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CHROMOSOMAL REPLICATIVE FORMS OF P1 AND P1dlac
DURING THE LYTIC CYCLE

by

John W. Bornhoeft

A Dissertation Submitted to the Faculty of the Graduate School
of Loyola University of Chicago in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy

May

1979

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VITA

John W. Bornhoeft was born in Lakewood, Ohio on April 24, 1943. He attended New Trier Twp. High School in Winnetka, Il., graduating in 1961. He received his Bachelor of Arts from Beloit College in 1965 and then did post-baccalaureate work in Anatomy and Physiology at Indiana University from 1965-1967. Virological problems were pursued as a Research Assistant in the Infectious Disease Laboratory, Department of Medicine, University of Illinois Medical School from 1967-1969.

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Ph.D studies in the Department of Microbiology at Loyola University of Chicago were begun in the fall of 1972. His dissertation research has been sponsored by Marvin Stodolsky, Ph.D. since early 1974.

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LIST OF ABBREVIATIONS

coef. -----coefficient

D-----distant

DS-----Direction of Sedimentation

EB-----Ethidium bromide

EDTA-----ethylenediamine tetraacetic acid

Escherichia coli (E. coli) genetic

functions and loci --

argF-----ornithine carbamoyltransferase

argI-----ornithine carbamoyltransferase

his-----histidine biosynthesis

lac-----lactose operon

mal-----maltose catabolism

metB-----cystathionine synthetase

proAB-----glutamate to glutamic semialdehyde

xyl-----xylose catabolism

G--a genome unit of single strand or duplex DNA

IEC---International Equipment Co.

IS1---insertion sequence 1, an 800 base pair deoxyribonucleotide sequence promoting chromosomal rearrangement

h-----hour

LCF---Lytic cycle chromosomal forms

M-----molecular weight

min-----minute

m.o.i.---multiplicity of infection

N.D.-----not detected

O.D. unit---optical density unit (absorbance)

PEG-----polyethylene glycol 6000

Pl-----genetic functions and loci-----

ban-----plasmid replication

cat----- IS1 sequence

cl-----lytic cycle repression

hfp-----origin of headful packaging

lys-----lysis

strA-----streptomycin resistance

sul-----sulfonamide resistance

tet-----tetracycline resistance

vir-----superinfection immunity

RSD-----relative sedimentation distance

S-----sedimentation coefficient

saccosyl-sodium lauroyl sarcosinate-30

sec-----second

soln-----solution

SSC-----standard sodium citrate

SDS-----sodium dodecyl sulfate

TCA-----trichloroacetic acid

TRIS-----tris (hydroxymethyl) aminomethane HCl

UV-----ultra violet light

INTRODUCTION

The temperate bacteriophage $\Phi 1$ was first isolated by Bertani (5) in 1951 from a lysogenic *Escherichia coli*. $\Phi 1$ virion populations contain three capsid size variants, $\Phi 1M$, (minute), $\Phi 1S$ (small), and $\Phi 1B$ (big) (60). Walker and Anderson (60) demonstrated that though the capsid size of the $\Phi 1$ virions does differ, their tail structures are indistinguishable. Only $\Phi 1B$ virions have plaque forming ability, while $\Phi 1B$ and $\Phi 1S$ are able to inject DNA into the host bacterium (60).

The genetic map of $\Phi 1$ bacteriophage is linear (47,59), a feature that has recently been found to be due to a site specific recombination function (51). The chromosomes from $\Phi 1B$ virions have a molecular weight of 66×10^6 daltons and contain a 12% terminal genetic repetition. The $\Phi 1$ virion chromosomes were formerly thought to be randomly permuted (1,29). However, Bachi and Arber (2) determined that about one third of the $\Phi 1$ virion chromosomes have one end at map unit 92, and only two thirds of the $\Phi 1$ virion chromosomes are comprised of a random, genetically permuted population (49). This permuted characteristic suggests that the virion chromosomes mature through the action of a headful packaging system (28) with encapsidation being initiated at map unit 92 for a third of the virion population (2,68).

