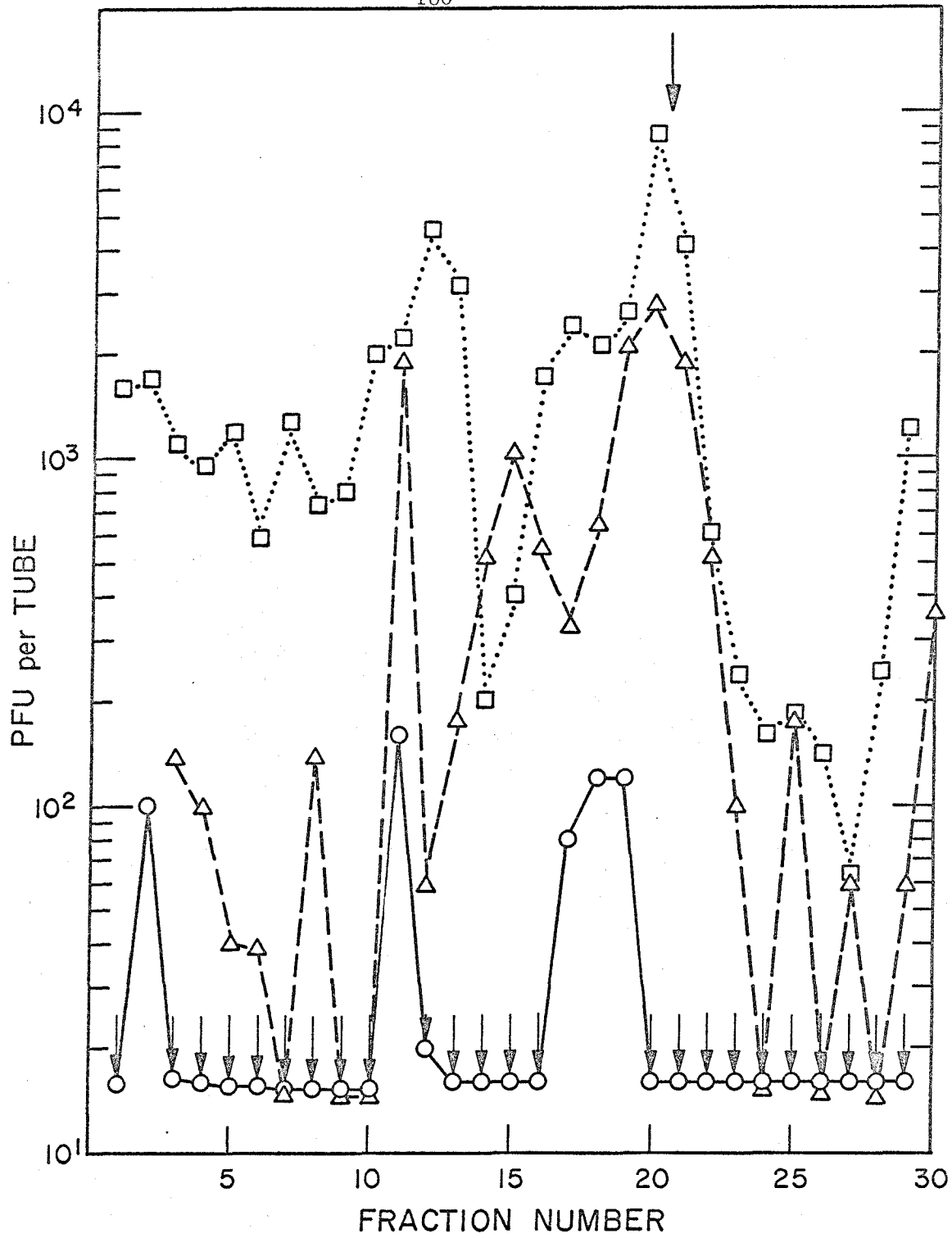
Figure 24

Figure 25. Spheroplast infectivity of component I molecules of different buoyant densities after infection with λc26 containing $^{32}P^{13}C^{15}N$. The DNA extracted 10 minutes after adding thymidine was sedimented through a sucrose gradient (Figure 24) and fractions containing component I were pooled, mixed with ^{3}H-E. coli DNA, b2b5c phage DNA, and CsCl and centrifuged to near equilibrium. A sample of ^{32}P $^{13}C^{15}N$-λc26 phage DNA was banded simultaneously. Fractions to be assayed in spheroplasts were first dialyzed to remove CsCl.

    (a)   X———X ^{3}H-E. coli DNA cpm

           ☐———☐   b2b5c spheroplast infectivity

    (b)   Phage DNA (λc26)   0———0 ^{32}P cpm

                               △———△   Spheroplast infectivity

    (c)   Component I   0———0 ^{32}P cpm

                          △———△   Spheroplast infectivity
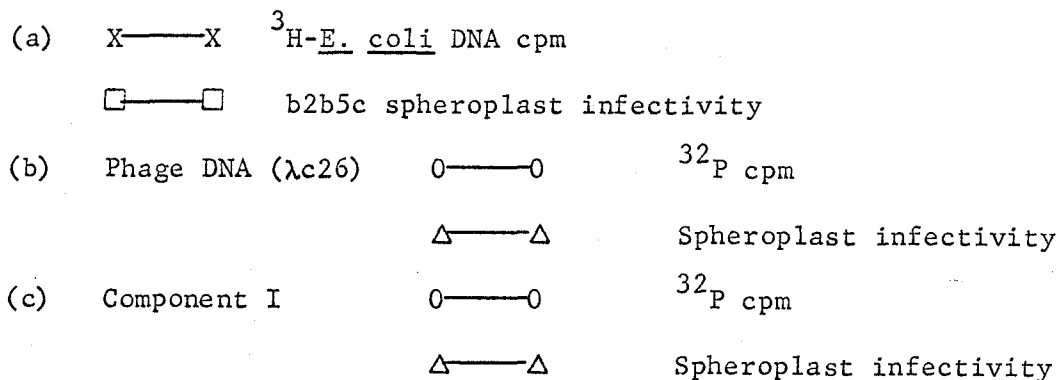
49 and 50 fractions were collected from the gradients containing phage DNA and component I, respectively. Only those fractions containing radioactivity or infectivity are shown.

Figure 25

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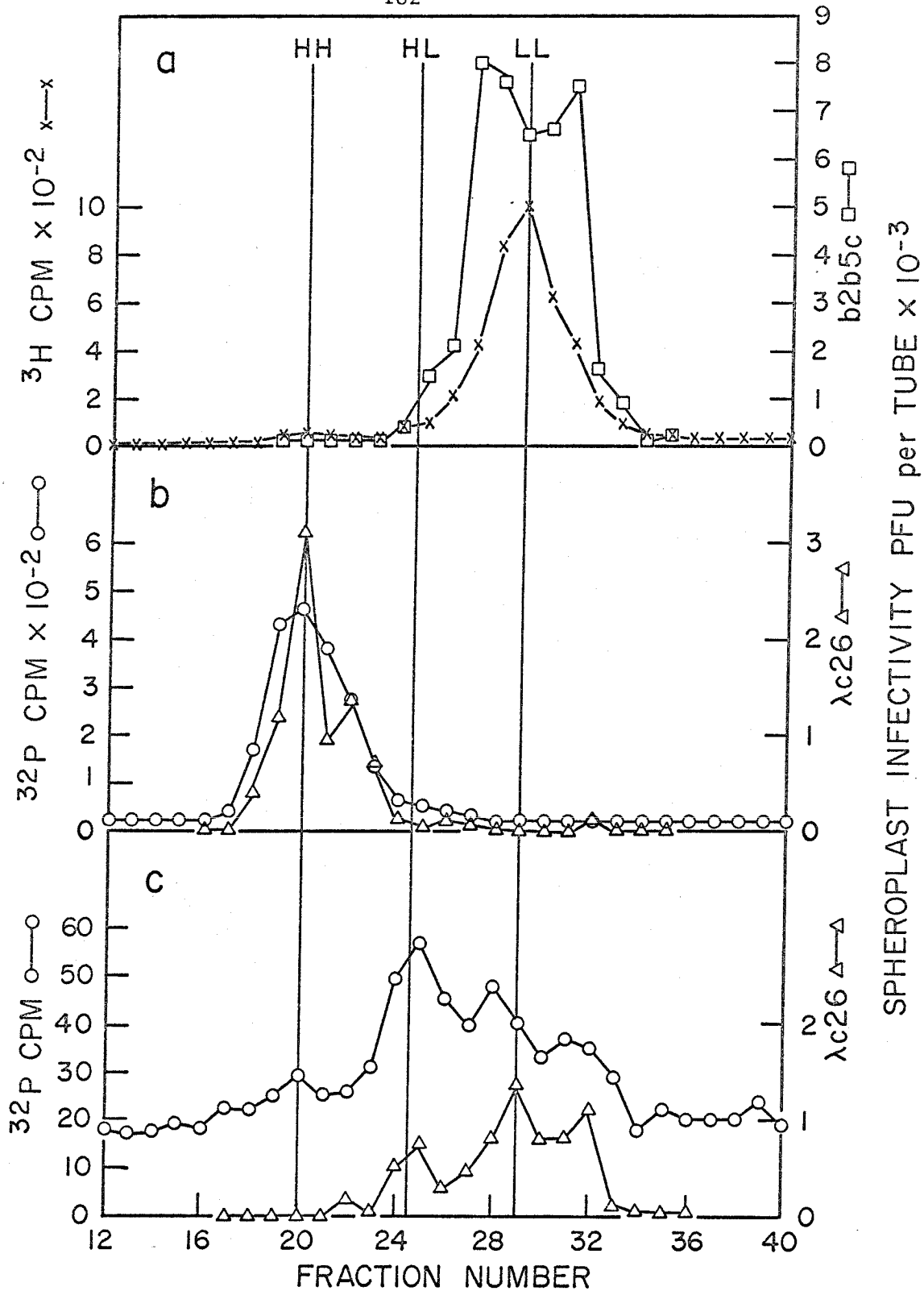
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Part III

VEGETATIVE LAMBDA DNA: PULSE-LABELED COMPONENTS

1. INTRODUCTION

Two distinct modes of phage DNA replication have been proposed.
The first, exemplified by ØX174, involves a circular replicative molecule
(21). The second has been suggested by an analysis of vegetative T4
DNA. The interpretation of the latter data is that linear concatenates
are the precursors to phage DNA. λ DNA synthesis also involves circular
replicative forms analogous to those found in ØX-infected cells (7).
Smith & Skalka (23), however, suggested that the precursor to phage
DNA was a concatenate of λ genomes. The possibility thus exists that
λ DNA synthesis involves both types of "replicative forms".

Certain differences in DNA replication, however, must exist between
ØX and λ, on the one hand, and T4 and λ on the other. The synthesis
of linear, double-stranded λ DNA from a circular template (that λ RF
can - directly or indirectly - initiate synthesis of phage DNA is shown
conclusively by its infectivity in the spheroplast assay (29)) must
differ in some respects from the production of single-stranded, circular
ØX DNA. Whatever mechanism produces phage-size DNA molecules must
differ in λ and T4 since deletions increase the length of terminally
heterozygous regions in T4 (24) - supporting the idea that "head-size"
pieces of T4 DNA are cut from a concatenate - but deletions in λ produce
DNA molecules whose length is decreased in proportion to the length of
deletion (15).

The experiments to be described were performed in an attempt to
identify the precursors to both RF I[1] and phage DNA, and to study in
a preliminary way the structure of "replicative intermediates". The
rationale of the experiments is to label λ-infected bacteria for periods
of time short enough that a significant fraction of the labeled molecules
will be only partially replicated. With longer exposures, more comple-
ted molecules will be present in the population and some relation between
incomplete (i.e. precursors) and completed molecules should become
evident.

This differs operationally from the usual "pulse-chase" experiment
since the isotope is not chased out and one relies on the changing rel-
ative amounts of the components to reveal their relationship. This
type of experiment was performed after considerable difficulty had been
experienced attempting to remove quantitatively nucleic acid precursors
after they had been phosphorylated within the cell.

The results confirm the general outline of λ DNA replication pre-
sented in Part II. RF I is synthesized almost exclusively at early

[1] Hereafter I will refer to the circular vegetative λDNA as λRF or
simply RF if there is no possibility of confusion with $\emptyset$X RF. As in $\emptyset$X,
RF I refers to the closed-circular (supercoiled) form and RF II to the
open-circular (one-nick) form. Unless stated otherwise, RF means RF I.
H-bonded circles refer to the form prepared by annealing phage DNA *in vitro*.

times in the latent period and phage DNA at late times. These results, together with the experiments described in Part II, indicate that RF I is not a major material precursor to phage DNA. However, RF II is labelled in a short pulse at both early and late times, indicating that it may be involved in the synthesis of both RF I and phage DNA. A heterogeneous component(s) sedimenting faster than RF II also seems to be a precursor to phage DNA. The inter-relationships of these components and their relation to the concatenate hypothesis are analyzed in the Discussion.

2. MATERIALS AND METHODS

Most of the techniques used have been described previously (29, 30). The procedure for preparing spheroplasts has been slightly revised; the revisions are included below.

(a) <u>Preparation of spheroplasts and assay of fractions from gradients</u>.

Typically, a 60 ml. culture of <u>E. coli</u> 1486/λ is grown in 3XD medium to 1-1.5 x 10^8 cells/ml and then centrifuged, resuspended, and converted to spheroplasts as described before. The use of younger cells (a suggestion of J. Newbold) has increased the reproducibility of the assay considerably. Usually, 1.2 ml of DNA is mixed with 0.3 ml of spheroplast stock. The infection is equally efficient when the DNA is dissolved in 0.05<u>M</u> tris, pH 8.1, or 0.05<u>M</u> phosphate buffer, pH 6.7. PAM can be added to the incubation mixture between 30 seconds and 30 minutes after mixing spheroplasts and DNA without influencing the assay efficiency.

Samples from sucrose gradients are assayed after a four-fold

dilution with buffer. The residual sucrose does not affect the assay

efficiency. Fractions from alkaline sucrose gradients are assayed

after neutralizing the alkali by dilution with low pH phosphage buffer

such that the final molarity of phosphage is below 0.05$\underline{M}$ and the pH

is 7 to 8.

