STRUCTURE AND SYNTHESIS OF BACTERIOPHAGE LAMBDA DNA

Thesis by

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In Partial Fulfillment of the Requirements
For the Degree
Doctor of Philosophy

California Institute of Technology
Pasadena, California
1967

(Submitted May 26, 1967)
I gratefully acknowledge the ever-constructive criticism of my thesis advisor, Dr. Robert Sinsheimer. He has made me feel at home in what might have been a strange ecological niche. Dr. Jean Weigle taught me that science, as well as mountaineering, is fun; she also introduced me to bacteriophage lambda for which I am grateful. I appreciate the opportunity I had to explore the world of Phycomyces under the tutelage of Dr. Max Delbrück. Dr. Jerome Vinograd has freely offered his sage advice and the use of his equipment.

My peers in the Biology Division have been an education in themselves. In particular I wish to thank John Kiger, Jr. with whose collaboration the experiments in Part IV were performed. The other graduate students in the Division have been no less skeptical and stimulating.

I have been supported at various times by the National Science Foundation, the Arthur McCallum Fund, and the National Institutes of Health. Their generous aid has made this work possible.

I wish to thank my wife Alison for being herself.
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 \( \lambda \). This distinction is undoubtedly due to its natural symbiotic relationship with a host, *E. coli* K12, whose genetics and physiology are so well characterized. As a typical temperate phage, \( \lambda \) 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. Superficially 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 \( \lambda \), 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 chromosome 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 \( \lambda \) 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), however, 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 chromosome 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 $5 \times 10^{-5}$ 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 function: 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 recombine 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 \( \lambda \) appears to be an isometric hexagon 550\( _\pm \) 25 Å in diameter. The tail, which is often curved, is about 1500 Å long. Empty phage heads often contain a core of proteinaceous material which is about 300 Å 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 \( \lambda \) contain in addition to normal phage particles, equal numbers of smaller "petit \( \lambda \)", 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$_2$O (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 characterization of the DNA isolated from purified phage have revealed the following 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 centrifugation, Hg(II) binding and Cs$_2$SO$_4$ 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 polynucleotide 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 displacement 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 structure 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 \( \lambda \) 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 (\( \Phi X 174, 23; \lambda \),22). If DNA or protein synthesis is blocked, transcription occurs from the early regions but not the late (37). Because the two strands of \( \lambda \) are separable (\textit{vide ante}) 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 T since it is C-poor. This agrees with the right half being the early half and its being light in a CsCl density gradient, \textit{i.e.} A T-rich. Genetic analysis of \( \lambda \) 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 \( \lambda \) 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 exclusion may exist between λ and ϕX174, both of which depend more extensively, 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). Similarly, 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 \(\lambda\) 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 \(\lambda\) 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 \(\lambda\) genome.

Campbell isolated sus mutants representing 18 cistrons (A - R). In addition to these there are the three classical clear-plaque mutants of \(\lambda\), 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 \(\lambda\) protein size of 300 amino acids, \(\lambda\) 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 \(\lambda\) cistrons have been identified, the specific functions of very few are known. The structural gene for only two proteins, endolysin and the \(\lambda\) 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 \(\lambda\) capsids, since no head structures are visible in electron micrographs of extracts made from induced, non-permissive lysogens for sus E (L. Siminovich, 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 \(\lambda\)-exonuclease, a thoroughly-characterized early enzyme (51) whose structural gene is between N and J (63) and \(\beta\)-protein, which is another \(\lambda\) 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 \(\lambda\)-specific RNA polymerase. Thus the early region of the genome would be transcribed by the \textit{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 \textit{E. coli} RNA polymerase to transcribe only the early region from phage DNA \textit{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 \(\lambda\) 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\textsuperscript{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, presumably 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 \textit{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

\textsuperscript{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 \( \lambda \) DNA. In the second section a spheroplast assay for \( \lambda \) DNA is described and the results obtained using this assay to study the biological activity of \( \lambda \) DNA extracted from phage or infected bacteria are presented. When the spheroplast assay is used to measure the biological activity of injected \( \lambda \) 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 \( \lambda \) 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 LAMBDNA 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 & Inghram, 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 b256 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$_2$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 b2b5e 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$_2$PO$_4$, pH 12.1, was used. The sample was denatured prior to centrifugation by 10 min incubation with 0.1 M Na$_2$PO$_4$, pH 12.4, 25°C. Centrifugation was for 3 hr at 25,000 rev./min at 6°C in a Spinco SW28 rotor.

The slow peak has the same sedimentation coefficient as DNA prepared from purified lambda phage b2b5e, 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 $\phi$X 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 b266 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).

The interest and advice of Dr Jean Weigle in this work are appreciated. This research was supported in part by grant RG 6965 from the U.S. Public Health Service.

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Received 22 September 1964

REFERENCES

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, λDNA 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 λDNA 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 H₂O.

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

**2XM9 medium** contains 14 g Na₂HPO₄, 6 g KH₂PO₄, 2 g NH₄Cl, 10 ml. 1M MgSO₄, 4 ml. 25% NaCl, and 40 ml. 10% glucose per liter. The glucose and MgSO₄ are added steriley 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.

A **dil** is 0.01M KPO₄, pH 7.0, 0.01M MgSO₄, and 10 g/ml. of bovine plasma albumin (11).
**TM** is 0.01M tris, pH 7.2, 0.01M MgSO₄. Bacteriophage stocks are stored and diluted into TM.

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

EDTA solutions are made with the disodium salt of ethylenediaminetetraacetic acid (Sigma Chemical Company).

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 0.25M tris, pH 8.1.

**(b) E. coli K12 strains**

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

CR34 is a thymidine-requiring derivative of C600 (17).

W3110 and W3110(λ) are prototrophs.

12/λ is a λ-resistant, gal₁⁻, gal₂⁻ derivative of 3101.

These strains were obtained from Dr. Jean Weigle.

1100 and 1486-23 were obtained from Dr. H. Hoffmann-Berling. Their endonuclease I activity (14) is only a few percent of that observed in the strain from which they were derived. Mutants resistant to λ were isolated by plating 10⁷ bacteria with 10⁶ 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 centrifugation 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), CHCl₃ 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 μ. 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 $4 \times 10^{10}$ pfu/ml. The virus is purified as described above. The stock contains approximately $6 \times 10^{-6}$ cts/min/phage particle. At this specific activity (1.0 c/13.5 mmoles $^{31}$P), there is $1 \times 32$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 $\lambda$DNA 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). $2-4 \times 10^9$ 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 0.1M tris, pH 9.0, 0.1M NaCl, 0.01M 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 extracted 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 consistently higher than recovery from infected sensitive cells. In one experiment 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) **Plaque assay for λ phage**

Plating bacteria are grown to saturation in TM, 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) **Intracellular phage assay**

0.10 ml. of the infected culture is mixed with 1.65 ml. of cold 0.05\(\text{M}\) tris, pH 8.1, containing 200 \(\mu\)g lysozyme, and 0.15 ml. 4% Na\(_2\)EDTA is added. The tube is kept cold and at the end of the experiment 0.1 ml. of 0.1M MgSO\(_4\) is added to stabilize the phage. Phage are assayed after freezing and thawing once.

(g) **Helper infectivity assay of λDNA**

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) **Spheroplast infectivity assay of λDNA**

Bacteria are grown with aeration at 37° C in 20 ml. of 3XD to an absorbance at 600 m\(\mu\) 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 1.5M 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 1.0M 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 °C.

For infection, 0.30 ml. of spheroplast stock is added to 1.20 ml. of a DNA solution in 0.05M tris, pH 8.1, and incubated 15 minutes at 30 °C. 1.50 ml. of PAM is then added and the suspension is incubated at 30 °C without aeration for 6 hours. Mature phage are measured after shaking 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 spheroplasts 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 immediately 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 infectivity 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/A or 12/A.

(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 3H are precipitated 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 H₂O. 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 precipitated 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, ì2/λ, and later 1486-23/λ and 1100λ 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. ì2/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 endonuclease I and in most experiments is more efficient than ì2/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 0.1-0.5M.