During bacteriophage infections the chromosome is injected and a lytic cycle may ensue. When vegetative replication is repressed, and lysogeny is established, the $\Phi 1$ prophage persists as a plasmid

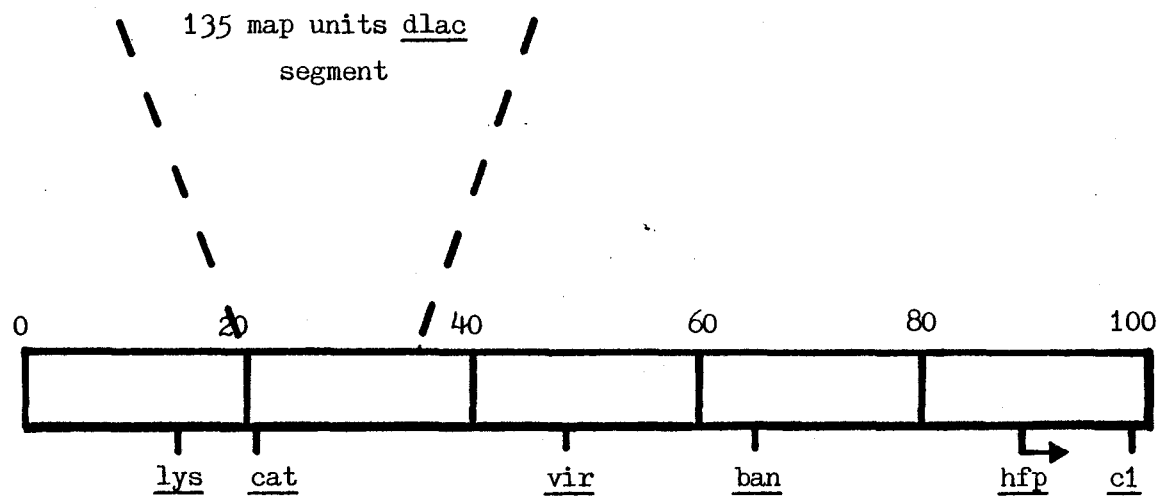
(1,30). The plasmid has a supercoiled 61×10^6 dalton chromosome which is presumably generated through a recombination of the ends of the terminally repetitious bacteriophage chromosomes (29).

The P1 plasmid usually does not integrate into the host chromosomes. However, since P1 virions mediate generalized transductions, P1 plasmid may on rare occasions integrate into the host chromosome. Alternatively, its headful packaging system may, on rare occasions, act on the host chromosomes during phage maturation (35). Luria *et al.*, (36) discovered that specialized lac⁺ transducing derivatives were sometimes generated during low frequency transduction processes. Rae and Stodolsky (42) subsequently determined that some P1dlac chromosomes are overlarge (too large to be encapsidated intact in a P1B capsid) and tend to overproduce small head (P1S) virions during the lytic cycle. It was established that the P1dlac plasmid of transductant PL0100 has a molecular weight of 133×10^6 daltons (6), which corresponds to 220 P1 map units; the P1dlac is missing 15 map units of P1 genes which have been replaced by about 135 map units of E. coli genes (68) (Figure 1).

Foreign genes of specialized transducing particles of P1 phage include cat (34), argF (52), pro (45,52), tet (38,48), and cat sul str (27). With the exception of genetic elements which are themselves transposons, these foreign genetic elements each have one site of union with P1 at the cat locus (68). The P1 cat site was demonstrated to encode the insertion sequence IS1 (68). Consequently, it was discovered that the P1 gene deletions of a P1dlac also extend from the cat site at 20 map units to approximately the 35 map

Figure 1. Genetic map of bacteriophage P1. One hundred physical map units corresponds to 61×10^6 daltons (29) or 92 kilobases (69). P1dlac has a 135 map unit segment of E. coli genes replacing a 10-15 map unit segment of the P1 chromosomes (68).

Abbreviations designating loci controlling P1 functions are: lysis (lys); an IS1 locus (cat); superinfection immunity (vir); plasmid replication (ban); origin of headful packaging (hfp) with the direction indicated; and lytic cycle repression (cl).



unit location (Figure 1) (68).

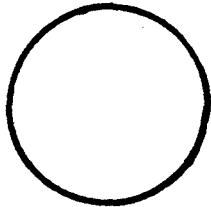
From an analysis of virion properties, Rae and Stodolsky (42) determined that the *Fl* gene deletions of *Fldlac* plasmid diminished the efficiency of virion production during the *Fldlac* lytic cycle. They, therefore, suggested that *Fl* and *Fldlac* lytic cycle intermediates might differ. They proposed that *Fldlac* plasmid has a block in the concatemer forming process (42). The proposed genetic block might be a cis or a trans dominant or recessive genetic defect. A cis dominant block could affect only the chromosome in which the mutation is encoded. A trans dominant block would affect a chromosome not containing the mutation. A recessive trait would be extinguished, or the phenotypic expression suppressed when a genetically complementing chromosome is present in the same bacterium.

The primary purpose of this research was to characterize *Fl* and *Fldlac* lytic cycle chromosomal forms (LCF). The LCF of several other phage systems have previously been determined. However, in the majority of cases the unit genomes were not as massive as those of the *Fl* and *Fldlac*.