(b) <u>Outline</u> <u>of</u> <u>a</u> <u>typical</u> <u>pulse</u> <u>experiment</u>

In all of the experiments presented in Results, <u>E. coli</u> AB2500

was infected with λc26 at a multiplicity of 2-5 pfu/cell at 37° C in

TM and then diluted into prewarmed (37°C) KG medium containing 5 μg/ml

thymine. The bacteria are pre-treated with 25 μg/ml of MC to inhibit

host DNA synthesis (30). About 5 minutes before adding ^{3}H-thymine,

uracil is added at 5 μg/ml to suppress uptake of label into RNA. At

the desired time radioactive thymine is added. Incorporation is stopped

by pipetting the culture into a heavy-wall glass centrifuge tube in a

dry-ice-acetone bath. The tube contains unlabeled thymidine at a final

concentration of 2 mg/ml. At the end of the experiment the frozen

cultures are thawed at 5°C and the infected bacteria are gently pelleted

by 4 minutes centrifugation at 4000 rev./min. The pellet is resuspended

in sucrose and the DNA extracted (30). Attempts to collect and wash

the cells by filtration usually result in losses of 50-90% of the in-

fected bacteria. Near the end of the latent period λ infective centers

become very sticky and difficult to resuspend from filter paper or a

centrifuge tube and some of the cells lyse. This situation might be

improved by the use of a lysis-defective mutant.

3. RESULTS

(a) Recovery of pulse-labeled DNA from infected cells

Since there has been considerable difficulty in the past recovering pulse-labeled DNA from infected (7, 22) or even uninfected (9) bacteria, the results of the following experiment will be described in some detail with regard to the recovery.

Cultures infected with λ (as described in Methods) were pulsed with ^{3}H-thymine (at a specific activity of 2.6 c/mmole) for 60, 120, and 300 seconds starting at 10 and 35 minutes after infection at $37°$C. The pulses starting at 10 minutes will be termed early, those starting at 35 minutes, late. From previous experiments these times correspond, respectively, to periods of synthesis of RF and phage DNA.

Table I indicates the radioactivity incorporated in each pulse by 5 ml. portions of the culture and compares the number of phage DNA equivalents synthesized with the number of active phage produced measured from the intracellular growth curve. The recoveries after collection of the cells and after phenol extraction of the lysates produced by lysozyme-EDTA, SDS, and pronase treatment of the infected cells (30) is also shown. The recoveries after phenol extraction (b) are apparently enhanced by the addition of sodium trichloroacetate to the DNA solution (final concentration 0.1$\underline{M}$, pH 8) prior to phenol extraction (26). These lysates were extracted very gently by rolling to produce

an emulsion. Even so, the recoveries are high enough to insure that conclusions drawn from an analysis of this DNA will apply to most of the pulse-labeled DNA in the cell.

In the late pulse a large number of λ DNA equivalents are synthesized (9 in 60 seconds). The fraction of partially replicated molecules will thus depend largely on the number of growing strands per cell.

As observed previously (7, 30), the rate of DNA synthesis is not constant at early times in the latent period but is approximately so later. Although this might imply a different mechanism of DNA synthesis at early and late times, such as an exponential versus a linear reaction, it could equally well imply that a different component in the reaction was rate-limiting at early and late times, or the establishment at late times of a steady state condition of synthesis and maturation of phage genomes. From 35-40 minutes the rate of DNA synthesis exceeds the rate of synthesis of active phage by about a factor of four. One might expect then to find a pool of free phage DNA in the cell, unlike the situation observed during ØX infection in which no free phage DNA is found (21). In the late five minute pulse the amount of phage DNA synthesized corresponds to about 35 λ equivalents. The burst size was only 90 pfu/cell, indicating that a large amount of the DNA was not put into active phage particles. (Weigle (personal communication) finds that λ lysates usually contain a variable, up to four-fold or higher, excess of full heads over tails. This might account for the "excess" DNA.)

(b) <u>Sedimentation</u> <u>analysis</u> <u>of</u> <u>pulse-labeled</u> <u>DNA</u>

(i) <u>At</u> <u>low</u> <u>ionic</u> <u>strength</u>, <u>pH</u> <u>10.5</u>

The distribution of the pulse-labeled (^{3}H) DNA described in Results

(a), and a sedimentation marker (^{32}P-phage DNA), after centrifugation

through sucrose gradients (in TE, pH 10.5) are shown in Figures 1 and 2.

The marker consists of a mixture of H-bonded circles and linear mole-

cules.   Only one-half of each fraction from the gradients containing

DNA labeled for 300 seconds was TCA precipitated and counted for radio-

activity, the remainder was used to assay for vegetative DNA infective

in the helper or spheroplast assay. DNA infective in the spheroplast

assay was measured both before and after denaturation of each fraction

from the gradient. The purpose of denaturation was to reveal the sedi-

mentation position of RF II on these gradients. Denaturation of RF II

produces single-stranded rings of λ DNA which are more infective in the

spheroplast assay than native DNA, thus an increase in the infectivity

after denaturation indicates the probable presence of RF II. RF I

usually has the same or slightly less infectivity after denaturation (31).

The sedimentation properties of the early, pulse-labeled DNA cor-

respond to those of RF I and RF II; that the fast-sedimenting DNA is

supercoiled and not a long, linear molecule (concatenate) is substantiated

in Results (c). The slow peak of ^{3}H- pulsed DNA co-sediments with the

marker H-bonded circular form and also with material which has the greatest

infectivity increase after denaturation, indicative of the position of

the <u>majority</u> of RF II. It is concluded that this pulse-labeled DNA is

also RF II. The amount of RF I increases relative to RF II as the

length of the pulse increases. At times near the end of the DNA eclipse

it is known that the ratio of RF I may be 5 or 10 to 1 (30). This

suggests a product-precursor relationship between RF I and RF II.   A

third minor component sedimenting about three times as fast as phage

DNA is also resolved on some of the gradients (fractions 4 and 5 in the

early and late 120 and 300 second pulse-labeled samples). It is either

non-infectious or present in too small quantities to be observed by

infectivity. Since bacterial DNA sediments only twice as fast as the

marker (extracted under the conditions used here), it is probably not a

linear molecule. The only forms of λDNA known to sediment this rapidly

are denatured single strands (neutralized and sedimented at high ionic

strength,(25) or denatured supercoils (30)).

Sedimentation of the late, pulse-labeled samples reveals an entirely

different distribution of components. Most of the ^{3}H in the extracts

labeled for 60 and 120 seconds sediments as RF II. Only in the extract

labeled for 300 seconds is a peak of linear phage DNA clearly resolved

(Figure 2). In addition to these components there is a large amount

of ^{3}H-DNA with a distribution of sedimentation coefficients sedimenting

faster than RF II. Although some of this material sediments nearly as

fast as RF I, very little, if any, behaves as supercoiled DNA (Results

(c)) when banded in a CsCl-ethidium bromide equilibrium density gradient.

The distribution of infectivity after sedimenting samples of the

early and late 300 second labeled extracts reveals three things. First,

the broad pattern of sedimenting material in the late sample is not an

artifact caused by front spreading since the infectivity on the same

gradient sediments homogeneously without evidence of front spreading

(the marker also behaves normally). Second, some of the material which

sediments faster than RF II apparently releases single-stranded rings

upon denaturation. This conclusion is based on the increased infec-

tivity after denaturation which occurs in the fractions ahead of RF II.

Third, there has been a 20-fold increase in the amount of helper-infective

phage DNA between 15 and 40 minutes, indicating that phage DNA synthesis is

occurring during the late pulse. The position of helper-infective DNA

corresponds to the position of a peak of pulse-labeled DNA on the same

gradient (Figure 2).

(ii) At low ionic strength, pH 10.5 and in 2.0M NaCl, pH 8.

The sedimentation rate of RF I is very sensitive to changes in

the ionic strength (30, 1), having a maximum $s_{20,w}$ at about $\mu = 10^{-2}$

(J. Kiger, personal communication) and decreasing at higher ionic

strengths. Denatured DNA is also more sensitive to changing salt con-

centrations than is native, linear DNA (25). To investigate the effect

of high and low ionic strength on the sedimentation behavior of the

DNA labeled in the 5 minute late pulse, portions were layered on sucrose

gradients containing (a) TE (0.01M tris, 0.01M EDTA), pH 10.5 or (b)

2.0M NaCl, TE, pH 8.1 and centrifuged for 6 hours at 25,000 rev./min

and 10 °C in the SW 25.1 rotor. Samples from the second phenol extrac-

tion, from which the recovery was considerable higher (Table 1, (b)),

were sedimented. Sedimentation markers, [32]P- b2b5c phage DNA and

purified ^{32}P-RF I (31), were admixed before centrifugation. The results

are shown in Figure 3. The marker ^{32}P-RF I sediments much slower at

the high ionic strength, as expected.

The low ionic strength gradient reveals that about 5% of the pulse-

labeled DNA sediments as RF I. This identification is verified in the

next section (Results (c)). Peaks corresponding to RF II and phage DNA

are also resolved. They are present in approximately equal proportions,

representing together about 50% of the pulse-labeled DNA. Most of the

remaining radioactivity on the low salt gradient sediments heterogeneously

ahead of RF II as was observed in Figures 1 and 2.

On the high salt gradient only the peak of phage DNA is clearly

resolved; the remaining radioactivity sediments heterogeneously, both

faster and slower than RF I. High ionic strength minimizes the dif-

ference in sedimentation rate between phage DNA and RF II (J. Kiger,

personal communication) and thus RF II is not resolved on this gradient.

Either the high ionic strength or the lower pH (or both) changes the sedi-

mentation properties of a large fraction of the late, pulse-labeled DNA

relative to those of RF I.

When the extract containing early, 300 second pulse labeled DNA

was sedimented through a sucrose gradient containing 2.0M NaCl the fast

peak sedimented only 1.6 times as fast as phage DNA, confirming its

identification as RF I.

(iii) <u>Sedimentation</u> <u>analysis</u> <u>of</u> <u>pulse-labeled</u> <u>samples</u> <u>extracted</u> <u>without</u>
<u>phenol</u>

Portions of the lysates just described were sedimented after treatment with pronase and SDS for two hours. At neutral pH the same sedimentation components just reported are observed and they are present in approximately the same proportions, suggesting certain components are not lost preferentially during phenol extraction. Attempts to sediment the non-phenol extracted lysates in alkali failed when most of the material pelleted on the bottom of the centrifuge tube.

(c) <u>Identification</u> <u>of</u> <u>pulse-labeled</u> <u>RF</u> <u>I</u> <u>by</u> <u>dye</u> <u>binding</u>

Radloff, Bauer & Vinograd (19) showed very elegantly that the preferential binding of the intercalating dye, ethidium bromide (Boots Pure Drug Co, Ltd., Nottingham, England), by linear or "nicked" circular DNA as compared to "supercoiled" DNA could be used as a technique to separate these forms. The supercoiled DNA binds less dye and hence has a greater buoyant density in a CsCl equilibrium density gradient than linear or open circular DNA. Phenol extracted pulse-labeled DNA was mixed with ethidium bromide and CsCl and centrifuged to near equilibrium. Phage DNA, ^{32}P- b2b5c, and purified ^{32}P-RF I were added before centrifugation. As shown in Figure 4 about 40% of the early 300 second pulse-labeled DNA on the gradient is RF I. No RF I is detectable in the late 60 second pulse sample, but about 5% of the late 300 second pulse DNA is RF I, as is shown by the inset to the bottom figure. The ^{3}H cpm

in RF I in the latter sample are not an artifact due to overlap from

the ^{32}P channel of the scintillation counter since the cpm in the ^{3}H

channel were corrected for this effect. That the correction is valid

is indicated by the absence of a ^{3}H peak in the dense position of the

gradient containing the late 60 second pulse-labeled DNA.

Because the recoveries of vegetative DNA from the gradients were

so low (see legend), the absence of RF I in the 60 second late pulse is

not proof that it was not labeled during this interval. The reason

for the low recoveries of the pulse-labeled DNA is unknown; the losses

were apparently not random since most of the ^{32}P marker DNA was recovered.

Single-stranded DNA might be expected to intercalate less dye than

native DNA, thus if the pulse-labeled material possessed regions of

single-strandedness it might have pelleted and not been recovered from

the gradients.

(d) <u>Synthesis</u> <u>of</u> <u>RF</u> <u>I</u>, <u>RF</u> <u>II</u>, <u>phage</u> <u>DNA</u> <u>and</u> <u>component</u> <u>X</u> <u>throughout</u>
<u>the</u> <u>latent</u> <u>period</u>

Synthesis of the components identified by the pulse experiment

just described was studied by pulse-labeling phage-infected bacteria

for sequential 5 minute intervals from 0 to 50 minutes as described in

Methods. For comparison, one portion of the culture was labeled con-

tinuously from 0 to 50 minutes. The total radioactivity incorporated

in the pulses was 80% of the amount incorporated continuously, indi-

cating a small lag before incorporation of the isotope during each pulse.

Samples of the phenol extracted lysates were centrifuged on low salt sucrose gradients (pH 8) with a marker DNA. The gradients are divided into three regions based on the distribution of sedimentation components: Region I contains material with the sedimentation rate of RF I. Region II contains the radioactivity which sediments ahead of the marker, but slower than RF I, ie RF II and component X - the heterogeneously-sedimenting material observed in Figures 1, 2, and 3. Region III contains material which sediments at the same rate as the marker (phage DNA). The cumulative amount of radioactivity in each of these regions is plotted in Figure 5. This plot shows the relative amount of the various components which would be _expected_ in the culture labeled continuously if each component labeled in the 5 minute pulses were stable and did not act as a precursor.

The results indicate that initially the rates of synthesis, and amounts, of RF I and RF II+X are equal (most of the latter was RF II in the early pulses). The decrease in the rate of synthesis of RF I after 20 minutes is accompanied by a very large increase in the rate of synthesis of phage DNA. The residual synthesis of RF I at late times is probably more apparent than real since it was shown in section (c) that very little RF I is synthesized at late times in the infection. Also, an alkaline sucrose gradient of the DNA labeled from 40-45 minutes revealed no RF I. These results predict that if all pulse-labeled components are stable the ratio of RF I : RF II+X : phage DNA would be 24 : 39 : 37 at 50 minutes. The ratio actually measured after sedimentation of the DNA labeled continuously from 0 to 50 minutes is

11 : 15 : 74.[1] The obvious implication is that RF II and component X

are precursors to phage DNA.

DISCUSSION AND CONCLUSIONS

Analyses of DNA pulse-labeled early in the latent period confirm

the conclusion reached previously (30). RF I and RF II, the closed and

open-circular forms, respectively, of λDNA are the primary components

which are synthesized initially after infection with λ. The increasing

ratio of RF I to RF II with longer periods of labeling, Figures 1 and

2, and (30) suggests that RF II is a precursor to RF I. No other "rep-

licative intermediates" are observed and it seems likely that shorter

pulses of higher specific activity will be required to elucidate further

the replication of RF I.

Sedimentation analyses of the DNA pulse-labeled late in the latent

period do not provide any evidence for a concatenate of λDNA. The

sedimentation patterns obtained bear no resemblance to those obtained

by Smith and Skalka (23). It is unlikely that this is a result of shear

breakage of the concatenate since bacterial DNA extracted by the same

[1]The amount of RF I predicted is probably exaggerated because of con-

tamination with X, which appears to overlap RF I in sedimentation rate.