(ii) Dilution of spheroplasts

If the spheroplasts are mixed with an equal volume of DNA in 0.05M 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) Linearity of the assay

Figure 2 shows the effect of various concentrations of DNA in the assay. The number of phage produced is approximately linearly proportional to the amount of DNA added between $5 \times 10^6$ and $10^{10}$ DNA molecules/spheroplast tube. The slight deviation from linearity in this experiment 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 $4 \times 10^{-5}$ 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 $\Phi 2\lambda$ spheroplasts (the spheroplast preparation was the same as that used to obtain Figure 2). $4.8 \times 10^{10}$ DNA molecules in 4.8 ml. of 0.05M 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 spheroplasts contrasts with the results obtained in spheroplasts infected with $\Phi X174$ 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 $2 \times 10^8$ 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. Apparently, 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) Single-burst experiment in spheroplasts

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 x 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 con-
tents 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 distri-
bution 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 x 10^{-5} phage/DNA molecule. In terms of infected
spheroplasts/DNA molecule the efficiency was 3 x 10^{-7}. If the satur-
ating 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 E. coli 1486-23 is about 300.

(vi) Competition experiments with E. coli DNA and RNA

When E. coli DNA is added to the incubation tube with λDNA there
is an inhibitory effect which depends on the concentration and secondary
structure of the \textit{E. coli} DNA. As shown in Figure 5, native \textit{E. coli} DNA inhibits the assay when its concentration in the incubation tube exceeds 1 \(\mu\text{g/ml}\). 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) \textit{E. coli} DNA inhibits the assay at concentrations as low as 0.05 \(\mu\text{g/ml}\).

Addition of total \textit{E. coli} RNA produces quite a different effect. There is an increase in the number of phage produced when 1-50 \(\mu\text{g/ml}\), of RNA are present. The maximum increase occurs when 5-10 \(\mu\text{g/ml}\). 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 \(\lambda^+\). One possible explanation for the enhanced efficiency with added \textit{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, \textit{E. coli} 1486-23 and 1100, were kindly supplied by Dr. H. Hoffmann-Berling and selected for resistance to \(\lambda\). Two separate cultures of each and of \(\lambda^+\) were grown and converted to spheroplasts as usual. \textit{E. coli} RNA at 8 \(\mu\text{g/ml}\). 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 \(\lambda^+\) 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 λ DNA

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$\lambda$ with 3110($\lambda$) for 10 minutes in $\lambda$ dil at 0°. This allows adsorption but not injection (2; personal observations). The other samples were taken 20 and 40 minutes after raising the temperature to 37° for 10 minutes to allow for injection, blending in a Waring blender 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 infectivity 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) Fractionation of the infectivity by sedimentation through a sucrose density gradient

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 spheroplasts. The results are shown in Figure 6. Two components contain most of the injected radioactivity with the faster, component I, containing 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 equiv-
alents 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 $^{32}\text{P}$ 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 infec-
tivity 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 $\lambda$-lysogen after infection with a cI mutant (c26). Similar experi-
ments have been performed after infection of the same strain with wild-
type $\lambda$ 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 infection 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 λDNA 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 $2.0 \times 10^9$ and $2.3 \times 10^9$ 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 $1.5 \times 10^9$ 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 demonstration 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 \( \lambda \) 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 extraction 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 \( \lambda \)DNA (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 degraded 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 spheroplast assay and the helper assay. The "notched" circles prepared in vitro by annealing phage DNA at low concentration are at least as infective 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 increase 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.

The continued encouragement and enthusiasm of J. Weigle are greatly appreciated. We wish to thank W. Dove for permission to mention his unpublished results. This research was supported in part by grants RG 6965 and GM 13554 from the United States Public Health Service.
TABLE 1

Efficiency of the spheroplast assay in endonuclease I⁺ and endonuclease I⁻ strains (pfu/DNA x 10⁶)

<table>
<thead>
<tr>
<th>Spheroplasts</th>
<th>Minus RNA</th>
<th>Plus RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ï²/ (+)</td>
<td>3.3</td>
<td>8.3</td>
</tr>
<tr>
<td>1100/ (-)</td>
<td>1.9</td>
<td>1.4</td>
</tr>
<tr>
<td>1486-23/ (-)</td>
<td>11.0</td>
<td>8.8</td>
</tr>
</tbody>
</table>

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)**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Helper assay</th>
<th>Spheroplast assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>20</td>
<td>0.046</td>
<td>1.5</td>
</tr>
<tr>
<td>40</td>
<td>0.022</td>
<td>0.5</td>
</tr>
</tbody>
</table>

*E. coli 3110(λ)* was grown to $3 \times 10^8$ 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**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>DNA concentration during circularization (μg/ml.)</th>
<th>Relative infectivity of circular DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Helper assay</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spheroplast assay</td>
</tr>
<tr>
<td>1</td>
<td>.072</td>
<td>.02</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>.29</td>
</tr>
<tr>
<td>3</td>
<td>8.0</td>
<td>.20</td>
</tr>
</tbody>
</table>

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. Effect of dilution of the spheroplasts on the efficiency of the assay. Spheroplasts of 12A were prepared as described in Methods. λb2b5c DNA at a concentration of 4 x 10⁹ 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 CHCl₃ 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., respectively, of DNA solution was added.

(b) To 0.1 ml. of spheroplasts, 0, 0.1, 0.3, 0.5 and 0.7 ml., respectively, of 0.05 M tris, pH 8.1 was added, followed by 0.1 ml. of DNA solution.
Figure 1
Figure 2. **Linearity of the spheroplast assay in T2/λ spheroplasts.**

λ b2c DNA at a concentration of $4 \times 10^{10}$ molecules/ml. was diluted $5^{1-5}$ times in 0.05 M tris, pH 8.1. Three 1.2 ml. portions of DNA at each concentration were mixed with three 0.3 ml. portions of spheroplasts prepared as described in Methods. 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 Methods 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 $4 \times 10^{-5}$. 
Figure 2
Figure 3. Growth of phage after infection of \( \text{II}A \) spheroplasts.

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 \( 1 \times 10^{10} \) molecules/ml. At 0 minutes the DNA was mixed with the spheroplasts. Aliquots were withdrawn at the indicated times, lysed, and assayed for phage. The arrows (↑) indicate values less than 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 \( 4 \times 10^{-5} \) at 6 hours.
Figure 3
Figure 4. **Single-burst experiment in spheroplasts of 12/8.**

Spheroplasts were prepared as in Methods. λb2b5c DNA, 60 ml., at a concentration of $2 \times 10^6$ molecules/ml. was mixed with 15 ml. of spheroplasts 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 contents 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. **Effect of added E. coli RNA or DNA on the efficiency of the spheroplast assay.** The incubation tube contained 0.3 ml. of 12/λ spheroplast stock and 1.2 ml. of 0.05 M tris, pH 8.1, containing $1 \times 10^{10}$ λb2b5c DNA molecules and *E. coli* RNA, native DNA, or denatured DNA. The concentration of the RNA and DNA was calculated from the absorbance at 260 μm 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.

- O---O 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 10⁹ 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 infectivity 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.

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x —— x  cts/min
0 —— 0  spheroplast assay
Δ —— Δ  helper assay
INFECTIVE PHAGE DNA EQUIVALENTS PER FRACTION

Spheroplast Assay X 10^8

Helper Assay X 10^5

Fraction Number

CPM per Fraction x-x
Figure 7. Infective DNA and intracellular phage during a sensitive infection.

A. E. coli CR34 was starved by aeration in TM for 60 minutes at 37° and then infected with about one $^{3}\text{H-}\lambda$ b2b5c per bacterium. After 10 minutes 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°C. 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 $^{3}\text{H}$ recovery.

B. AB2500 was grown to 6 x 10$^8$ cells/ml. in K medium plus thymidine, centrifuged, resuspended in 1/5 volume TM at 37°C, and infected with $^{32}\text{P-}\lambda$ c26 at a multiplicity of 5 pfu/bacterium. After 5 minutes at 37°C 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 Methods.

More than 99% of the pfu adsorbed during 5 minutes at 37°C, 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 produced by an equivalent number of $^{32}\text{P}$ 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 infectivity of each sample is recorded.