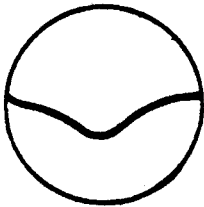
The exceptions are the T even-phages which have chromosomes in the $130-160 \times 10^6$ dalton range (25). These phages degrade host chromosomes so that the infected cells contain only phage LCF. T4 is the most studied T even phage and was found to have a duplex linear DNA that is permuted (53,57) and terminally repetitious (53). During lytic cycle replication T4 is replicated by generalized recombination (4,17,64) (Figure 2G), firewheel replicative forms (4, 20,63,64) (Figure 2E), and/or multi forked rolling circle replication

Figure 2. Some chromosomal forms which might be generated during a bacteriophage P1 lytic cycle (18,20,57,63).

A
Monomer



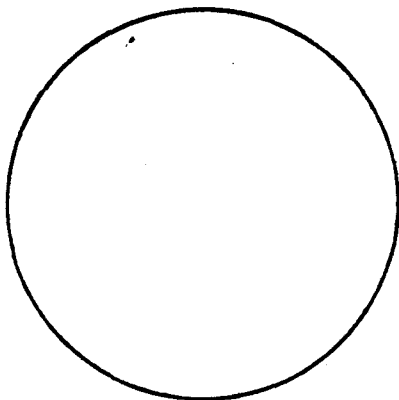
C
Theta



G
Linear



H
Concatemer
(Recombination)



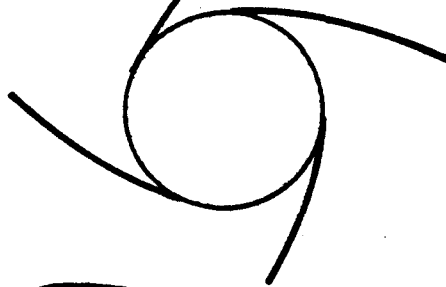
B
Multiforked Rolling Circle



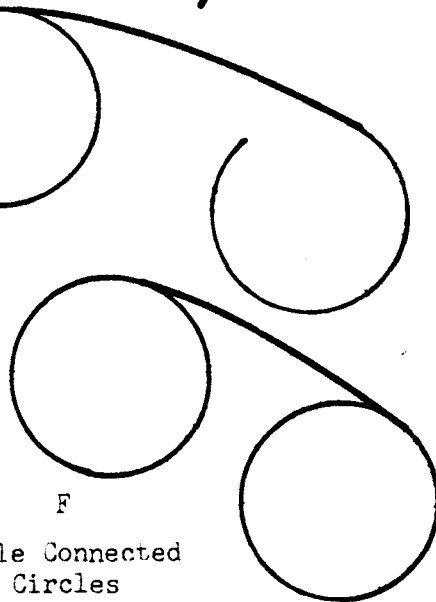
D
Rolling Circle



E
Firewheel



F
Double Connected
Circles



(18) (Figure 2B). Recombination between homologous regions of the T4 chromosomes promotes some strand elongation. Recombinants also form when the end of one molecule recombines with an internal homologous region of another molecule (17). Werner (64) postulated that intramolecular recombination occurred between branches of rolling circle or firewheel replicative forms (Figure 2F). Whatever the form of T4 replication, the concatemers formed do not exceed two unit genomes in size (64) probably because headful packaging is concomitant with chromosomal replication.

Bacteriophages lambda and T7 have chromosomes shorter than that of P1. Their chromosomes are non-permuted and have repetitious ends (57). Both phages have been shown to circularize on entering the host cell and to generate concatemers during the lytic cycle (16,39,57,58,62). The concatemers generated during the lambda phage lytic cycle are tails of rolling circle replicative forms (16,39,58,62) that do not exceed 2-6 unit genomes in size (62). They are preferred substrates for the capsid packaging system (16). T7 and lambda phages vary in the final processing of the virion DNA. During the packaging process, a lambda packaging nuclease makes staggered cuts at specific nucleotide sequences that generate the non-permuted repetitious DNA molecules with single strand staggered ends (24,37); the T7 packaging nuclease cuts in a staggered manner at a specific nucleotide sequence, but DNA polymerase uses the longer DNA strand as a template for a shorter strand synthesis that yields non-permuted and terminally repetitious chromosomes with duplex ends (37).