Thus the real amount of X predicted may be larger by as much as 10%,(the

amount of "RF I" synthesized after 30 minutes), and RF I will be correspon-

dingly decreased. There are approximately equal amounts of RF II and X

between 30 and 50 minutes.

procedure sediments uniformly at a rate twice as fast as phage DNA, the rate of Smith and Skalka's "concatenate". It seems that under the conditions used in these experiments a concatenate is not an essential component of the replication process of λ DNA.

The precursors to phage DNA sediment heterogeneously faster than mature phage DNA (Figures 1, 2, 3, and 5). Part of the precursor DNA has the sedimentation properties of RF II: it sediments with H-bonded circles of λ DNA and with material which releases infective DNA upon denaturation (presumably single-stranded rings). This is not conclusive evidence that the pulse-labeled DNA itself contains single-stranded rings since the infectivity represents the behaviour of the bulk of the DNA whereas the pulse-labeled material is only a small fraction of the total DNA on the gradients. The heterogeneous material has a range of sedimentation coefficients between (and perhaps including) that of RF I and RF II. At high ionic strength some of it sediments faster than RF I.

The sedimentation rate of the precursors is more sensitive to ionic strength than phage DNA but less sensitive than RF I. The net effect, however, is difficult to evaluate due to the heterogeneity. High ionic strength appears to make the heterogeneous material sediment more uniformly and some of it more slowly (Figure 3). However, part of this effect may have been caused by the lower pH used on the high salt gradients. That is, low salt (0.01$\underline{M}$) and moderately high pH (10.5) may have caused incipient denaturation of some of the pulse-labeled DNA, increasing its sedimentation rate.

RF I may be synthesized in small quantities even very late (35-40

minutes, lysis occurred at 50 minutes) in the latent period, as revealed

by CsCl equilibrium density gradient centrifugation in ethidium bromide

(Figure 4). This is further evidence that RF II is synthesized late in

the infection. Such late synthesis of RF I is not always observed,

however, and it cannot be ruled out that late synthesis of RF I is simply

due to asychrony in the culture. The small amount of late RF I synthesis,

taken together with the evidence that the amount of RF I does not decrease

significantly after phage DNA synthesis begins (30), indicates that it

is not a major precursor to phage DNA. Pulse-chase experiments quali-

tatively support this conclusion, although the interpretation of these

experiments is difficult since some radioactivity was incorporated

during the chase.

A hypothetical model for λ DNA replication

Although it may seem premature to present a model for λ DNA repli-

cation at this stage of experimentation, it might serve a useful purpose

if it clarifies the present state of knowledge and focuses attention

on those areas about which we know least. Any model or theory should

also serve as a source of inspiration for further experimentation and

predict specific results which will either confirm the theory and lead to

a more precise formulation, or refute the theory and produce a re-evaluation

of the data. The hypothetical nature of the model shown in Figure 6 is

emphasized. The main assumptions on which the model is based are that

RF II is a precursor to both RF I and phage DNA and, moreover, only

circular forms of λ DNA are able to replicate. Such a role is also

implicated for ØX RF II (12).

Steps which are not supported by direct physical data are indicated

by dashed lines. The postulated source of the enzymes involved, either

phage (designated by a λ) or host (designated by E. coli) specified,

is also included.

Agreement between theory and experiment

Step 1, the conversion from linear to H-bonded circular phage DNA,

appears from Gellert's work in vitro to be a prerequisite for the for-

mation of covalent circles (8). H-bonded circles do not result simply

from injection, however, since cells from which uninjected phage have

been removed by blending still may contain linear DNA (unless phenol

extraction of the infected complexes disrupts the H-bonded circles).

The rate of circularization, determined from the rate of loss of helper

infectivity, is quite variable and may depend on the physiological state

of the cells.

Ogawa & Tomizawa (17) presented evidence suggesting that the two

"nicks" in the H-bonded circles are not healed simultaneously, hence

this is shown as two steps, 2 and 3. The enzyme discovered by Gellert

is probably responsible for repairing "nicked" DNA in general since it

is found in uninfected cells. An enzyme with similar properties, poly-

nucleotide ligase, has also been found in T4-infected cells by Weiss

and Richardson (27).

A λ specific DNA polymerase has not been demonstrated (18) but
indirect evidence suggests the participation of a λ function for DNA
replication. Lambda DNA synthesis occurs in 30 μg/ml. of CAM but not
in 100 μg/ml. suggesting a CAM resistant (structural ?) λ protein
necessary for DNA replication (30) and at least three λ-loci control
some step prior to DNA synthesis (sus N, O, P; 10).

The CAM resistant λ protein is indicated at step 4, the many-fold,
semi-conservative replication of λRF (30)^2, although it may also be
required for synthesis of phage DNA. This protein might be analogous
to the replicator protein proposed by Lark (11) to attach the repli-
cating DNA to the cell membrane. An E. coli or λ DNA polymerase would
then be responsible for reproducing RF I. RF II is probably a precur-
sor to RF I (steps 4a and 4b) since it is labeled to a greater extent
than RF I in a short pulse (Figures 2 and 4) but does not accumulate
as extensively (30).

Step 5, lysogenization by circular λ DNA, was proposed by Campbell
(3) from genetic evidence. Lysogenization may occur without DNA rep-
lication (2).

Steps 6a-6d are the most hypothetical in the model, although the
evidence is fairly strong that RF II is involved somehow in the synthesis
of phage DNA. It is postulated that a late λ enzyme, a DNA polymerase,
is responsible for synthesizing a strand of DNA complementary to the

[1]Semi-conservative replication of those RF I molecules containing
parental label is all that has actually been demonstrated.

"nicked" strand of RF II (step 6a). As synthesis progresses the closed

strand of RF II is left without a complement, becoming a single-stranded

ring upon completion of replication. This step of replication would

be asymmetric since only one strand of the duplex is copied; and semi-

conservative since the nicked strand acquires a new complement. Asym-

metry is proposed because: 1. RF II molecules are labeled more rapidly

in a pulse than linear DNA; and 2. No DNA polymerase with a $3' \longrightarrow 5'$

specificity is required as is imposed for symmetric replication. One

model for the replication of RNA bacteriophages is also asymmetric and

semi-conservative (13). In the latter case, of course, the displaced

single-strand becomes the viral strand.

The single-stranded DNA ring produced by step 6a is converted to

a double-stranded RF II by a host enzyme (step 6b). The discovery that

single-stranded rings (but not linear strands) of λDNA are infective

(31) suggests that this step can occur. Single-stranded rings of λ DNA

have not been demonstrated during a normal infection, however. This

scheme for synthesizing phage DNA, though requiring two DNA polymerases,

requires only one specificity in the direction of replication.

On the basis of the model, the change in sedimentation properties

with ionic strength (Figure 3) would be due to the partial single-stranded

character of the pulse labeled DNA shown in step 6a. This might also

explain the poor recovery of the pulse-labeled DNA from the ethidium

bromide density gradient (Figure 4). The presence of single-stranded

rings in DNA sedimenting faster than RF II is suggested by Figure 2 in

which denaturation increases the amount of DNA able to infect spheroplasts

in this region of the gradient.

Once step 6, phage DNA synthesis, commences it is assumed that
conversion of RF I to RF II occurs to only a slight extent; otherwise
RF I would appear to be a precursor to phage DNA. It might be simplest
to assume that only those RF II molecules at a replication "site" at
the time a λDNA polymerase is made become involved in the synthesis
of phage DNA (the number of sites is assumed to be smaller than the
number of RF molecules; 28). The reverse of step 4, namely conversion
of RF II to RF I, occurs only rarely after phage DNA synthesis begins
(Figure 4).

The predicted mechanism of RF replication is exponential. The rate
of DNA synthesis, measured by incorporation of ^{3}H-thymine into λ-infected
cells, is also exponential early in infection; that is, the rate in-
creases continually from 0 to 25 minutes after infection (30). However,
the model predicts a linear mechanism of phage DNA replication (analogous
to Luria's stamping machine model; 14). The fact that the rate of DNA
synthesis is constant during the period of phage DNA synthesis is thus
consistent with the model. The rate of DNA synthesis will reflect the
mechanism only if the amount of template, and not the amount of enzyme,
is rate limiting. Other interpretations of the rates of DNA synthesis
are, of course, also possible. Luria's analysis of the clone size dis-
tribution of T4 mutants was in agreement with a geometric process of
gene duplication (14) but a comparable analysis has not been made with λ.

Predictions from the model

Although the model predicts synthesis of phage DNA by a semi-con-
servative mechanism, it predicts that phage DNA does not replicate;
once a linear molecule is made it is removed from the vegetative pool
(although it could be re-cycled if it were closed once again). All
the replicating DNA is circular. A λ concatenate might appear if excess
phage genomes are present. The ends could join (step 1) and covalent
linkages might be established (steps 2 and 3). The model also predicts
that only linear, not circular strands, would be labeled in a late
pulse.

If single-stranded regions are produced during replication (step 6),
these should be easily detectable and would provide a critical test
of this step in the model. Fractionation of pulse-labeled DNA by column
chromatography using column material which detects single-strandedness,
such as benzoylated-napthoylated cellulose (20), would be appropriate.

Since several steps in the model are predicted to be catalyzed by
host enzymes, studies of λ DNA replication in E. coli strains able to
adsorb λ but not allowing phage growth would be very rewarding. Denhardt,
Dressler and Hathaway (6) have begun work in this area with ØX, as has
Dowell (5). According to the model one might expect that host mutants
which do not convert ØX single strands to ØX RF would be able to rep-
licate λ RF without producing phage DNA.

TABLE 1

Recovery of pulse-labeled DNA and the rate of synthesis of DNA

and active phage

Time	Early (10 min)			Late (35 min)		
Pulse length (sec)	60	120	300	60	120	300
Total ^{3}H-thymine incorporation (cpm)	54000	159000	540000	292000	500000	1070000
Recovery of ^{3}H-DNA after collecting cells (%)	65	57	71	34	41	48
Recovery of ^{3}H-DNA after phenol extraction (a)	50	45	43	59	51	41
(b)	-	-	-	78	66	71
DNA equivalents/IC	1.8	5.3	18	9.5	16	35
Active phage/IC	-	-	-	1.8	3.6	9

The total cpm incorporated is per 5 ml. culture. The ^{3}H-thymine

specific activity was 2.6 c/mmole and the number of DNA equivalents is

based on this figure, the thymine content of λDNA (4), and the known

counting efficiency. At this specific activity there are about 2 x 10^{-4}

disintegrations/min/ DNA or approximately 0.3 disintegrations/day/ DNA.

The total incorporation was measured by TCA precipitation of a portion of

the culture immediately after the pulse. The recoveries after phenol ex-

traction are based on the amount recovered after collection of the cells.

The recovery is enhanced in (b) by the addition of sodium trichloro-

acetate to the lysate (final concentration 0.1$\underline{M}$) prior to phenol extraction.

Figure 1. <u>Sedimentation properties of vegetative λDNA labeled with</u>
^{3}<u>H-thymine for 60 and 120 seconds at 10 and 35 minutes</u>. One-half ml.

portions of the lysates (phenol extracted, Table 1 a) were mixed with

^{32}P- b2b5c phage DNA and layered on sucrose gradients containing TE,

pH 10.5, and centrifuged for 5 hours at 25000 rev./min and 6°C in the

SW 25.1 rotor. One ml. of 55% CsCl (density = 1.7 g/cm^3) was layered

on the bottom of the sucrose gradients to prevent the DNA from pelleting.

All of each fraction (about 0.85 ml) was TCA precipitated and counted

in a Beckman scintillation counter. The cpm in the ^{3}H channel were

not corrected for a 2% overlap from the ^{32}P channel, causing a small

amount of skewing of the slow-sedimenting peak of early, 60 second

pulsed DNA toward the top of the gradient. Recovery of ^{3}H pulse-

labeled DNA layered on the gradient: 60 seconds, early, 100%; 60 seconds,

late, 71%; 120 seconds, early, 75%; 120 seconds, late, 71%.

X——X ^{3}H cpm   0——0 ^{32}P cpm

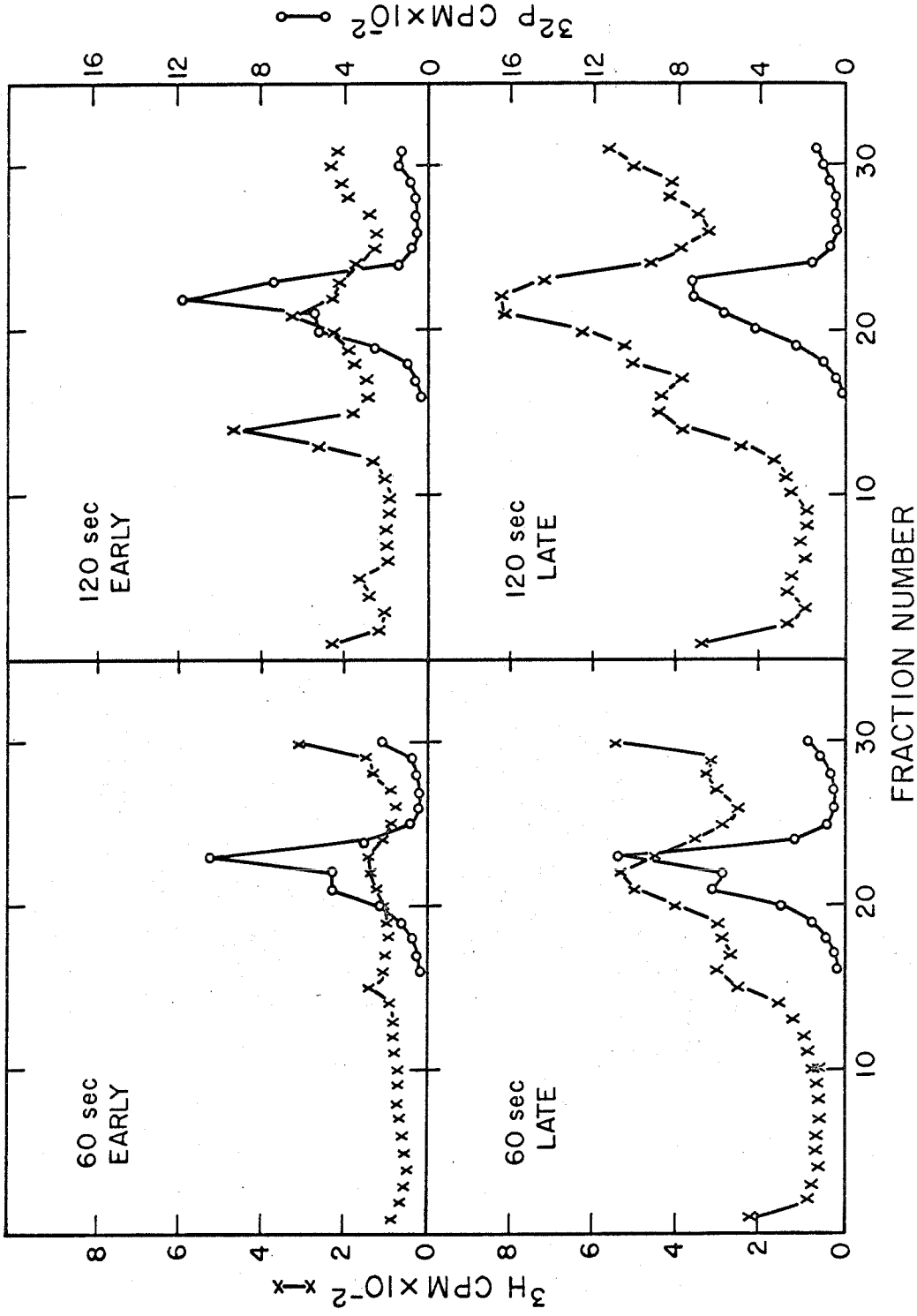Figure 1

Figure 2. <u>Sedimentation</u> <u>and</u> <u>biological</u> <u>properties</u> <u>of</u> <u>the</u> <u>DNA</u> <u>extracted</u>