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

- $\bullet\bullet$ intracellular phage
- $\triangle\triangle$ helper assay
- $\circ\circ$ spheroplast assay
REFERENCES FOR PART I

Part II

VEGETATIVE LAMBDA DNA:

PHYSICAL CHARACTERIZATION AND REPLICATION
1. INTRODUCTION

In contrast to the considerable effort that has been expended studying 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 identified 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 tertiary 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 replication 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), substantiating 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 replication(33). This might be involved in attaching the DNA to the membrane (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 encapsulated (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 \( \lambda \) DNA has been detected \textit{in vivo} during three distinct phases of the \( \lambda \) 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 \( \lambda \) 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 \( \lambda \)-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 \textit{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 H₂O 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 sedimentation 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 complete 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 synthesized after infection of a susceptible 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 λ DNA synthesized under these conditions reveals that before intracellular phage are produced, 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 sedimentation 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 completely 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 3H-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; chloramphenicol¹ was a gift of Parke, Davis Co.; mitomycin C¹, Nutritional Biochemicals; ¹⁵^N, Biorad Laboratories, Richmond, California; ¹³C-glucose, D-glucose ¹³C, 42 atom % ¹³C (uniformly labeled), Merck, Sharp and Dohme, Ltd., Montreal, Canada.

¹Abbreviations 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$_2$·6H$_2$O, 12.1 g Trizma base, 0.046 g KH$_2$PO$_4$, 1.0 ml. of 0.1M CaCl$_2$, and 0.02 g Na$_2$SO$_4$. The pH is adjusted to 7.4 before autoclaving. This is supplemented with $^{12}$C-glucose and $^{14}$NH$_4$Cl or $^{13}$C-glucose and $^{15}$NH$_4$Cl 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 x $10^8$ 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 m$m$ (about 4 x $10^8$ 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 13C-15N is about 0.032 g/cm³ greater than the density of phage containing 12C and 14N. 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 x 10⁹ 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 SDS¹ (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 M tris, 0.02 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 M tris, 0.001 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-$\lambda$b2b5c 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 M \( \text{K}_3\text{PO}_4 \) 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 \(^3\text{H}-\text{DNA} \) by this method is 23% (in the Beckman counter), compared to a value of 30% obtained simply by drying a small sample of \(^3\text{H}-\text{thymidine} \) 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) **Lysogenization**

This procedure is adapted from that of Fry (15) and Sechaud (45). *E. coli* 3110 is grown to 2 x 10⁹ 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. λc⁴ 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 lysogenized 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 lysogenized. (They seemed to be "preinduced.") Even with this precaution,
we have never been able to lysogenize more than 70% of a thymine-requiring 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 containing 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. $^3$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 $^3$H-thymidine containing media, are pulsed with radioactive 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 \( \Phi X \) 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 (\( \lambda \)) was infected as described in the legend to Figure 5 with (a) \( ^3H-\lambda \text{vir} \), (b) \( ^{32}P-\lambda c26 \), or (c) a mixture of \( ^3H-\lambda \text{vir} \) and \( ^{32}P-\lambda c26 \).

The infection with \( \lambda \text{vir} \), both alone and in the mixed infection, produced a burst size of 50-100 \( \lambda \text{vir} \) pfu/cell. In the mixed infection, less than 2 pfu/cell were \( \lambda c26 \), in agreement with the observation of Thomas & Bertani (57) that in a mixed infection with a heteroimmune phage, \( \lambda \text{vir} \) in this experiment, and a homoimmune phage, \( \lambda c26 \), the homoimmune phage is still repressed despite the replication of the heteroimmune phage.

(When \( \lambda \text{vir} \) infects a \( \lambda \)-lysogen as here, it, too, is apparently initially repressed, as evidenced by a lower initial rate of \( \lambda \)-mRNA synthesis (49).)

The sedimentation of the \( ^3H-\lambda \text{vir} \) 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 \( ^{32}P-\lambda \text{DNA} \), 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-λ DNA marker at pH 12.6. These properties are identical to those of component I (2) isolated from homoinmune infected 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 centrifuge 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 homimmune lysogenic cells is shown in Table 2. The ratio of the distance sedimented by component I to the distance sedimented by a marker phage DNA is recorded. The alkaline sucrose gradients contained 0.10 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 respective 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) Shear resistance of component I

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 expectation is at least partially realized.

Intracellular \(^{32}\)P-\(\lambda\) DNA was obtained from \textit{E. coli} 3110(\(\lambda\)) superinfected with \(^{32}\)P-\(\lambda\)c26. High molecular weight \textit{E. coli} DNA containing \(^{3}\)H-

\(^2\)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 \(\times\) \(10^6\) 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 \(\times\) \(10^6\) 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) Buoyant density of component I in alkaline CsCl

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). E. coli CR34 was infected with $^{32}$P-λc26 and the extracted DNA was sedimented on a neutral sucrose gradient. Fractions containing component I were pooled and mixed with $^3$H-E. coli DNA and the density adjusted to about 1.76 g/cm$^3$ with CsCl. The buffer was 0.05 M K$_3$PO$_4$-KOH, pH 12.8 (measured in CsCl). The solution was centrifuged for 60 hours at 17$^\circ$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 $^{32}\text{P}$-DNA has a buoyant density in alkaline CsCl about 0.02-0.03 g/cm$^3$ heavier than the $^3\text{H-}E.\ \text{coli}$ 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 $\lambda$DNA 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.

$E.\ \text{coli} \ 3110(\lambda)$ was grown and infected with $^{32}\text{P}\lambda c 26$. Thirty minutes after infection the bacteria were harvested, converted to spheroplasts, and lysed by dilution into distilled H$_2$O. A $^3\text{H-}\lambda$DNA 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 λDNA, 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 λDNA 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 effect 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 culture is due to bacterial DNA synthesis. The amount of $^3$H-thymidine incorporated by the MC-treated, uninfected culture is reduced to about 3% of the incorporation 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) **Effect of MC pretreatment on phage development and $^3$H-thymidine incorporation in $\lambda$-infected E. coli AB2497$^{+}$**

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 untreated cultures.

As may be noted in Tables 3a and 3b, a large fraction of bacteria survived $\lambda$ 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 $\lambda$ was used, the surviving bacteria could not be lysogenic. This is not an uncommon result in our experience. 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 $\lambda$-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<sup>-</sup> 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) Inhibition of DNA synthesis in uninfected hcr<sup>+</sup> and hcr<sup>-</sup> cells and the capacity to support λ growth as a function of MC concentration

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 <sup>3</sup>H-thymidine. After 45 minutes aeration at 37° the amount of acid insoluble <sup>3</sup>H-thymidine incorporated was determined both before and after hydrolysis with 0.5M NaOH (14 hours at 37° C).

A similar experiment using the same concentrations of MC was performed with λc26-infected AB2497(hcr<sup>+</sup>) 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 hcr<sup>-</sup> 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 \(\lambda\) is drastically reduced between 1.0 and 5.0 \(\mu\)g/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 \(\mu\)g 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 thereafter 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 ³H-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 control 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 3H-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 3H-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 $\lambda cI$

(i) **In the absence of CAM**

A culture of MC-treated *E. coli* AB2500 was infected with $^{32}$P-$\lambda c26$ 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, incubated for ten minutes, and blended in a Waring Blender (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 incorporation 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 removed from the phenol-buffer interface. At 70 and 85 minutes about 90% of the total incorporated $^3$H was recovered, suggesting that the DNA synthesized 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 extracting 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 M NaOH and 0.30 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 $\lambda$-infected cells obtained by Smith & Skalka (52) after a similar analysis.

The results described above indicate that late in an infection with $\lambda$ about 10-15% of the total (extractable) DNA in the cell has the sedimentation 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 distribution of $^3$H-containing vegetative DNA and spheroplast-infectious DNA is shown in Figure 16. Before centrifugation $\lambda b5$ 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 $\lambda$ 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 3H-thymine during the infection, but the culture containing 100 μg/ml. of CAM contained only 1H-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 32P (contributed by the infecting phage), 3H-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, 3H-containing λc26 DNA was added as a sedimentation marker (no 3H 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 deletion 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 $\lambda$-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 recovery 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 extraction 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 $\lambda$DNA 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 vegetative 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 $\lambda$ 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 \textit{E. coli} chromosome. Phage were prepared (as described in Methods) which contained radiophosphorus and heavy isotopes of carbon and nitrogen ($^{13}\text{C}$ and $^{15}\text{N}$). The DNA of such phage is more dense than is DNA which has been synthesized in medium containing $^{12}\text{C}$ and $^{14}\text{N}$ 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.