<u>from</u> <u>300</u> <u>second</u> <u>pulse-labeled</u> <u>infected</u> <u>bacteria</u>. The conditions for

sedimentation are given in the legend to Figure 1. One-half ml. of the

phenol extracted lysates from the infected cultures labeled for 300

seconds at 10 and 35 minutes was sedimented. One-half of each fraction

was TCA precipitated and counted. Helper infectivity was assayed by

mixing 50 μl. of either individual fractions (16-25) or pooled frac-

tions (1-5, 6-10, 11-15, and 26-30) with 0.2 ml. of helper-infected

cells. The infectivity of native DNA in the spheroplast assay was

measured by adding 0.2 ml. of each fraction to 0.6 ml. of 0.05 $\underline{M}$ tris,

pH 8.1 and mixing with 0.2 ml. of spheroplast stock. The infectivity of

denatured DNA in the spheroplast assay was measured by adding 50 μl. of

0.5$\underline{N}$ NaOH to 0.2 ml. of each fraction. After 15 minutes at 37° 0.60 ml.

of 0.05 $\underline{M}$ tris, pH 8.1, 0.05$\underline{M}$ NaH$_2$PO$_4$, pH 4.0 was added. The final

pH was about 7.5 The recoveries of ^{3}H layered on the gradient were:

early pulse, 73%; late pulse, 63%.

X———X	^{3}H cpm
0———0	^{32}P cpm
$\triangle$———$\triangle$	helper infectivity
$\square$———$\square$	spheroplast infectivity, native
●———●	spheroplast infectivity, denatured

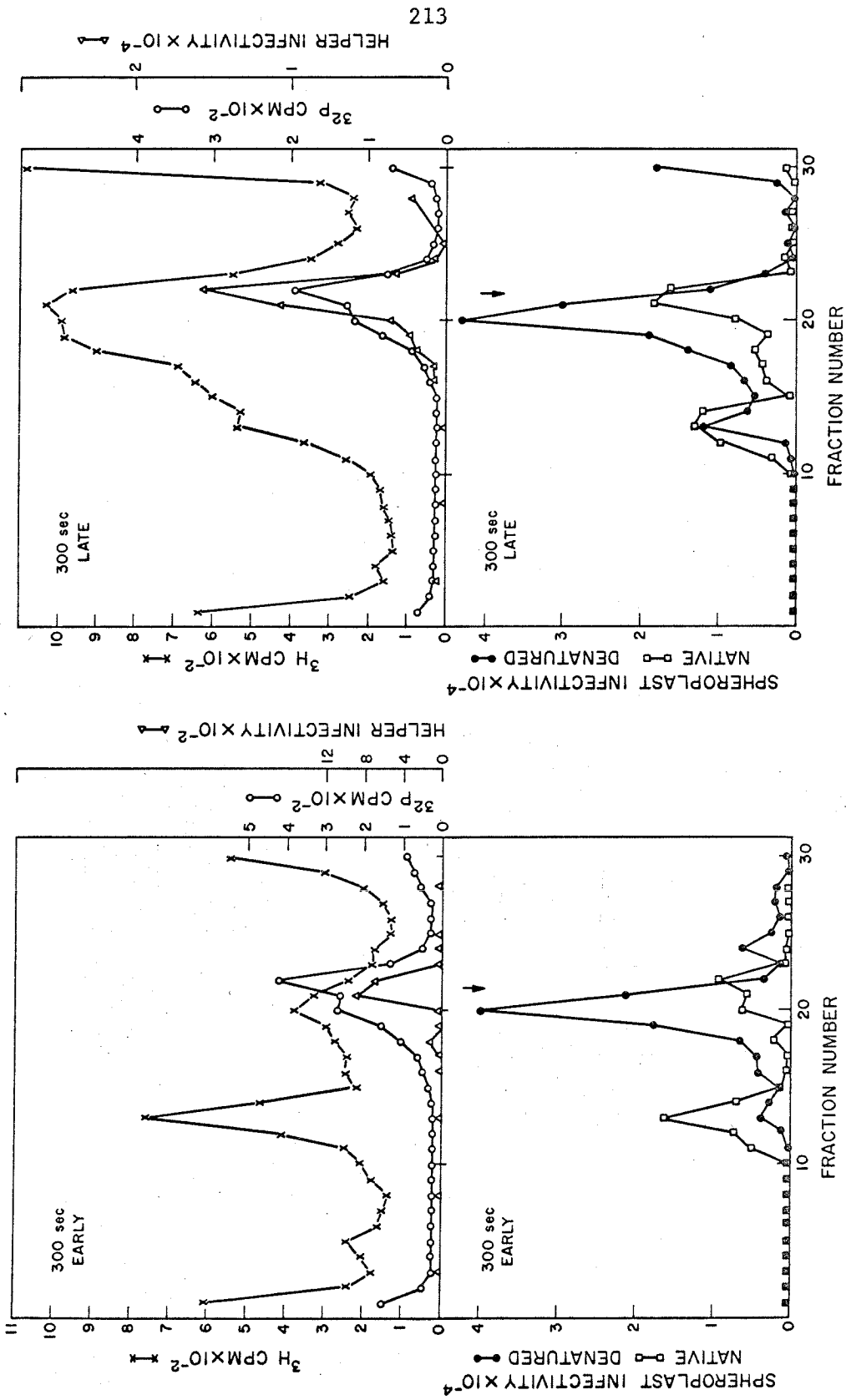Figure 2

Figure 3. <u>Sedimentation</u> <u>of</u> <u>late</u> <u>300</u> <u>second</u> <u>pulse-labeled</u> <u>DNA</u> <u>at</u> <u>low</u>

<u>and</u> <u>high</u> <u>ionic</u> <u>strength</u>. Purified ^{32}P phage DNA and RF I were added

as sedimentation markers prior to centrifugation for 6 hours at 25,000

rev./min at about 10 .

(a) 5-20% sucrose in TE (0.01$\underline{M}$ tris, pH 10.5, 0.001$\underline{M}$ EDTA).

Recovery: ^{3}H, 65%; ^{32}P, 69%.

(b) 5-20% sucrose in TE, pH 8.1, 2.0$\underline{M}$ NaCl. Recovery: ^{3}H, 63%;

^{32}P, 71%.

X———X ^{3}H cpm   0———0 ^{32}P cpm

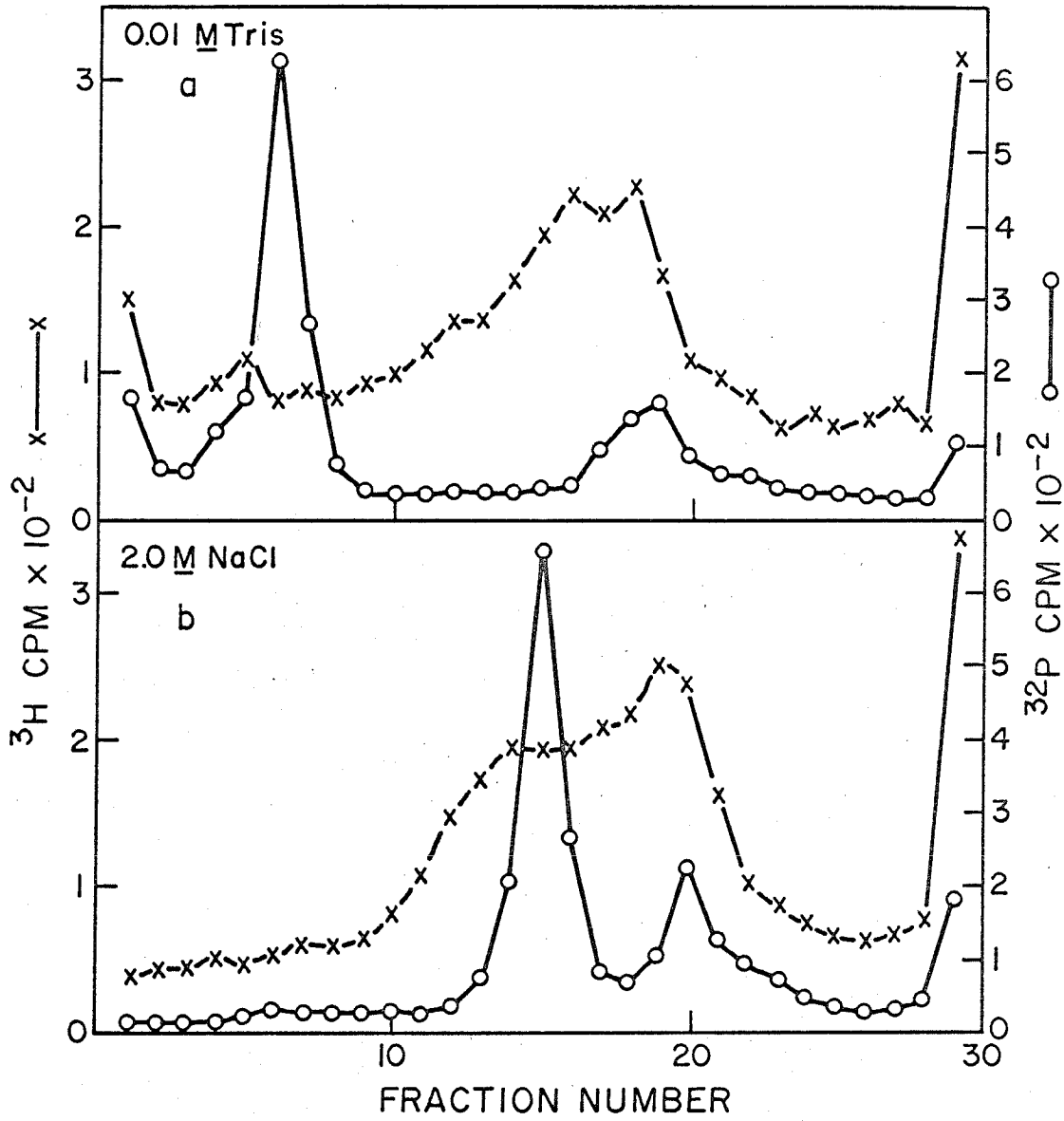Figure 3

Figure 4. <u>CsCl</u> <u>equilibrium</u> <u>density</u> <u>gradient</u> <u>centrifugation</u> <u>of</u> <u>early</u> <u>and</u>

<u>late</u> <u>pulse-labeled</u> <u>DNA</u> <u>in</u> <u>ethidium</u> <u>bromide</u>. Portions of the lysates

to be analyzed were mixed with ^{32}P-phage DNA, ^{32}P-RF I, an aqueous solu-

tion of ethidium bromide (Boots Pure Drug Co., Nottingham, England)

and solid CsCl to produce a final concentration of 50 µg/ml. ethidium

bromide and a density of 1.56 g/cm^3. The solution was centrifuged to

near equilibrium (32 hours at 30,000 rev./min and 20). Three-drop

fractions were collected, TCA precipitated with carrier, and counted

in the scintillation counter. Recoveries of radioactivity layered on

the gradient: late, 60 second, ^{3}H, 20%, ^{32}P, 79%; late, 300 second,

^{3}H, 40%, ^{32}P, 78%; early, 300 second, ^{3}H, 58%, ^{32}P, 80%. The cpm in the

^{3}H channel of the scintillation counter were corrected for a 2% over-

lap from the ^{32}P channel.

X——X ^{3}H cpm   0——0 ^{32}P cpm

Figure 4

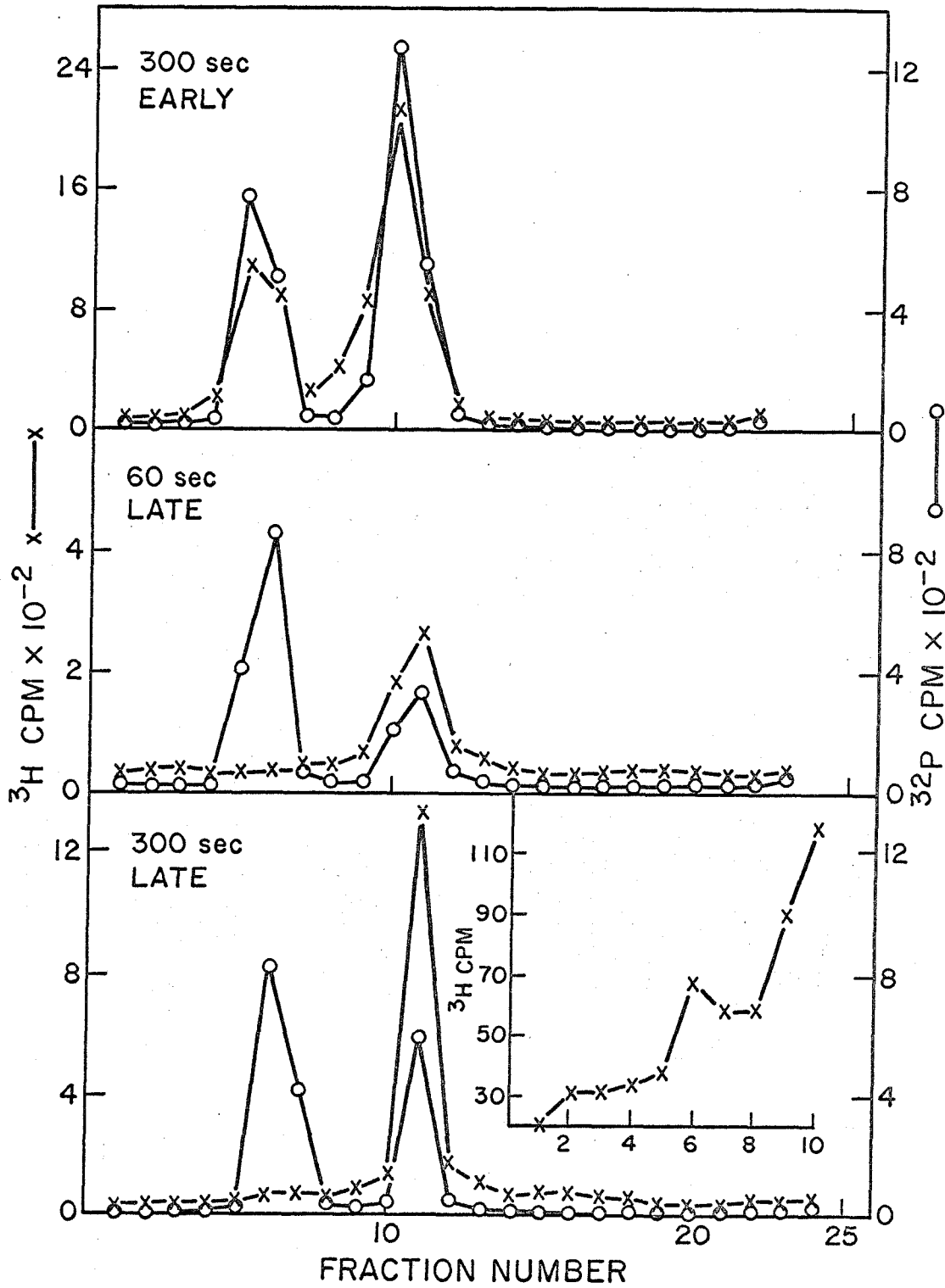Figure 5. <u>Cumulative</u> <u>synthesis</u> <u>of</u> λ-<u>specific</u> <u>DNA</u> <u>components</u> <u>predicted</u>
<u>from</u> <u>sequential</u> <u>5</u> <u>minute</u> <u>pulses</u>. Equal portions of lysates from 5

minute pulse-labeled cultures and also from a culture labeled contin-

uously from 0-50 minutes, were layered onto 5-20% sucrose gradients

in TE and centrifuged as usual. The amount of radioactivity in the

three regions described below was measured and the cumulative sum de-

termined at five minute intervals. (The 0-5 and 20-25 minute samples

were lost.)

    Region I: RF I   0————0

    Region II: RF II and component X (all material sedimenting between

            phage DNA and the RF I position)   X————X

    Region III: Phage DNA (radioactivity which co-sedimented with the
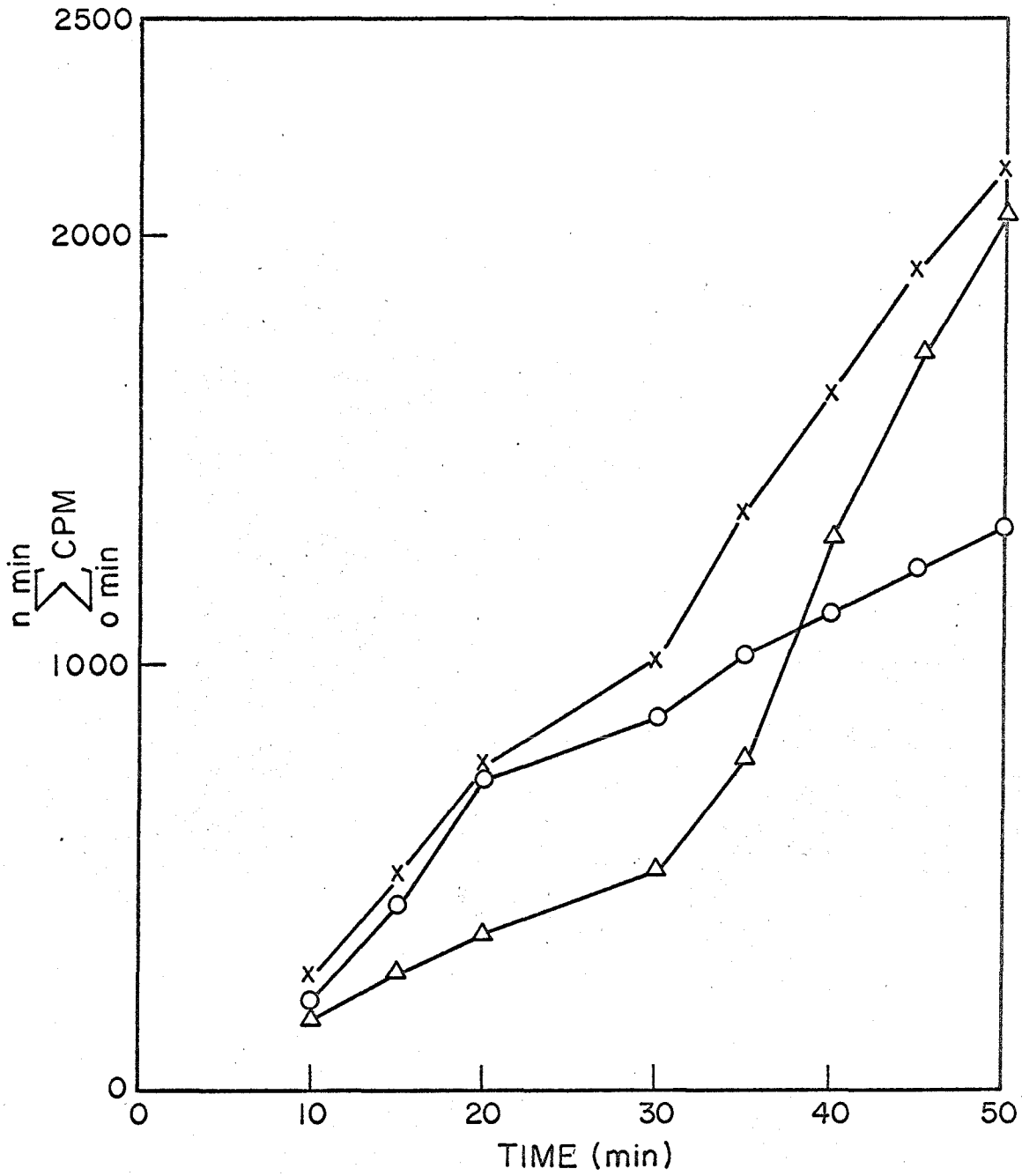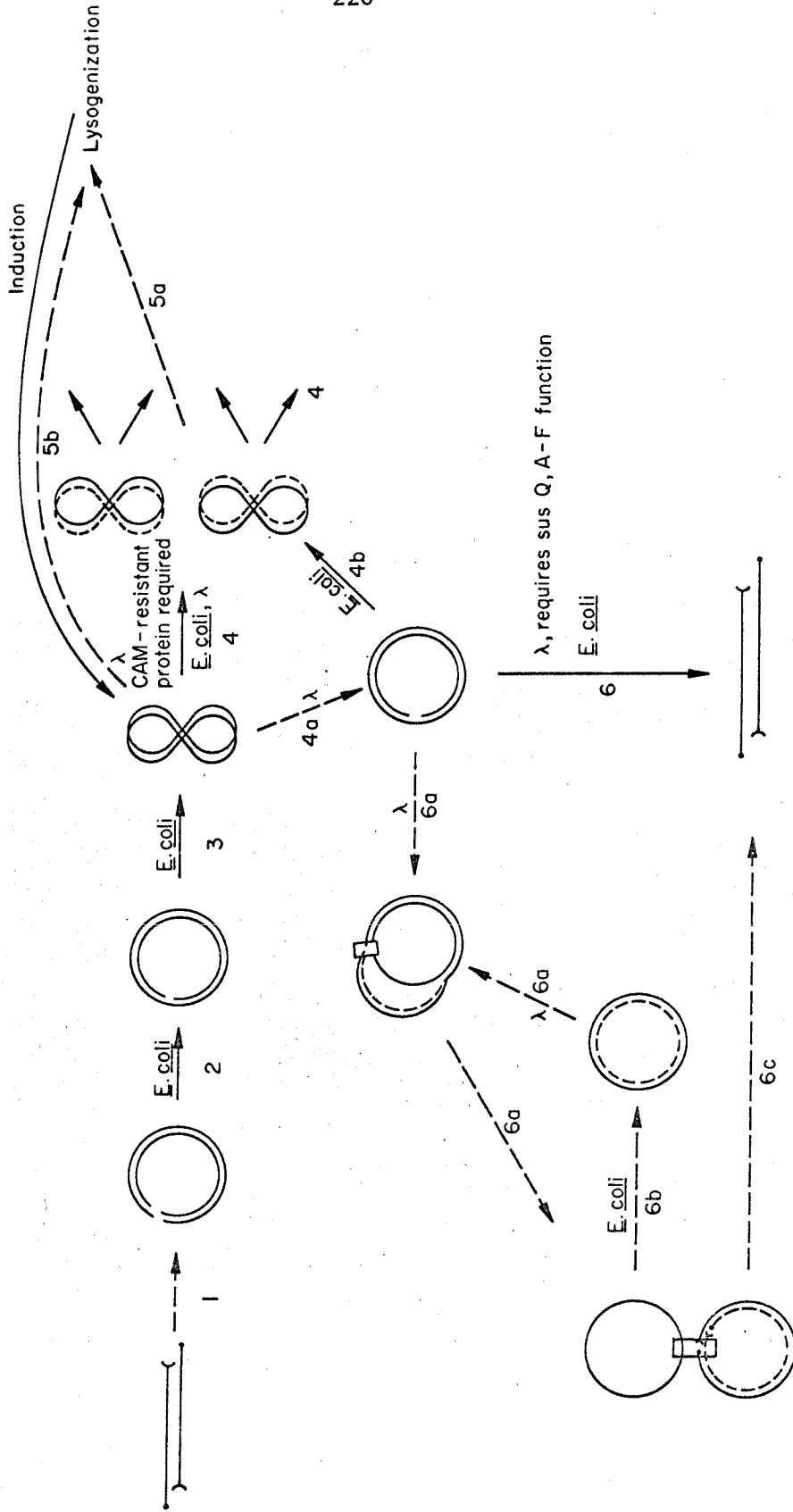
            phage DNA marker)   Δ————Δ

Figure 5

Figure 6

Hypothetical model for lambda DNA replication

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Part IV

PURIFICATION OF INTRACELLULAR LAMBDA DNA

AND ITS INFECTIVITY AFTER DENATURATION

1. INTRODUCTION

The possession of biological activity and evidence for a high degree of both regional and temporal control of RNA transcription make λRF a natural choice for studying DNA replication and transcription in vitro. In addition to its usefulness for biological studies, the supercoiled form of λDNA is potentially useful for a variety of physical studies concerned with the origin and consequences of DNA circularity since it is the highest molecular weight closed-circular DNA presently available. Before such studies can be undertaken the DNA must be available in large quantities in a purified form. The purpose of the first section of Part IV is to describe a method for the semi-large scale purification of RF I.

The second section is devoted to an investigation which began when it was discovered that upon denaturation of intracellular λDNA, its infectivity, measured in the spheroplast assay, increased substantially. In contrast, the infectivity of phage DNA may decrease by a factor of 100 under the same conditions. The increased infectivity of the intracellular DNA had two possible non-exclusive explanations: either RF I was more infective in the denatured than the native configuration, or the single-stranded rings produced by denaturation of RF II were able to infect spheroplasts and do so more efficiently than native DNA. The experiments to be described support the second interpretation. RF I has the same or slightly less infectivity after denaturation whereas denaturation of RF II is accompanied by a large increase in infectivity.

The denatured, infective DNA from RF II sediments at the rate expected
of single-stranded rings of λ DNA.

2. MATERIALS AND METHODS

Procedures and materials not described previously in this thesis
are included below.

(a) Chemicals

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$\underline{M}$
Na_3citrate-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, Ltd., Nottingham, England.

(b) pH measurement

A radiometer titrator with a G 222B 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 $Ca(OH)_2$, is within 0 - + .04 pH units, and the
precision is about $\pm$ 0.05 pH units, in the range pH 12-13.

(c) DNA denaturation

Buffers for alkaline titration of DNA are prepared by mixing 5.0 ml. of $0.20\underline{M}$ K_2HPO_4, x ml. of $1.0\underline{M}$ 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 also used occasionally and these will be described as they occur.

(d) Electron microscopy

DNA is observed in the electron microscope after preparation essentially according to the procedure of Kleinschmidt and Zahn (14). The electron microscope facilities were graciously made available by Dr. A. Hodge.

(e) Preparation of intracellular λ DNA

(i) E. coli W3110 lysogenic for the strain of phage to be used in the infection is grown to about 1×10^9 cells/ml. in K medium (unless stated otherwise) at 37° and 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 Blendor, followed by centrifugation to collect the cells.

(ii) The cell paste from a one liter culture is then resuspended in 10 ml. of cold 25% sucrose in 0.04 $\underline{M}$ tris, pH 8.1, blended in a Waring Blendor to disperse the cells, and treated according to the procedure of Godson (7). The cells are converted to spheroplasts by the addition of 1.0 ml. of lysozyme (6.4 mg/ml., grade 1 dissolved in 0.25$\underline{M}$ tris, pH 8.1) and 1.0 ml. of 2% (w/v) EDTA. After three minutes on ice, during which time the cells are converted to spheroplasts, a solution containing 1.1 ml. of 0.1 $\underline{M}$ $MgSO_4$ and 1.5 ml. of 5% (w/v) Brij (in 0.01 $\underline{M}$ 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 very viscous, gelatinous pellet which contains cell membranes and DNA.

(iii) The supernatant solution is brought to 1XSSC by the addition of 20XSSC and phenol extracted once. The aqueous phase is collected after centrifugation at 5000 rev./min in a Sorvall centrifuge GSA rotor. Phenol is removed from the aqueous solution by ether extraction, and the ether removed by bubbling nitrogen gas through the solution.

(iv) At this point several independent procedures have been used to purify further the intracellular λ DNA.

(1) NaCl is added to 1.0$\underline{M}$ and the solution is stored in the refrigerator overnight. The precipitated RNA is removed by centrifugation and discarded. DNA does not precipitate in 1$\underline{M}$ NaCl. The solution is diluted

to 0.4<u>M</u> NaCl with H_2O and passed through a methylated albumin-
Kieselguhr column, previously washed with 0.4<u>M</u> NaCl, as described by
Sueoka and Cheng (22). The column is 4.7 cm wide and is filled to a
height of 1.6 cm. Material in the column is eluted with a 250 ml.
linear 0.4 to 1.0<u>M</u> NaCl gradient (buffered with 0.05<u>M</u> sodium phosphate,
pH 6.7). Fractions containing DNA, which elutes at 0.75<u>M</u> NaCl, are
combined and dialyzed <u>versus</u> 0.01<u>M</u> tris, 0.001<u>M</u> EDTA(TE), pH 8.1.

(2) The preparation is treated with 10 μg/ml. of RNase at 37° for one
hour and then brought to pH 5.4 with HCl and passed through a Sephadex
G-100 column equilibrated with 0.01<u>M</u> Na_3 citrate, 0.02<u>M</u> NaCl, pH 5.4.
The material is eluted with the same buffer. The DNA comes out in the
void volume while the RNase digestion products are retarded in their
passage through the column. RNase eluting with the DNA in the void
volume can be removed by shaking the pooled DNA - containing fractions
with IRC-50 (which has been heated to 60° for 10 minutes and washed thor-
oughly). The pH is raised to 8 with KOH.