\textit{E. coli} CR34 was infected with $^{32}\text{P}$,$^{13}\text{C}$,$^{15}\text{N}$-\lambda c26 in light ($^{12}\text{C}$,$^{14}\text{N}$), $^{1}\text{H}$-thymine medium, so that the progeny DNA (except those molecules containing contributions from the parent) would be "light" and nonradioactive. 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 containing the unresolved components II and III. A control culture was infected with $^{32}\text{P}$ $^{12}\text{C}$,$^{14}\text{N}$-\lambda c26 and treated similarly. CsCl was added to the pooled fractions to raise the density to 1.70 g/cm$^3$, a density reference $^{3}\text{H}$-\textit{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 $^{32}\text{P}^{13}\text{C}^{15}\text{N-\lambda c26}$ DNA (extracted from mature virus particles) and $^3\text{H-}\text{E. coli}$ DNA. ($^{32}\text{P}^{12}\text{C}^{14}\text{N-\lambda c26}$ DNA has the same density as $\text{E. coli}$ DNA (Figure 20b)). Figures 19b and 19c demonstrate the density distribution of component I, containing parental $^{32}\text{P}$ and $^{13}\text{C}^{15}\text{N}$, 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 $^{32}\text{P}$ is found in DNA which has either the density of $^{13}\text{C}^{15}\text{N}$ 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 $H^{(13}\text{C}^{15}\text{N})$ and $L^{(12}\text{C}^{14}\text{N})$ DNA.

The density distribution of those components II and III which contain $^{32}\text{P}$ 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"$\lambda$ in the presence of $^3\text{H-thymine}$. $\text{E. coli}$ AB2500 was grown and treated with MC as described previously to inhibit host
DNA synthesis. After thoroughly washing the bacteria with TM, $^{32}\text{P}\,^{13}\text{C}\,^{15}\text{N-}$\text{λc}26 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. $^3\text{H}$-thymine at a concentration of 2.5 $\mu$g/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 incubation at 30 ºC. Samples of the extracts were sedimented on neutral sucrose gradients as usual. The distributions of $^{32}\text{P}$, $^3\text{H}$-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, $^3\text{H-E. coli}$ 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 addition 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 $^3H$$^{12}C$$^{14}N$-DNA and one strand of $^{32}P$$^{12}C$$^{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 $^{32}\text{P}$ is still associated with $^3\text{H}$ after the treatment with alkali. The peak of $^3\text{H}$ 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 $\lambda c26$ per bacterium and aerated an additional 30 minutes in the absence of thymidine. The
phage used in the infection contained $^{32}\text{P}$ and also $^{13}\text{C}$ and $^{15}\text{N}$ so the parental DNA could be separated from the progeny duplexes by CsCl density gradient centrifugation. The intended purpose of the thymidine starvation 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 observed 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 spheroplast-infected DNA precedes that of DNA infective in the helper assay.

Samples of the infected cell extracts were mixed with a DNA sedimentation 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 ($\lambda$c26) is well resolved from the $^{3}$H-E. coli DNA and the b2b5c infectivity. The $\lambda$c26 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 (designated 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 $\lambda$ (J. Weigle and R. D'Ari, personal communication), this might be the explanation for this observation. Alternatively, the light radioactivity could be due to breakdown of some of the phage DNA involved in abortive infections and re-incorporation 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 \textit{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 \(\lambda\) and \textit{E. coli} DNA (31). Also, no chemical modifications of the phage DNA composition which would allow it to be distinguished from \textit{E. coli} DNA have been found. Initial efforts to study synthesis of \(\lambda\)DNA 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 \( \lambda \) and host DNA synthesis after induction of \( \lambda \)-lysogens. They concluded that host DNA synthesis continued approximately unabated after temperature or MC induction (using hcr\(^+\) bacteria).

However, the levels of \( \lambda \) 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 \( \lambda \)DNA. 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 \( \lambda \) 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 \( \lambda \)DNA 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 production 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 repression caused by the λcI mutants tested, however, was much smaller than that observed after P22 infection. Conceivably, part of the difference 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 synthesized. 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,
appears to occur, 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 3H-thymine into
hybrid component I which was synthesized in 1H-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 synthe-
sis of component I.

Some gratifying parallels between the processes of infection with
ØX174 and \( \lambda \) may be noted in these experiments. In summary these include:
1. Synthesis of (ØX174) or conversion to (\( \lambda \)) a double-stranded, circular
replicative form (ØXRF or \( \lambda RF \)). The conversion is resistant to 100 \( \mu g/ml \).
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 \( \lambda \) (65, 44). In the case of \( \lambda \), 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 \( \lambda RF \)
occurs specifically in the early part of the latent period, before syn-
thesis of mature phage begins. In both instances, RF replication occurs
in the presence of 30 \( \mu g/ml \), but not 100 \( \mu g/ml \), 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 \( \lambda \), sus N, O and P, are also blocked
completely in DNA synthesis (3, 10, 28). Since this protein is resistant
to 30 \( \mu g/ml \) 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 repli-
cation of ØXRF (Denhardt, personal communication). 3. Synthesis of
mature viral DNA occurs after net synthesis of ØXRF or \( \lambda RF \) ceases (47;
Figures 15 and 16). CAM at 30 \( \mu g/ml \). inhibits synthesis of viral DNA
(47, 11; Figure 17) as do some mutations of φX (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 λ DNA, since these mutants do synthesize λ DNA 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 synthesis 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 φX 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 λ RF and the late synthesis of mature viral DNA. The multiplication of λ RF 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 λ RF 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

<table>
<thead>
<tr>
<th>Extraction procedure</th>
<th>cpm/ml</th>
<th>Helper Infectivity</th>
<th>Spheroplast Infectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IC/ml x10^-7</td>
<td>IC/32Pdpm</td>
</tr>
<tr>
<td>SDS-Pronase</td>
<td>3.0 x 10^5</td>
<td>5.3, 5.0</td>
<td>180, 170</td>
</tr>
<tr>
<td>SDS-Pronase - Phenol</td>
<td>1.9 x 10^5</td>
<td>3.5, 3.2</td>
<td>180, 170</td>
</tr>
</tbody>
</table>

\(^{32}\)P-\(\lambda b2b5c\) 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

<table>
<thead>
<tr>
<th>Ionic Strength</th>
<th>pH = 8.1</th>
<th>pH = 12.8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K12S</td>
<td>K12(λ)</td>
</tr>
<tr>
<td>0.01</td>
<td>1.9</td>
<td>1.9, 1.9*</td>
</tr>
<tr>
<td>0.10</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>0.20</td>
<td></td>
<td>3.4</td>
</tr>
<tr>
<td>0.60</td>
<td></td>
<td>3.1</td>
</tr>
<tr>
<td>1.0</td>
<td>1.6</td>
<td>1.5-1.6*</td>
</tr>
</tbody>
</table>

*Bode & Kaiser, (2)
### TABLE 3

**Effect of Mitomycin C (MC) Pretreatment on the Production of Infective Centers and Phage**

(a) *E. coli AB2500, hcr-

<table>
<thead>
<tr>
<th>Infected culture</th>
<th>Colony formers per ml.</th>
<th>Infective centers per ml.</th>
<th>Unadsorbed pfu per ml.</th>
<th>% pfu adsorbed</th>
<th>Phage yield per ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before infection</td>
<td>After MC and infection</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minus MC</td>
<td>$2.4 \times 10^8$</td>
<td>$1.0 \times 10^8$</td>
<td>$2.2 \times 10^8$</td>
<td>$6.0 \times 10^8$</td>
<td>$40%$</td>
</tr>
<tr>
<td>Plus MC</td>
<td>$2.4 \times 10^8$</td>
<td>$5.0 \times 10^3$</td>
<td>$1.5 \times 10^8$</td>
<td>$5.4 \times 10^8$</td>
<td>$46%$</td>
</tr>
</tbody>
</table>

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 ³H-thymidine were added to the cultures and samples taken to measure TCA-insoluble ³H-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⁺

<table>
<thead>
<tr>
<th>Infected culture</th>
<th>Colony formers per ml.</th>
<th>Infective centers per ml.</th>
<th>Unadsorbed pfu per ml.</th>
<th>% pfu adsorbed</th>
<th>Phage yield per ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before infection</td>
<td>After MC and infection</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minus MC</td>
<td>$2.6 \times 10^8$</td>
<td>$1.4 \times 10^8$</td>
<td>$1.3 \times 10^8$</td>
<td>$1.6 \times 10^8$</td>
<td>$84%$</td>
</tr>
<tr>
<td>Plus MC</td>
<td>$2.6 \times 10^8$</td>
<td>$1.7 \times 10^5$</td>
<td>$2.4 \times 10^7$</td>
<td>$1.8 \times 10^8$</td>
<td>$82%$</td>
</tr>
</tbody>
</table>

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>
<thead>
<tr>
<th>Source of DNA</th>
<th>Normalized Specific Infectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phage</td>
<td>1.00</td>
</tr>
<tr>
<td>Thymine starved cells infected and deprived of thymine for 30 min</td>
<td>0.079</td>
</tr>
<tr>
<td>Same, 10 minutes after adding thymidine</td>
<td>0.13</td>
</tr>
<tr>
<td>Same, 20 minutes after adding thymidine</td>
<td>3.00</td>
</tr>
</tbody>
</table>

The efficiency of this assay was quite low, thus the values obtained varied within the range shown.
<table>
<thead>
<tr>
<th></th>
<th>RELATIVE DISTANCE SEDIMENTED</th>
<th>BUOYANT DENSITY IN ALKALINE CsCl</th>
<th>TWISTED MOLECULES IN ELECTRON MICROGRAPHS</th>
<th>SHEAR RESISTANCE RELATIVE TO VIRAL DNA</th>
<th>SEPARABILITY OF STRANDS</th>
<th>INFECTIOUS AFTER DENATURATION</th>
<th>INCREASED BUOYANT DENSITY IN ETHIDIUM BROMIDE-CsCl*++</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIRAL DNA</td>
<td>1.0</td>
<td>1.0</td>
<td>~1.770</td>
<td>NO</td>
<td>SAME</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>COMPONENT I</td>
<td>1.9</td>
<td>3.5</td>
<td>~1.790</td>
<td>YES</td>
<td>MORE RESISTANT</td>
<td>NO</td>
<td>YES</td>
</tr>
</tbody>
</table>

* 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. CsCl equilibrium density gradient centrifugation of $^{32}$P.$^{13}$C.$^{15}$N-$\lambda$c26. A small portion of purified heavy phage were mixed with vir, h and $\lambda$b2b5c (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. $^{32}\text{P}$- b2b5c DNA extracted with SDS and pronase was mixed with phenol extracted $^3\text{H-}\lambda$DNA and centrifuged for 6 hours at 25000 rpm and 6°C in the SW 25.1 rotor. The sucrose gradients contained 0.01 M tris 0.001 M EDTA, pH 10.5. The DNA extracted from wild type phage ($^3\text{H}$) sediments about 9% faster than the DNA from the deletion mutant ($^{32}\text{P}$), as expected from its higher DNA content.

![Diagram]

\[\text{O---O}^{32}\text{P cpm}\]

\[\text{X---X}^3\text{H cpm}\]
Figure 3. Sedimentation of intracellular λDNA extracted with or without phenol. C600(b5) was infected with $^{32}\text{P}$- b2b5c and cultured for 40 minutes at $37^\circ$ 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 $^3\text{H}$-λDNA sedimentation marker onto pH 10.5 sucrose gradients and centrifuged for 6 hours at 25000 rpm in the SW 25.1 rotor.

C——0 $^{32}\text{P}$ cpm

X——X $^3\text{H}$ cpm
Figure 3
Figure 4. Buoyant density of intracellular ADNA extracted without phenol. The DNA extracted as described in the legend to Figure 3b was mixed with phenol extracted phage DNA and centrifuged to near equilibrium 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 radioactivity found at the top of the gradient (Fraction 25) after centrifugation as due to DNA trapped in the precipitate. Density increases from right to left.

\[
\begin{align*}
0 & \quad 0 \quad \text{32P cpm} \\
\times & \quad \times \quad \text{3H cpm}
\end{align*}
\]
Figure 5. Sedimentation of intracellular λDNA through sucrose gradients at neutral and alkaline pH. E. coli 3110(λ) was grown in KG medium, centrifuged, resuspended in three portions of TM at 0°C, and infected with (a,b) ³H-λvir, (c) ³²P-λc26, or (d) a mixture of ³H-λvir and ³²P-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 Blender 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 10⁸ 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 M NaOH, 0.90 M NaCl. ³²P-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 ³H-λvir DNA radioactivity and helper infectivity. b: Intracellular ³H-λvir DNA and ³²P-λ phage DNA, pH 12.8; c: Intracellular ³²P-λc26 DNA radioactivity and helper infectivity. d: Intracellular ³H-λvir and ³²P-λc26 DNA from the mixed infection.

\[\begin{align*}
\_\_\_\_\_x & \quad ³H \text{ cts/min} \\
\_\_\_\_\_0 & \quad ³²P \text{ cts/min} \\
\Delta & \quad \Delta \quad \text{Infectivity in the helper assay}
\end{align*}\]
Figure 5
Figure 6. The effect of shear on the sedimentation rate of intracellular λDNA and E. coli DNA. Intracellular 32P-λc26 DNA from the experiment described in the legend to Figure 2 was mixed with high molecular weight 3H-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: 3H 140%; 32P 80%. b: Sheared, recovery 3H 50%; 32P 20%.

\[
\begin{align*}
0 & \quad 32P \text{ cts/min} \\
X & \quad 3H \text{ cts/min} \\
\Delta & \quad \Delta \text{ Helper infectivity}
\end{align*}
\]
Figure 6
Figure 7. **Buoyant density of component I in alkaline CsCl.** 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 M K$_3$PO$_4$ with KOH added to bring the pH to 12.8.

a: $^{32}$P component I, and $^3$H-E. coli DNA were centrifuged for 60 hours at 17°C at 30,000 rev./min.

- $^{32}$P cts/min
- $^3$H cts/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.

- $^{32}$P cts/min
- $^3$H cts/min
- 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$_2$O. The solution was immediately mixed with $^3$H-phage DNA, layered on a neutral sucrose gradient, and centrifuged for 7 hours at 25,000 rev./min at 3°C.

0---0 $^{32}$P cts/min
X---X $^3$H cts/min
Figure 9. **Effect of mitomycin C pretreatment on $^3$H-thymidine incorporation and phage growth.** 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. *E. coli* AB2500 hcr⁻ b. *E. coli* 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 10. DNA and phage synthesizing capacity after MC treatment of hcr⁺ and hcr⁻ cells. E. coli AB2497 or AB2500 were treated with MC as described in Methods. After washing by filtration the cultures were resuspended in KG medium containing ³H-thymidine and aerated at 37° for 45 minutes. Acid insoluble ³H-thymidine incorporation in each sample was measured by TCA precipitation before and after hydrolysis with 0.5N 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⁻

ollect colony-forming ability, hcr⁺

3H-thymidine incorporation, hcr⁻

3H-thymidine incorporation, hcr⁺

□, before alkaline hydrolysis; ■, after alkaline hydrolysis, hcr⁻. 

Δ, before alkaline hydrolysis; ▲, after alkaline hydrolysis, hcr⁺.