(3) A step gradient of CsCl in 15% sucrose containing 0.04<u>M</u> tris, pH 8
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 SW 25.1 rotor at 25000 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 after centrifugation. This procedure concen-
trates the DNA in the middle of the CsCl gradient. Fractions containing

DNA are pooled and dialyzed against TE.

(v) The procedures described above give preparations containing three species of λ DNA; open circular DNA (RF II or component II), closed-circular DNA (RF I or component I), and component III, linear phage DNA. The closed circular DNA is purified free of components II and III by CsCl equilibrium density gradient centrifugation in the presence of ethidium bromide (17). The preparation is brought to 100 μg/ml. ethidium bromide, the density adjusted to 1.56 g/cm^3 with solid CsCl, and then centrifuged for 2-3 days at 37000 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 in two density layers, the heavier of which contains the supercoils. Fractions are collected as usual. The free and inter-calated dye can be removed from the DNA solution by passage through a cation exchange resin as described by Radloff, Bauer & Vinograd (17).

(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

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

Each E. coli cell contains an amount of DNA equivalent to about

100 - 300 λ genomes, depending on the number of bacterial chromosomes.

We have taken advantage 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 centrifugation,

while much of the cytoplasmic contents of the cell is released. Godson

and Sinsheimer (8) used the technique, which we have adapted, to pre-

pare E. coli polyribosomes which are released from the cell by gentle

lysis. In order to test the efficacy of this method for separating

intracellular λ DNA from the bulk of the E. coli DNA, the experiment

described below was performed.

E. coli 3110(λ) was grown to a cell concentration of 1 x 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 isotope and resuspended

in 20 ml. of non-radioactive medium. Deoxyadenosine enhances the incor-

poration of exogenous thymidine in a non-thymine-requiring host (2).

When the cell density had reached 2.7 x 10^{9} cells/ml., ^{32}P-λc26 was

added at a multiplicity of about 7 pfu/cell. After further growth for

30 minutes the cells were collected by centrifugation, blended and treated

as described in Methods. A portion of the 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$\underline{M}$ and both fractions were

phenol extracted once, then the phenol removed by ether, and the ether

removed by bubbling N_2 gas through the solutions. Portions of the pellet

and supernatant fractions were TCA precipitated before and after phenol

extraction, the precipitates collected on filters and counted in a

scintillation counter to determine the ^{3}H and ^{32}P activities in the

pellet and supernatant fractions. The results are shown in Table 1.

The recovery of ^{32}P-λ DNA in the supernatant was 31%. About 99.6%

of the ^{3}H-$\underline{E.\ coli}$ DNA was in the pellet fraction.

The specific activity of the ^{3}H-$\underline{E.\ coli}$ DNA in the pellet, determined

from the amount of ^{3}H in that fraction and the amount of DNA determined

chemically by the indole procedure (13), was 1.7 x 10^4 cts/min/µg DNA.

The specific activity of the ^{32}P-λ DNA was calculated to be 6.4 x 10^4

cts/min/µg DNA from the ^{32}P content and the absorbance at 260 mµ of

the phage preparation. Using these values and the total amount of ^{3}H

and ^{32}P recovered in the pellet plus supernatant fractions, the ratio

of bacterial DNA to λ DNA before centrifugation of the lysate was 10.4µg:

1.0 µg. The ratio in the phenol extracted supernatant after centri-

fugation was 0.065 µg E. coli DNA : 1.0 µg λ DNA, a purification relative

to E. coli DNA of 160-fold.[1] The phenol extracted supernatant contained

8.8 µg of λ DNA and 0.57 µg of bacterial DNA. That is, 93% was phage DNA.

[1]This includes a preferential loss of coli DNA during phenol extraction.

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

The DNA obtained from the MAK column was examined in the electron microscope after spreading the DNA on a water-air interface in the presence of cytochrome C according to the method of Kleinschmidt and Zahn (14) (see Methods)(Plate 1). Three types of DNA molecules, distinguishable by their unique configurations, are 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% supercoils. To exclude the possibility that the tightly twisted molecules observed are 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 (9) and examined in the electron microscope as described above. As can be seen in Plate 2, no tightly twisted circular molecules are observed. Thus the prediction from sedimentation properties that component I is a tightly twisted, circular form of λ DNA, analogous to ØX 174 RF (4, 18) or polyoma viral DNA (24) is substantiated by direct observation.

(b) <u>Separation</u> <u>of</u> <u>closed</u> <u>circular</u> λ <u>DNA</u> <u>from</u> <u>linear</u> <u>and</u> <u>open</u>

<u>circular</u> <u>DNA</u>

Preparations of intracellular λ DNA almost (93%) free of <u>E. coli</u>

DNA can be obtained by the methods just described. However, these

preparations still contain three species of λ DNA: linear, open-circular,

and closed-circular molecules. These can be separated into two classes

consisting of (a) linear plus open-circular DNA and (b) closed-circular

DNA by CsCl density gradient centrifugation in ethidium bromide as

described by Radloff, Bauer & Vinograd (17). Since the aforementioned

publication contains a figure showing the separation of these intra-

cellular components of λ DNA prepared by us (using method 1) another

illustration seems redundant. An example of the separation obtainable

by this method is also shown in Figure 4 of Part III of this thesis.

(c) <u>A note</u> <u>on</u> <u>the</u> <u>recovery</u> <u>of</u> <u>closed-circular</u> <u>DNA</u>

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 ob-

tained has only been 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 banding in ethidium bromide to separate

closed-circular DNA from other forms of DNA, typically only 25-30% of

the DNA is supercoiled. The step or steps at which the apparent degra-

dation of component I to component II occurs have not been identified.

The most obvious step at which the recovery could be improved is in the lysis procedure. In fact, with the advent of the CsCl-ethidium bromide technique, the gentle lysis procedure may not even by necessary. It remains to be seen whether the presence of the excess bacterial DNA would require several re-fractionations in ethidium bromide, which in themselves might result in a loss of component I equivalent to that suffered in the Godson procedure. The degradative loss of component I is partially inherent in the large size of the molecule relative to other closed-circular DNA species such as ØX RF or polyoma DNA. Thus an average of one phosphodiester scission per 30×10^6 daltons of DNA would degrade 63% of λ component I but less than 10% of ØX RF I.

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

The circular intracellular forms of λ DNA are infective in a spheroplast assay (26). After alkaline denaturation of extracts containing intracellular λ 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 an increased ability to infect spheroplasts. Double-strandedness seemed to be a prerequisite to infection with λ DNA since denatured phage DNA was not infective (or was much less so than native DNA) in either the helper assay (12) or the spheroplast assay described by Brody, et al. (3). Since the closed-circular form of polyoma DNA denatures at a higher pH than the open-circular

form (23), it was hoped that λ component II would behave similarly so

that it could be selectively denatured and subsequently separated from

the native component I. Thus it was necessary to determine the pH at

which the infectivity increase occurred. As it turned out, the experi-

ments to be described below have no further bearing on the denaturation

of component I since they indicate that the increased infectivity ob-

served is due to single-stranded rings of λ DNA which are released by

denaturation of component II. The infectivity of component I remains

unchanged or decreases slightly after incubation at a pH which denatures

component II.

(i) Alkaline titration

Intracellular λ DNA prepared by method 2 was further purified by

sedimentation through a neutral sucrose gradient. Fractions containing

components I and II were pooled together, dialyzed, the volume reduced

to 3 ml., and then dialyzed against SSC/100. Portions of this solution

were alkali denatured as described in Methods. After reneutralization

the samples were assayed in spheroplasts. The same experiment was

repeated with b2b5c phage DNA. Figure 2 shows the biological activity

of the intracellular (iλ) 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 the 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

range. The pH-induced activation curve is quite sharp, unlike the de-

naturation of polyoma DNA component I observed by hyperchromicity (23).

(ii) Buoyant density and infectivity

In order to determine whether the infectivity present after treating the intracellular DNA with alkali was due to material with the buoyant density of native or denatured DNA, a sample of a preparation containing b2b5c intracellular DNA made 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 ^3H-λc26 DNA, was added and the density raised to 1.71 g/cm^3 with CsCl. As a control, a sample of the preparation which had not been incubated with alkali was treated similarly. The two samples were then centrifuged for 37 hours at 30000 rev/min and $20°C$. After deceleration, two-drop fractions were collected from a hole punched in the bottom of the tube and assayed for radioactivity and infectivity. One-half of each fraction from the gradient containing native DNA was first denatured and then assayed. The results are shown in Figure 3.

The radioactivity (^{32}P) and infectivity (i^{b5}) on the gradient con-taining _denatured_ intracellular DNA are present in two peaks. The density difference between the marker, native ^3H-λDNA, and the heavy ^{32}P peak is about 0.015 g/cm^3, the density difference expected if the heavy peak represents denatured DNA. The light peak is presumed to arise from renatured (or undenatured) component I since it has a native density after treatment with alkali and it is present to the same extent as sedimen-tation component I in this preparation.

The specific infectivity is highest in the light peak. Assuming that this is component I, it appears that it is more infective per molecule than DNA in the heavy peak. However, since the latter contains both denatured components II and III, these data alone do not determine the relative infectivities of component I and II. They do suggest that some denatured form of intracellular λDNA is more infective than denatured phage DNA. This is not denatured component I since a sample from the heavy peak sedimented through an alkaline sucrose gradient at the same rate as phage DNA.

Alkali causes a large increase in infectivity on the gradient containing undenatured intracellular DNA (Figure 3b). This fact and the approximately equal amounts of infectivity in the native and denatured regions of the gradient containing intracellular DNA denatured prior to centrifugation suggests that both component I and some form of denatured DNA are more infective after denaturation. The enhanced infectivity of I is contradicted by the results in the next sections, however. Part of the discrepancy may be due to the apparent loss of infectivity of the denatured DNA during centrifugation. The DNA denatured prior to centrifugation is only 30% as infective as the DNA denatured after centrifugation.

(iii) Identification of the native λ DNA component which produces infective DNA upon denaturation

Results presented in Part III (Figure 2) of this thesis indicate that it is component II which increases in infectivity after denaturation.

The sucrose gradients on which the increased infectivity of component
II was recorded also contained component I. The infectivity of component I
is the same before and after denaturation on one of the gradients refer-
red to, and decreases several-fold on the other. The infectivity of
component II increases about 8-fold after denaturation on both gradients.
These results were obtained with non-purified λDNA extracted from
infected sensitive cells.

Similar results are obtained with purified intracellular λDNA
prepared as described in Methods. The DNA whose infectivity is to be
described was obtained using procedure 3 of Methods. DNA referred to
as component I was obtained by pooling fractions containing the fast-
sedimenting peak of radioactivity from a neutral sucrose gradient. DNA
referred to as component II was obtained from the leading edge of the
slow sedimenting peak of radioactivity on the same gradient. Linear
phage DNA was prepared by phenol extraction of a sample of the ^{32}P-
phage used in the preparation of the intracellular DNA described above
so the specific activity of the phage and intracellular DNA would be
the same. These three preparations were then dialyzed against 1.0mM
tris, pH 7.4, 0.05M NaCl. Portions were subsequently denatured in 0.20N
NaOH for 10 minutes at 37°C and reneutralized with HCl. The specific
infectivity of the native and denatured DNA in the spheroplast assay is
shown in Table 2. The specific infectivities recorded are the pfu/cts/min
obtained from the linear range of the assay and normalized to native phage
DNA.

As can be observed in Table 2, the infectivity of component II but not component I, increases after denaturation. The magnitude of the increase in the case of component II is about 30-fold. Phage DNA infectivity decreases about 25-fold after denaturation. The residual infectivity of the phage DNA is probably due to renaturation of some of the molecules (3).

(iv) Identification of the strand in component II which is infective

The foregoing results indicate that it is component II which increases in infectivity after denaturation. Denaturation of component II produces two components which differ about 15% in their sedimentation rate in alkali (16; J. Kiger, personal communication). These two components are presumed to be linear and circular single-strands of λDNA. Denaturation of phage DNA produces only linear strands, and these have little if any infectivity in the spheroplast assay (Figure 3).

Alkali denatured component II and alkali-denatured phage DNA (prepared as described in Results d(iii)) were sedimented through alkaline sucrose gradients containing $0.05\underline{M}$ K_3PO_4 and enough KOH to make the final pH 12.2. The samples were centrifuged in the same rotor (an SW 25.3, Spinco) for 15 hours at 25000 rev/min and $6°C$. After deceleration, fractions were collected into 0.4 ml. of $0.03\underline{M}$ phosphate buffer, pH 4, so that the final pH of the combined sucrose solution and buffer was about 8. Each fraction was then assayed for radioactivity and infectivity in the spheroplast assay. The results are shown in Figure 4.

The infectivity in the gradient containing denatured component II

sediments about 1.11 times as fast as the peak of radioactivity and

about 1.16 times as fast as the peak of residual infectivity associated

with phage DNA in Figure 4b. The peak of radioactivity on the gradient

containing component II is apparently due to contamination of the com-

ponent II preparation with component III since (1) it has very little

infectivity associated with it, and (2) it sediments at the rate of

denatured linear strands. The normalized, specific infectivity of the

denatured component II on the gradient is 6.1 and that of denatured phage

DNA is 0.044.

I conclude from these experiments that circular single-stranded

λ DNA is able to infect spheroplasts, and do so more efficiently than

native forms of λDNA. It has been known for some time that circular,

but not linear single-strands of viral $\emptyset$X 174 DNA can infect spheroplasts

(6) and the same is true for the small phage fd (15). The infectivity

of single-stranded rings of polyoma DNA, which is normally double-stranded,

is also suggested by the work of Dulbecco and Vogt (5). In fact, this

data and that of Weil (25) suggests that the single strands produced

by denaturation of polyoma component II are also more infective than

the native DNA.

Increases in infectivity upon denaturation of RF II of $\emptyset$X 174 have

been studied by Jaenisch, Hofschneider & Preuss (10) with conclusions

similar to those proposed above.