X—X phage capacity, hcr⁺
Figure 11. $^3$H-thymidine incorporation into E. coli 3110 infected with $\lambda^+$, cII, and cI. E. coli 3110 was grown in K medium to $2 \times 10^9$ 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 multiplicity 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 $\mu$g 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 $\lambda c^+$ (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. H-thymine incorporation and phage growth in MC-treated E. coli AB2500 infected with λcI, λcII, or λcIII. Cells were grown to 2 x 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 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 13. *DNA synthesis in MC-treated E. coli AB2500 infected with λ in the presence of 0, 30 and 100 μg/ml. of chloramphenicol (CAM).* 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 KB 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 3H-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).

\[\begin{align*}
00 & \quad 0 \text{ μg/ml. CAM} \\
\square\quad & \quad 30 \text{ μg/ml. CAM} \\
\triangle\quad & \quad 100 \text{ μg/ml. CAM} \\
\times\quad & \quad \text{uninfected, 0 μg/ml. CAM}
\end{align*}\]
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.

\[ \begin{align*}
X -- X & \quad 3^H \text{ cts/min} \\
\Delta -- \Delta & \quad \text{helper-infective DNA} \\
\bigcirc -- \bigcirc & \quad \text{intracellular phage}
\end{align*} \]
Figure 14
Figure 15. Sedimentation analysis of vegetative λDNA at various stages of infection. Extracts of cells (containing the nucleic acids from about 4 x 10⁸ cells) prepared as described in Figure 10 were layered on 5-20% sucrose gradients containing (a-e) TE, pH 8, or (f) 0.3 M NaOH, 0.3 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.

\[
\begin{align*}
0 \quad & - \quad 0 & \quad ^{32}P \text{ parental DNA cts/min} \\
X \quad & - \quad X & \quad ^{3}H\text{-vegetative DNA cts/min} \\
\Delta \quad & - \quad \Delta & \quad \text{helper infectivity}
\end{align*}
\]
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 fraction 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 spheroplasts was added. The remainder of the bioassay was performed as described in Methods.

- □□□ marker $^{15}$DNA spheroplast infectivity
- X□X $^3$H cts/min
- △△△ vegetative iλDNA spheroplast infectivity
Figure 16
Figure 17. Sedimentation analysis of vegetative λDNA synthesized in the presence of CAM. 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 extracted 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.

```
O——0   parental $^{32}$P cts/min
X——X   $^3$H cts/min
△——△   helper infectivity
□——□   spheroplast infectivity
```
Figure 18. *Sedimentation analysis of vegetative DNA extracted at various times with or without phenol*. 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 e was for only 4-5 hours compared to 6 hours for the others.
Figure 18
Figure 19. CsCl equilibrium density gradient centrifugation of $^{32}\text{P}^{13}\text{C}^{15}\text{N}$-λ phage DNA and component I extracted 30 minutes after infection.

a. Phenol extracted DNA from purified heavy phage was mixed with E. coli $^3\text{H}$-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 pycnometrically as described in Methods.

b. and c. Component I was isolated after sucrose gradient sedimentation of extracts made 30 minutes after infection of CR34 with $^{32}\text{P}^{13}\text{C}^{15}\text{N}$-λc26 in light, nonradioactive medium. A density marker, $^3\text{H}$-E. coli DNA, was added before centrifugation. The results in (b) and (c) are from two similar experiments.

0—0 $^{32}\text{P}$ cts/min
X—X $^3\text{H}$ cts/min
□□□□ density
Figure 20. CsCl equilibrium density gradient centrifugation of $^{32}\text{P}^{13}\text{C}^{15}\text{N}$-components II and III, $^{32}\text{P}^{12}\text{C}^{14}\text{N}$-component I.

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 $^3\text{H-}E.\text{ coli}$ DNA had been added.

b. Component I obtained as described in Figure 14 but radioactive, light phage were used in the infection. It was mixed with CsCl and $^3\text{H-}E.\text{ coli}$ DNA before centrifugation.

\[
\begin{array}{ccc}
0 & 0 & 32\text{P cts/min} \\
X & X & 3\text{H cts/min}
\end{array}
\]
Figure 21. Sedimentation analysis of vegetative λDNA. E. coli 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/m mole. 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
Δ——Δ helper infectivity
Figure 22. CsCl equilibrium density gradient centrifugation of isolated component I. 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). $^3H$-E. coli DNA was added as a density marker to the latter sample before centrifugation since in this case the progeny DNA was not labeled with $^3H$. 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 scintillation counter.

a. Component I from "thymine starved" cells; $^{32}P$, $^{13}C$, $^{15}N$-parental DNA; E. coli DNA density marker. b. Component I, 10 minutes after addition of $^3H$-thymine. c. Component I, 15 minutes after addition of $^3H$-thymine.
Figure 23. Stability of HL DNA after alkaline denaturation. Components I and II were isolated from neutral sucrose gradients and centrifuged (separately) to equilibrium in neutral CsCl density gradients. The fractions containing HL₁ were pooled and dialyzed to remove CsCl, and then denatured with alkali and reneutralized as described in the text. Pooled HL₁ was treated identically. The reneutralized solutions were mixed with CsCl to bring the final density to approximately 1.71 g/cm³, 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 different experiments in which the vegetative DNA was labeled with ³H-thymine at different specific activities, hence the ratio ³²P/³H is different for HL₁ and HL₁. The parental DNA contained ³²P, ¹³C, and ¹⁵N.

a. Component II.  b. Component I.

X——X  ³H cts/min
0——0  ³²P cts/min
density
Figure 24. *Spheroplast infectivity associated with component I at various stages of infection*. 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).

Δ-----Δ 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 $^{32}\text{P}^{13}\text{C}^{15}\text{N}$. 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 $^3\text{H-E. coli}$ DNA, b2b5c phage DNA, and CsCl and centrifuged to near equilibrium. A sample of $^{32}\text{P}^{13}\text{C}^{15}\text{N}$-λc26 phage DNA was banded simultaneously. Fractions to be assayed in spheroplasts were first dialyzed to remove CsCl.

(a) $\text{X} \text{X} 3\text{H-E. coli DNA cpm}$

\[\text{b2b5c spheroplast infectivity}\]

(b) Phage DNA (λc26) $0 \text{ 0 32P cpm}$

\[\text{Spheroplast infectivity}\]

(c) Component I $0 \text{ 0 32P cpm}$

\[\text{Spheroplast infectivity}\]

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
REFERENCES FOR PART II


Part III

VEGETATIVE LAMBDA DNA: PULSE-LABELED COMPONENTS
1. INTRODUCTION

Two distinct modes of phage DNA replication have been proposed. The first, exemplified by \( \Phi X174 \), 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. \( \lambda \) DNA synthesis also involves circular replicative forms analogous to those found in \( \Phi X \)-infected cells (7). Smith & Skalka (23), however, suggested that the precursor to phage DNA was a concatenate of \( \lambda \) genomes. The possibility thus exists that \( \lambda \) DNA synthesis involves both types of "replicative forms".

Certain differences in DNA replication, however, must exist between \( \Phi X \) and \( \lambda \), on the one hand, and T4 and \( \lambda \) on the other. The synthesis of linear, double-stranded \( \lambda \) DNA from a circular template (that \( \lambda \) 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 \( \Phi X \) DNA. Whatever mechanism produces phage-size DNA molecules must differ in \( \lambda \) 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 \( \lambda \) 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 completed 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 relative 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 presented 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 ØX RF. As in ØX, 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) Preparation of spheroplasts and assay of fractions from gradients.

Typically, a 60 ml. culture of E. coli 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 0.05M tris, pH 8.1, or 0.05M 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 phosphate buffer such that the final molarity of phosphate is below 0.05M and the pH is 7 to 8.