Table 1

Recovery of Intracellular λ DNA in the Godson Procedure

	cpm/50μ1 ^{3}H-E. coli DNA	cpm/50μ1 ^{32}P-λ 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

Table 2

Specific Infectivities of λ - DNA

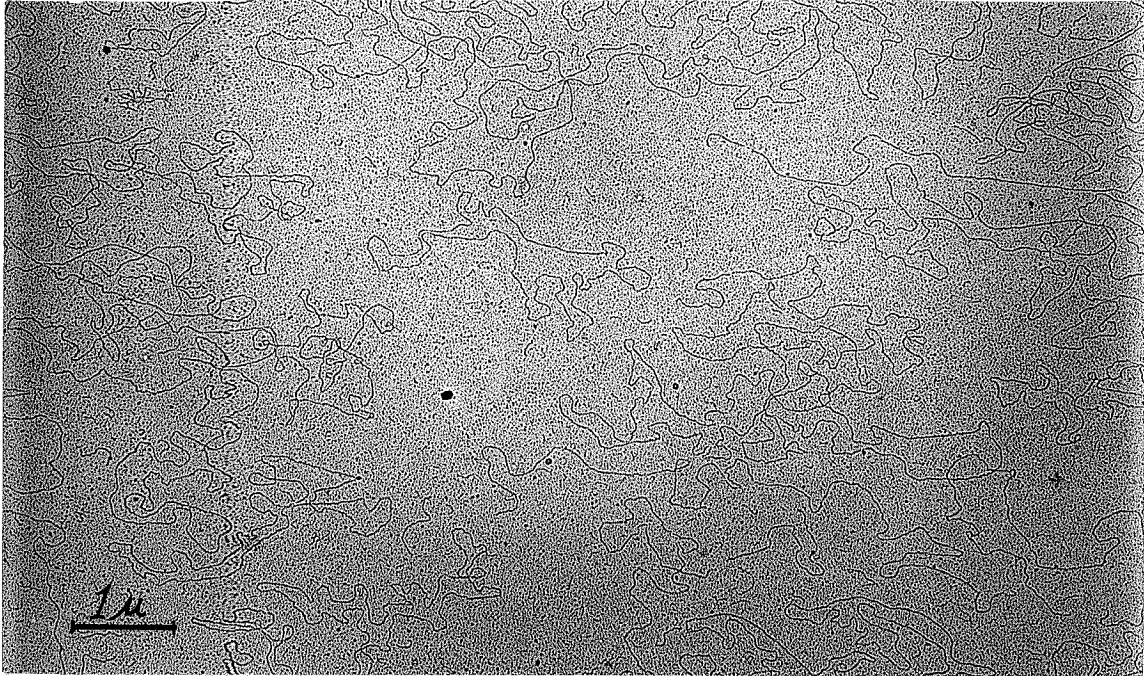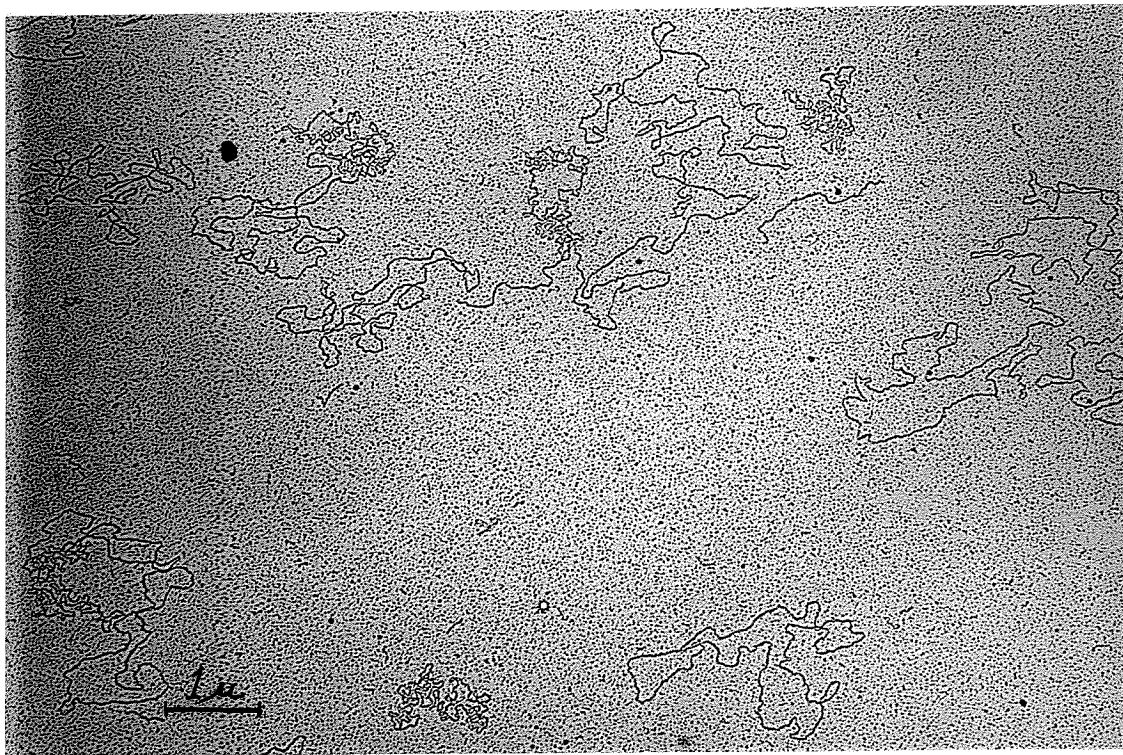|		
Component I	native	3.3
	denatured	1.6
Component II	native	0.49
	denatured	14.7
Phage DNA	native	1.00
	denatured	0.041
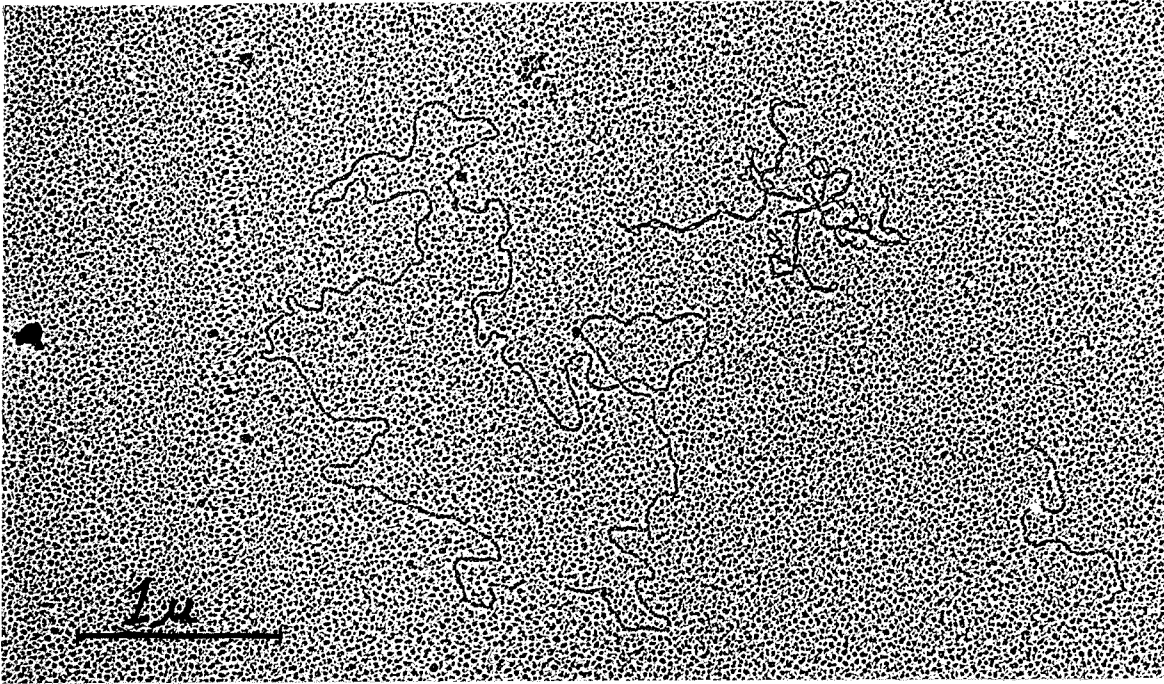
(a)
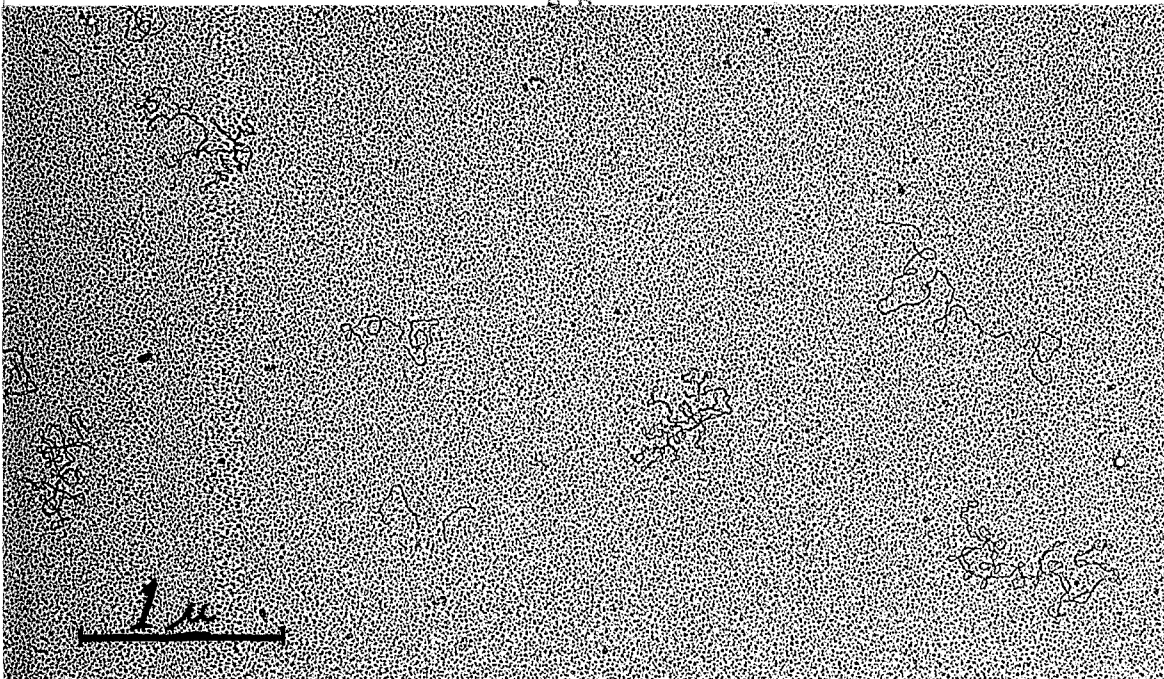
(b)
PLATE I

Purified intracellular lambda DNA. (a) X25,500 (b) X16,000

(a)

(b)

PLATE   II

Annealed phage DNA. (a) and (b) X12,900

Figure 1. <u>Separation</u> <u>of</u> <u>RNA</u> <u>and</u> ^{32}P-<u>intracellular</u> λ<u>DNA</u> <u>by</u> <u>chromato-</u>
<u>graphy</u> <u>on</u> <u>methylated</u> <u>albumin-Kieselguhr</u>. The nucleic acids prepared by
method 1 were passed through a methylated albumin-Kieselguhr column
using a linear 0.4-1.0 $\underline{M}$ NaCl gradient. Portions of each fraction were
put on planchets, dried, and counted in a Nuclear Chicago Low Back-
ground counter. The absorbance was measured in a Beckman model DU
spectrophotometer. Fractions 31-38, containing the DNA, were combined,
dialyzed, and reduced in volume to determine the amount of ^{3}H-<u>E. coli</u>
DNA present. The NaCl molarity was determined from the refractive index.

0———0 NaCl molarity   ——— ^{32}P cpm   – – – – A_{260}

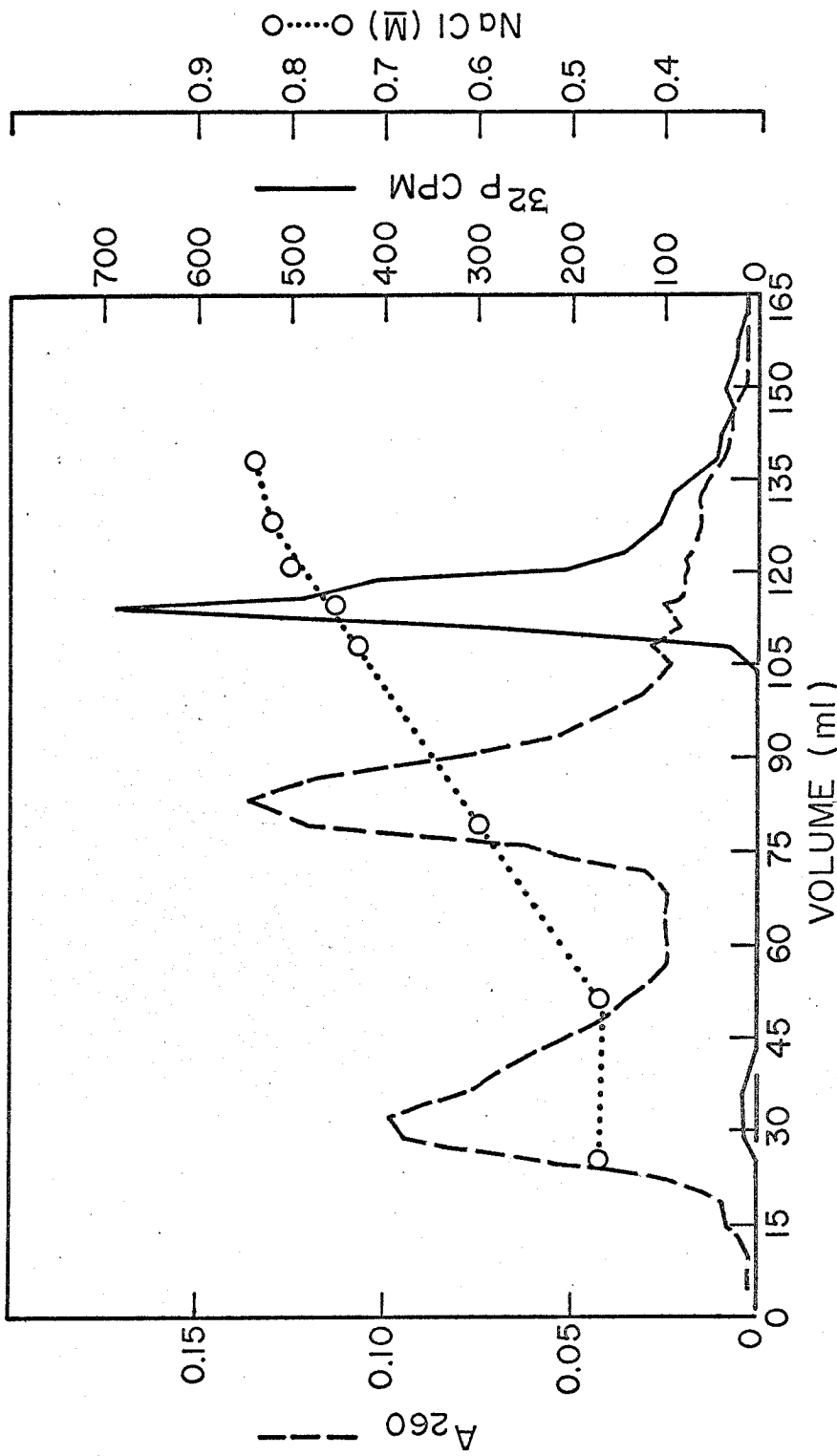Figure 1

Figure 2. <u>Alkaline</u> <u>titration</u> <u>of</u> <u>the</u> <u>biological</u> <u>activity</u> <u>of</u> <u>intra-</u>

<u>cellular</u> λ <u>DNA</u> <u>and</u> <u>phage</u> <u>DNA</u>. Intracellular λ DNA (from λc26 phage)

purified by procedure 2 of Methods was further purified by sedimentation

through a neutral sucrose gradient. Fractions containing component I

and II were pooled together, dialyzed, the volume reduced to 3 ml. by

flash evaporation, and finally dialyzed against SSC/100. Different

0.3 ml. portions of this DNA solution were mixed with 1.7 ml. of an al-

kaline buffer described in Methods. The pH was measured on a Radiometer

titrator and then the sample was incubated for 15 minutes at 37° C.