(b) Outline of a typical pulse experiment

In all of the experiments presented in Results, E. coli 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) KC 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 ³H-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 infected 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 \( \lambda \) (as described in Methods) were pulsed with \( ^{3}H \)-thymine (at a specific activity of 2.6 c/mmol) for 60, 120, and 300 seconds starting at 10 and 35 minutes after infection at 37\(^{\circ}\)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 0.1M, 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 λDNA 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 ΦX 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) **Sedimentation analysis of pulse-labeled DNA**

(i) **At low ionic strength, pH 10.5**

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 molecules. Only one-half of each fraction from the gradients containing DNA labeled for 300 seconds was TCA precipitated and counted for radioactivity, 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 sedimentation 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 correspond 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 majority 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 infect-
ivity 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 $^{32}\text{P}}$-RF I (31), were admixed before centrifugation. The results are shown in Figure 3. The marker $^{32}\text{P}}$-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 difference 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 sedimentation 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) **Sedimentation analysis of pulse-labeled samples extracted without phenol**

 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) **Identification of pulse-labeled RF I by dye binding**

 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) **Synthesis of RF I, RF II, phage DNA and component X throughout the latent period**

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 continuously from 0 to 50 minutes. The total radioactivity incorporated in the pulses was 80% of the amount incorporated continuously, indicating 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, i.e. 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
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 $\lambda$DNA are the primary components which are synthesized initially after infection with $\lambda$. 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 "replicative 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 $\lambda$DNA. 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

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1The amount of RF I predicted is probably exaggerated because of contamination 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 correspondingly 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 \( \lambda \) 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 \( \lambda \) 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 (0.01M) 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 asynchrony 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 qualitatively 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 replication 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 ΦX 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 formation 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, polynucleotide 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, 0, 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 replicating 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 precursor 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 replication (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

¹Semi-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. Symmetry 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'→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 λ DNA 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 increases 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 distribution 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-conservative 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 0X, as has Dowell (5). According to the model one might expect that host mutants which do not convert 0X single strands to 0X RF would be able to replicate λ RF without producing phage DNA.
TABLE 1

Recovery of pulse-labeled DNA and the rate of synthesis of DNA and active phage

<table>
<thead>
<tr>
<th>Time</th>
<th>Early (10 min)</th>
<th>Late (35 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulse length (sec)</td>
<td>60  120  300</td>
<td>60  120  300</td>
</tr>
<tr>
<td>Total $^3$H-thymine incorporation (cpm)</td>
<td>54000  159000  540000</td>
<td>292000  500000  1070000</td>
</tr>
<tr>
<td>Recovery of $^3$H-DNA after collecting cells (%)</td>
<td>65  57  71</td>
<td>34  41  48</td>
</tr>
<tr>
<td>Recovery of $^3$H-DNA after phenol extraction</td>
<td>50  45  43</td>
<td>59  51  41</td>
</tr>
<tr>
<td>DNA equivalents/IC</td>
<td>1.8  5.3  18</td>
<td>9.5  16  35</td>
</tr>
<tr>
<td>Active phage/IC</td>
<td>-  -  -</td>
<td>1.8  3.6  9</td>
</tr>
</tbody>
</table>

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 $2 \times 10^{-4}$ 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 extraction are based on the amount recovered after collection of the cells. The recovery is enhanced in (b) by the addition of sodium trichloroacetate to the lysate (final concentration 0.1M) prior to phenol extraction.
Figure 1. Sedimentation properties of vegetative ADNA labeled with \(^3\text{H}\)-thymine for 60 and 120 seconds at 10 and 35 minutes. One-half ml. portions of the lysates (phenol extracted, Table 1 a) were mixed with \(^{32}\text{P}\)-b2b5c phage DNA and layered on sucrose gradients containing TE, pH 10.5, and centrifuged for 5 hours at 25000 rev./min and 6\(^\circ\)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 \(^3\text{H}\) channel were not corrected for a 2% overlap from the \(^{32}\text{P}\) 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 \(^3\text{H}\) pulse-labeled DNA layered on the gradient: 60 seconds, early, 100%; 60 seconds, late, 71%; 120 seconds, early, 75%; 120 seconds, late, 71%.

\[ \begin{align*} 
&\text{X—X} \quad ^3\text{H} \text{ cpm} \quad 0—0 \quad ^{32}\text{P} \text{ cpm} 
\end{align*} \]
Figure 2. Sedimentation and biological properties of the DNA extracted from 300 second pulse-labeled infected bacteria. 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 fractions (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 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 0.5N NaOH to 0.2 ml. of each fraction. After 15 minutes at 37° 0.60 ml. of 0.05 M tris, pH 8.1, 0.05N NaH₂PO₄, pH 4.0 was added. The final pH was about 7.5. The recoveries of ³H layered on the gradient were:

<table>
<thead>
<tr>
<th>X—X</th>
<th>³H cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>O—O</td>
<td>³2P cpm</td>
</tr>
</tbody>
</table>

Δ—Δ helper infectivity

▌▌▌▌ spheroplast infectivity, native
▌▌▌▌ spheroplast infectivity, denatured
Figure 3. Sedimentation of late 300 second pulse-labeled DNA at low and high ionic strength. 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 (0.01M tris, pH 10.5, 0.001M EDTA). Recovery: $^3$H, 65%; $^{32}$P, 69%.

(b) 5-20% sucrose in TE, pH 8.1, 2.0M NaCl. Recovery: $^3$H, 63%; $^{32}$P, 71%.

X——X $^3$H cpm 0——0 $^{32}$P cpm
Figure 3
Figure 4. 

CsCl equilibrium density gradient centrifugation of early and late pulse-labeled DNA in ethidium bromide. Portions of the lysates to be analyzed were mixed with $^{32}$P-phage DNA, $^{32}$P-RF I, an aqueous solution 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°C). 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% overlap from the $^{32}$P channel.

\[ X\ldots X \quad ^3H \text{ cpm} \quad O\ldots O \quad ^{32}P \text{ cpm} \]
Figure 4
Figure 5. **Cumulative synthesis of λ-specific DNA components predicted from sequential 5 minute pulses.** Equal portions of lysates from 5 minute pulse-labeled cultures and also from a culture labeled continuously 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 determined at five minute intervals. (The 0-5 and 20-25 minute samples were lost.)

Region I: RF I

Region II: RF II and component X (all material sedimenting between phage DNA and the RF I position)

Region III: Phage DNA (radioactivity which co-sedimented with the phage DNA marker)
REFERENCES FOR PART III


29. Young, E. This Thesis, Part I.

30. Young, E. This Thesis, Part II.

31. Young, E. This Thesis, Part IV.
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 0.01M Na₃citrate-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 ± 0.05 pH units, in the range pH 12-13.
Buffers for alkaline titration of DNA are prepared by mixing 5.0 ml. of 0.20M K₂HPO₄, x ml. of 1.0M KOH, and H₂O 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.

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.

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 x 10⁹ cells/ml. in K medium (unless stated otherwise) at 37°C 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 un.injected phage are removed from the bacteria by blending at full speed in a Waring Blender, 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 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 l dissolved in 0.25M 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 con-
taining 1.1 ml. of 0.1 M MgSO$_4$ and 1.5 ml. of 5% (w/v) Brij (in 0.01 M
tris, pH 7.2) is added with mixing. The viscous solution is transferred
to a polycarbonate screw cap centrifuge tube and centrifuged for 15
minutes at 30,000 rev./min in a Spinco 30 rotor. The non-viscous super-
natant 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 χDNA.

(1) NaCl is added to 1.0M and the solution is stored in the refrigerator
overnight. The precipitated RNA is removed by centrifugation and dis-
carded. DNA does not precipitate in 1M NaCl. The solution is diluted
to 0.4M NaCl with H₂O and passed through a methylated albumin-Kieselguhr column, previously washed with 0.4M 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 1.0M NaCl gradient (buffered with 0.05M sodium phosphate, pH 6.7). Fractions containing DNA, which elutes at 0.75M NaCl, are combined and dialyzed versus 0.01M tris, 0.001M 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 0.01M Na₃ citrate, 0.02M 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 thoroughly). The pH is raised to 8 with KOH.

(3) A step gradient of CsCl in 15% sucrose containing 0.04M 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 concentrates 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 λDNA; 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/cm³ 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 intercalated 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 $^{32}\text{P}$-intracellular $\lambda$ DNA and $^3\text{H}$-E. coli DNA

Each E. coli cell contains an amount of DNA equivalent to about 100 - 300 $\lambda$ genomes, depending on the number of bacterial chromosomes. 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 prepare E. coli polyribosomes which are released from the cell by gentle lysis. In order to test the efficacy of this method for separating intracellular $\lambda$ DNA from the bulk of the E. coli DNA, the experiment described below was performed.

E. coli 3110($\lambda$) was grown to a cell concentration of $1 \times 10^8$ cells/ml. in 20 ml. of K medium at 37°C. Deoxyadenosine at a final concentration of 250 $\mu$g/ml and 0.1 mc of $^3\text{H}$-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 incorporation of exogenous thymidine in a non-thymine-requiring host (2). When the cell density had reached $2.7 \times 10^9$ cells/ml., $^{32}\text{P}$-$\lambda$c26 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 $^3\text{H}$ and $^{32}\text{P}$ 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 0.2M 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 $^3\text{H}$ and $^{32}\text{P}$ activities in the pellet and supernatant fractions. The results are shown in Table 1. The recovery of $^{32}\text{P}-\lambda$ DNA in the supernatant was 31%. About 99.6% of the $^3\text{H}-\text{E. coli}$ DNA was in the pellet fraction.

The specific activity of the $^3\text{H}-\text{E. coli}$ DNA in the pellet, determined from the amount of $^3\text{H}$ in that fraction and the amount of DNA determined chemically by the indole procedure (13), was $1.7 \times 10^4$ cts/min/µg DNA. The specific activity of the $^{32}\text{P}-\lambda$ DNA was calculated to be $6.4 \times 10^4$ cts/min/µg DNA from the $^{32}\text{P}$ content and the absorbance at 260 µν of the phage preparation. Using these values and the total amount of $^3\text{H}$ and $^{32}\text{P}$ recovered in the pellet plus supernatant fractions, the ratio of bacterial DNA to $\lambda$DNA before centrifugation of the lysate was 10.4µg: 1.0 µg. The ratio in the phenol extracted supernatant after centrifugation was 0.065 µg E. coli DNA : 1.0 µg $\lambda$DNA, a purification relative to E. coli DNA of 160-fold.\textsuperscript{1} The phenol extracted supernatant contained 8.8 µg of $\lambda$DNA and 0.57 µg of bacterial DNA. That is, 93% was phage DNA.

\textsuperscript{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 \( \lambda \) 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 \( \lambda \) 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 \( \lambda \) DNA, analogous to \( \Omega X \) 174 RF (4, 18) or polyoma viral DNA (24) is substantiated by direct observation.
(b) Separation of closed circular \( \lambda \) DNA from linear and open circular DNA

Preparations of intracellular \( \lambda \) DNA almost (93%) free of \( E. \ coli \) DNA can be obtained by the methods just described. However, these preparations still contain three species of \( \lambda \) DNA: linear, open-circular, and closed-circular molecules. 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 intracellular components of \( \lambda \) 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) A note on the recovery of closed-circular DNA

Starting with \( 10^{14} \) phage particles (100 \( A_{260} \) units or 5 mg of DNA) which would be used to infect a 5 liter culture, the best recovery obtained has 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 degradation 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 be 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 $30 \times 10^6$ daltons of DNA would degrade 63% of $\lambda$ component I but less than 10% of ØX RF I.

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

The circular intracellular forms of $\lambda$ DNA are infective in a spheroplast assay (26). After alkaline denaturation of extracts containing intracellular $\lambda$ DNA a reproducible but variable increase in the infectivity is observed. This increase was originally attributed to a loss of the ordered secondary structure of component I without concomitant separation of the strands, resulting in an increased ability to infect spheroplasts. Double-strandedness seemed to be a prerequisite to infection with $\lambda$ DNA since denatured phage DNA was not infective (or was much less so than native DNA) in either the helper assay (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 experiments to be described below have no further bearing on the denaturation of component I since they indicate that the increased infectivity observed 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\textsuperscript{b5}) as a function of pH. The midpoint of the transition is pH 11.8 ± 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 $^{3}$H-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 ($^{1}b_{5}$) on the gradient containing denatured intracellular DNA are present in two peaks. The density difference between the marker, native $^{3}$H-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 sedimentation 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 referred 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 32p-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/cots/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 0.05M K₃PO₄ 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 0.03M 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 component 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 λ DNA. It has been known for some time that circular, but not linear single-strands of viral ΦX 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 ΦX 174 have been studied by Jaenisch, Hofschneider & Preuss (10) with conclusions similar to those proposed above.
Table 1

Recovery of Intracellular \( \lambda \) DNA in the Godson Procedure

<table>
<thead>
<tr>
<th></th>
<th>cpm/50( \mu l ) ( ^{3} \text{H-E. coli} ) DNA</th>
<th>cpm/50( \mu l ) ( ^{32} \text{P-\lambda DNA} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysate supernatant</td>
<td>294</td>
<td>7,700</td>
</tr>
<tr>
<td>Lysate pellet</td>
<td>67,980</td>
<td>17,000</td>
</tr>
<tr>
<td>Phenol extracted supernatant</td>
<td>97</td>
<td>5,660</td>
</tr>
<tr>
<td>Phenol extracted pellet</td>
<td>26,202</td>
<td>11,500</td>
</tr>
</tbody>
</table>
Table 2

Specific Infectivities of \( \lambda \) - DNA

<table>
<thead>
<tr>
<th>Component</th>
<th>State</th>
<th>Specific Infectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Component I</td>
<td>native</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>denatured</td>
<td>1.6</td>
</tr>
<tr>
<td>Component II</td>
<td>native</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>denatured</td>
<td>14.7</td>
</tr>
<tr>
<td>Phage DNA</td>
<td>native</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>denatured</td>
<td>0.041</td>
</tr>
</tbody>
</table>
PLATE I

Purified intracellular lambda DNA. (a) X 25,500  (b) X 16,000
PLATE II
Annealed phage DNA. (a) and (b) X12,900
Figure 1. Separation of RNA and $^{32}\text{P}$-intracellular $\lambda$DNA by chromatography on methylated albumin-Kieselguhr. The nucleic acids prepared by method 1 were passed through a methylated albumin-Kieselguhr column using a linear 0.4-1.0 M NaCl gradient. Portions of each fraction were put on planchets, dried, and counted in a Nuclear Chicago Low Background 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 $^{3}\text{H-}E.\text{ coli}$ DNA present. The NaCl molarity was determined from the refractive index.

0——0 NaCl molarity  --- $^{32}\text{P}$ cpm  ----- $A_{260}$
Figure 2. Alkaline titration of the biological activity of intracellular λ DNA and phage DNA. 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 alkaline 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 0.05M tris, pH 8.1. Phage DNA (λb2b5c) was denatured in the same buffers and assayed identically.

0—-0  iλ intracellular DNA

0—-0  i b5  phage DNA
Figure 3. Distribution of spheroplast infectivity of native and denatured intracellular λ DNA after centrifugation in a CsCl equilibrium density gradient.

(a) λ b2b5c intracellular DNA was denatured with NaOH, pH 12.2, for 15 minutes at 25° and then neutralized with NCl. Native 3H-i λ DNA was added and the density raised to 1.71 g/cm³ 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 µl. of the CsCl solution. Regions of the gradient not shown did not contain significant amounts of radioactivity.

\[ \text{3H cpm} \text{ λ phage DNA} \quad \text{X-----X infectivity, λ phage DNA} \]
\[ \text{32P cpm, intracellular} \quad \text{○○○○ infectivity, b5 intracellular DNA} \]

(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.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>X—X</td>
<td>$^3$H cpm, phage DNA</td>
</tr>
<tr>
<td>0—0</td>
<td>$^{32}$P cpm, intracellular DNA</td>
</tr>
<tr>
<td>△—△</td>
<td>infectivity, native intracellular DNA</td>
</tr>
<tr>
<td>•—•</td>
<td>infectivity, denatured intracellular DNA</td>
</tr>
</tbody>
</table>
Figure 3

Fraction 14 to 39

DENSITY 1.79
CPM 32P 1.77

Spheroplast Infectivity, pfu per Tube

p5

Native

Denatured

10^4
10^2
10^1
10^0
10^-1
Figure 4. Distribution of $^{32}$P-DNA (●—●) and infectivity (X—X) after sedimentation through a 5-20% sucrose gradient containing 0.05M K$_3$PO$_4$ and enough KOH to make the pH 12.2. The samples were denatured prior to centrifugation by incubation at 37° for 15 min in 0.2N 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
REFERENCES FOR PART IV


27. Young, E. This thesis, Part II.

28. Young, E. This thesis, Part III.