The pH was adjusted to about 8 with HCl and portions of each sample

were assayed in the spheroplast assay after dilution with 0.05$\underline{M}$ tris,

pH 8.1. Phage DNA (λb2b5c) was denatured in the same buffers and assayed

identically.

0———0 iλ intracellular DNA

●———● i^{b5} phage DNA

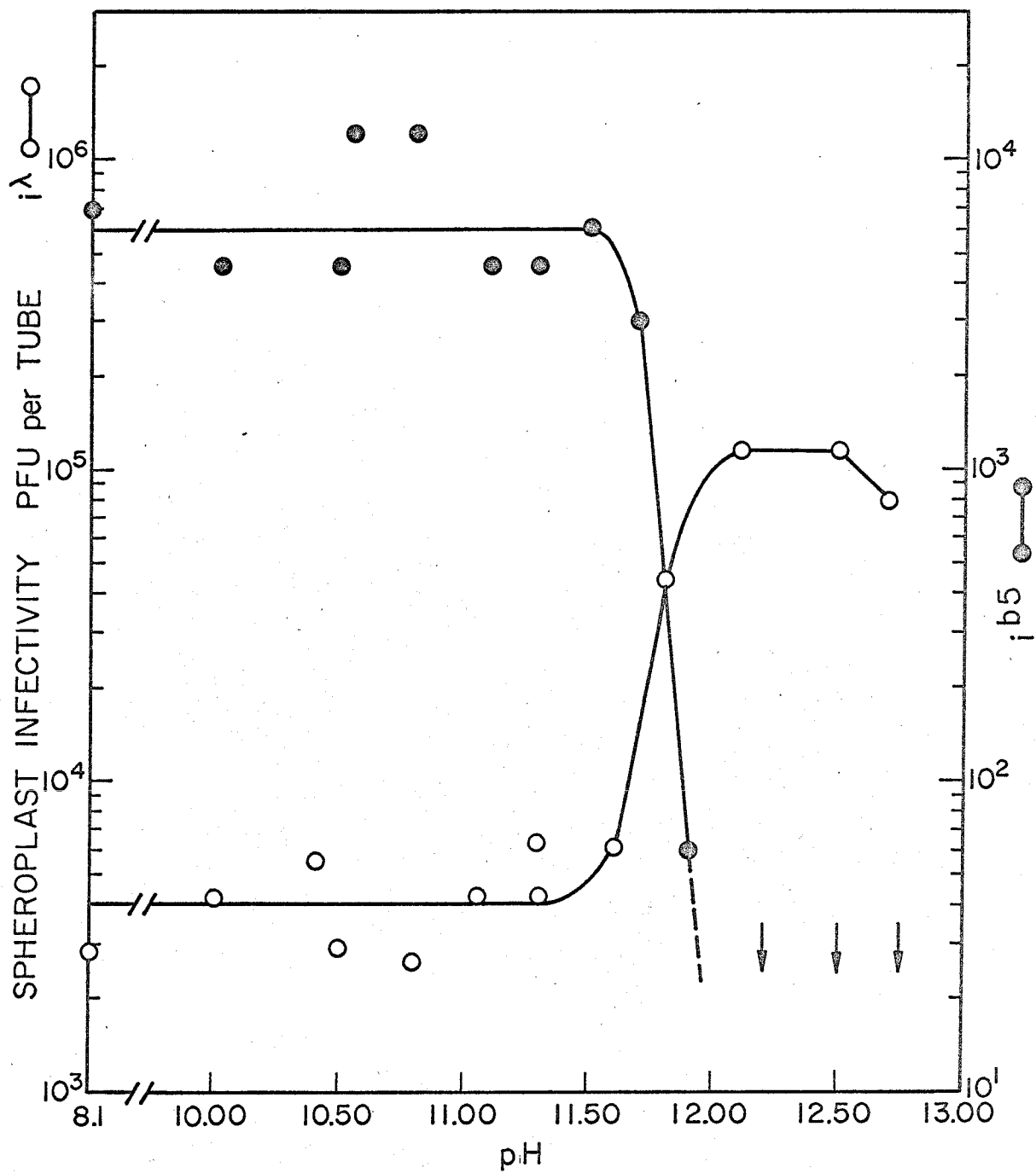Figure 2

Figure 3. <u>Distribution</u> <u>of</u> <u>spheroplast</u> <u>infectivity</u> <u>of</u> <u>native</u> <u>and</u> <u>denatured</u> <u>intracellular</u> λ <u>DNA</u> <u>after</u> <u>centrifugation</u> <u>in</u> <u>a</u> <u>CsCl</u> <u>equilibrium</u> <u>density</u> <u>gradient</u>.

(a) λ b2b5c intracellular DNA was denatured with NaOH, pH 12.2, for 15 minutes at 25° and then neutralized with NC1. Native ^{3}H-i λ DNA was added and the density raised to 1.71 g/cm^3 with CsCl. Centrifugation was for 37 hours at 30000 rev./min at 20° C. Two-drop fractions were collected into buffer and assayed for radioactivity by liquid scintillation counting (without TCA precipitation). Those fractions containing the peaks of radioactivity were dialyzed (19) against 0.01M tris, pH 8, and subsequently assayed in the spheroplast assay. The density gradient was measured by collecting fractions into paraffin oil and weighing 50 μ 1. of the CsCl solution. Regions of the gradient not shown did not contain significant amounts of radioactivity.

X——X ^{3}H cpm iλ phage DNA   X-----X infectivity, iλ phage DNA

0——0 ^{32}P cpm, intracellular ●——● infectivity, i^{b5} intra-
DNA   cellular DNA

(b) Native intracellular DNA was centrifuged as described above (a). After dialysis of the fractions containing radioactivity each was divided into two equal portions, one of which was denatured with NaOH (pH 12.2-12.5) and neutralized 15 minutes later. Native and denatured portions

(Figure 3 cont)

of each fraction were assayed in the spheroplast assay, using the same

spheroplast stock as was used in (a) above.

X————X ^{3}H cpm, phage DNA

0————0 ^{32}P cpm, intracellular DNA

Δ————Δ   infectivity, native intracellular DNA

●————●   infectivity, denatured intracellular DNA
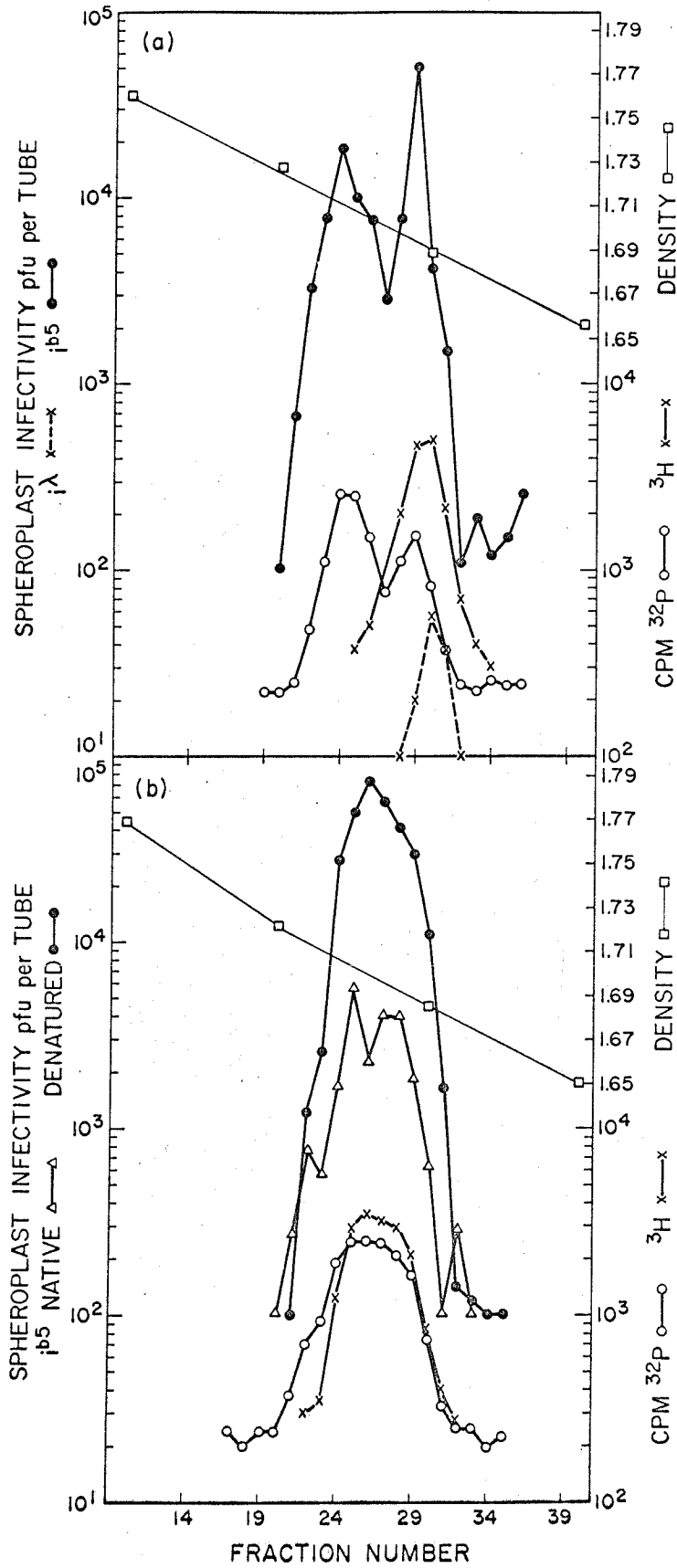
Figure 3

Figure 4. <u>Distribution of</u> ^{32}P- <u>DNA</u> (●————●) <u>and infectivity</u> (X————X)
<u>after sedimentation through a 5-20% sucrose gradient containing 0.05M</u>
<u>K</u>$_3$<u>PO</u>$_4$ <u>and enough KOH to make the pH 12.2.</u> The samples were denatured
prior to centrifugation by incubation at 37° for 15 min in 0.2<u>N</u> NaOH.
Centrifugation was for 15 hours at 25000 rev./min at 6° in a Spinco
SW 25.3 rotor. Samples (a) and (b) were sedimented simultaneously in
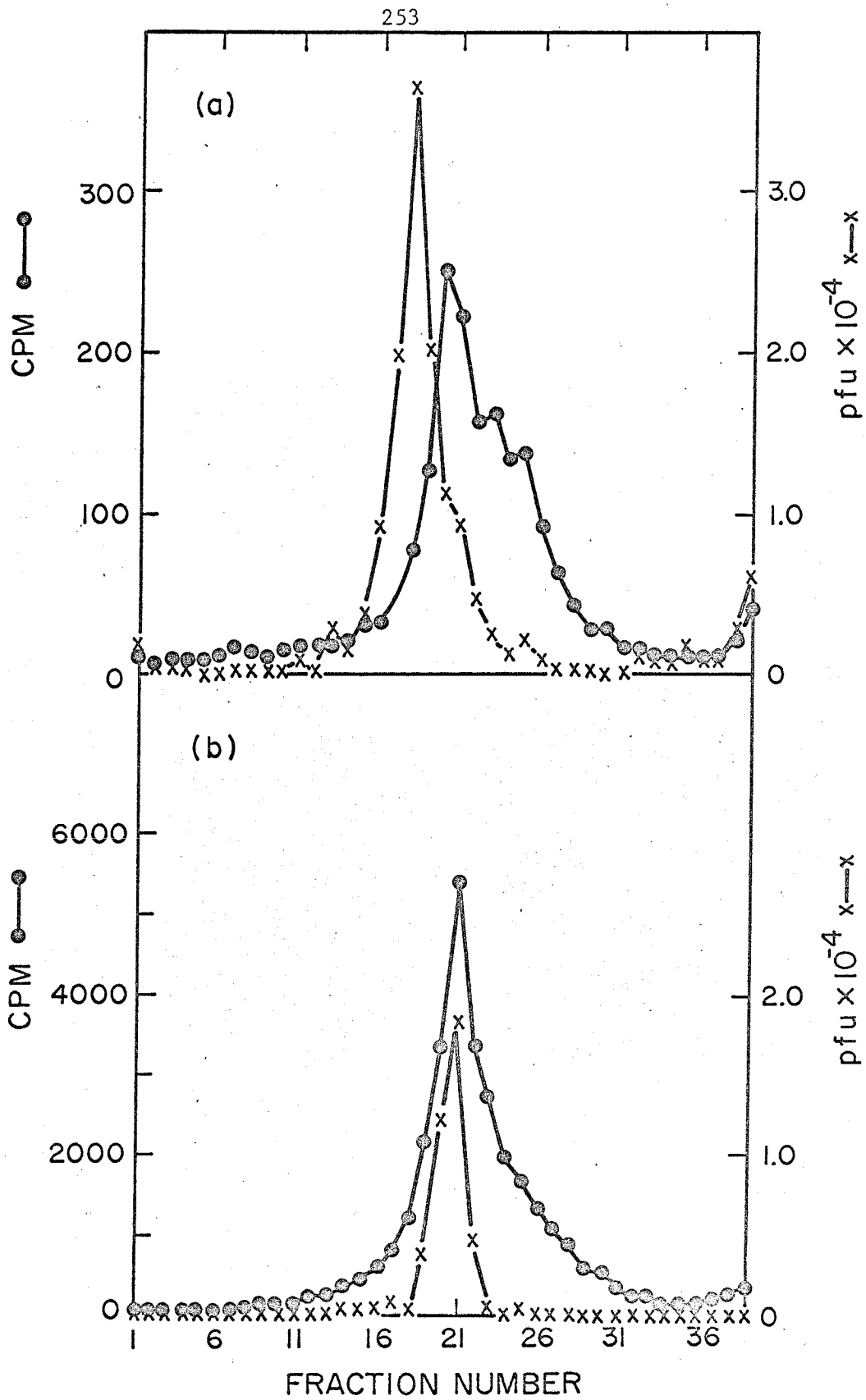the same rotor. (a) is component II DNA. (b) is phage DNA.

Figure 4

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