Bacteriophage P22 and P1 have several similarities. Both phages are temperate and their chromosomes are duplex, linear, permuted and have repetitious ends (57). However, P22 has a molecular weight of only $26-27 \times 10^6$ daltons (7). Both bacteriophages have linear genetic maps (7,59,68) and are circularized after entering the host bacterium through recombination of their repetitious ends (7,29). The LCF of P22 have been characterized. P22 generates concatemers by means of a rolling circle replicative form (Figure 2B) and/or generalized recombination (7,20) (Figure 2G-H). Like P1 (29), concatemers are processed into virions by a headful packaging system (7). The size of the LCF concatemers is thought to be in the 2-5 unit genomes size but certainly does not exceed 10 unit genomes in size (7). The P22 concatemers appear only late in the lytic cycle (7). An accumulation of supercoils is observed later in the lytic cycle, however, these supercoils are infrequently used as virion DNA precursors (57).

The lytic cycle intermediates of P1 could, in principle, include any of the replicative forms found for the above bacteriophages and shown in Figure 2. Sedimentation velocity gradients provide one means of characterizing lytic cycle intermediates. (15, 43,66) The most compact form of DNA is the covalently closed supercoil. A supercoil sediments 1.8 times faster than linear DNA of the same mass in a neutral sucrose gradient (12). All other chromosomal forms of the same mass are expected to have intermediate relative sedimentation rates. Thus, neutral sucrose gradients are useful for assessing the mass of a lytic cycle form irrespective of

its molecular conformation.

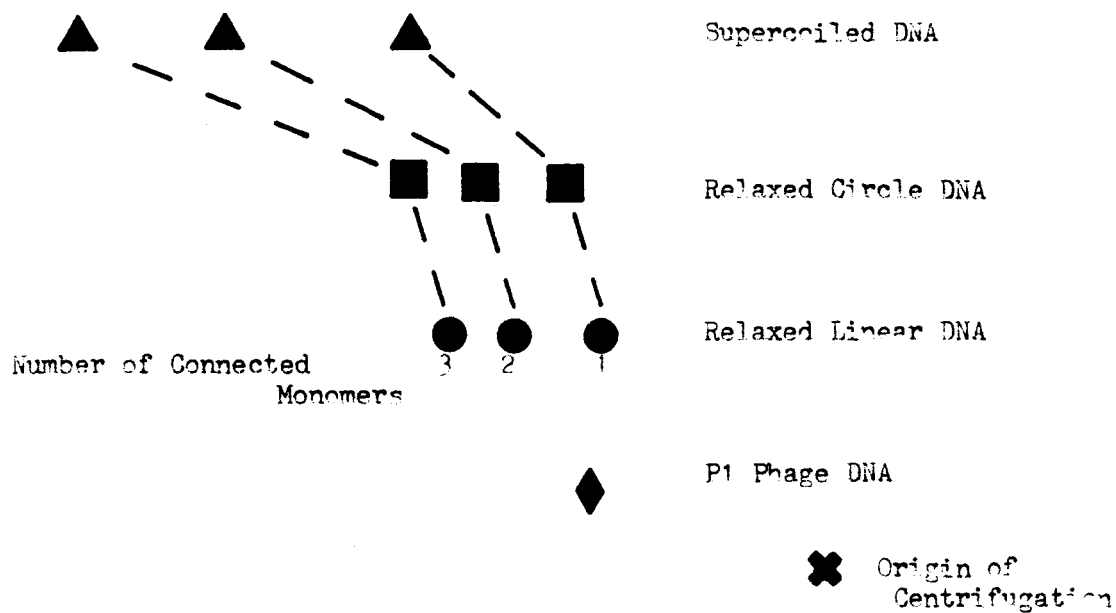
Upon denaturation by alkali treatment, all chromosomal forms except supercoils are separated into constituent strands (12). Because of its double covalently closed structure, strands of a supercoil are intertwined. This denatured complex retains the native mass and sediments 3-4 times faster than free constituent single strands (12). Figure 3 shows the relative positions of single strand and supercoiled chromosomal forms expected upon isokinetic sedimentation. Single strand concatemers up to several unit genomes long sediment less rapidly than the monomeric supercoil. This sedimentation system accordingly provides a means for unambiguously recognizing single strand concatemers sedimenting more slowly than monomer supercoils.

Isopycnic and rate zonal centrifugation (isokinetic) were used to study the lytic cycle intermediates of Pl and Pl_{dlac} plasmids. Svedberg and Pedersen developed the theory of macromolecular sedimentation (55). They outlined the physical properties that affect the sedimentation rate or coefficient of any moiety being centrifuged. The sedimentation velocity per unit of centrifugal force is designated the sedimentation coefficient and is expressed in Svedberg units ($1 \text{ S} = 10^{-13} \text{ sec.}$).

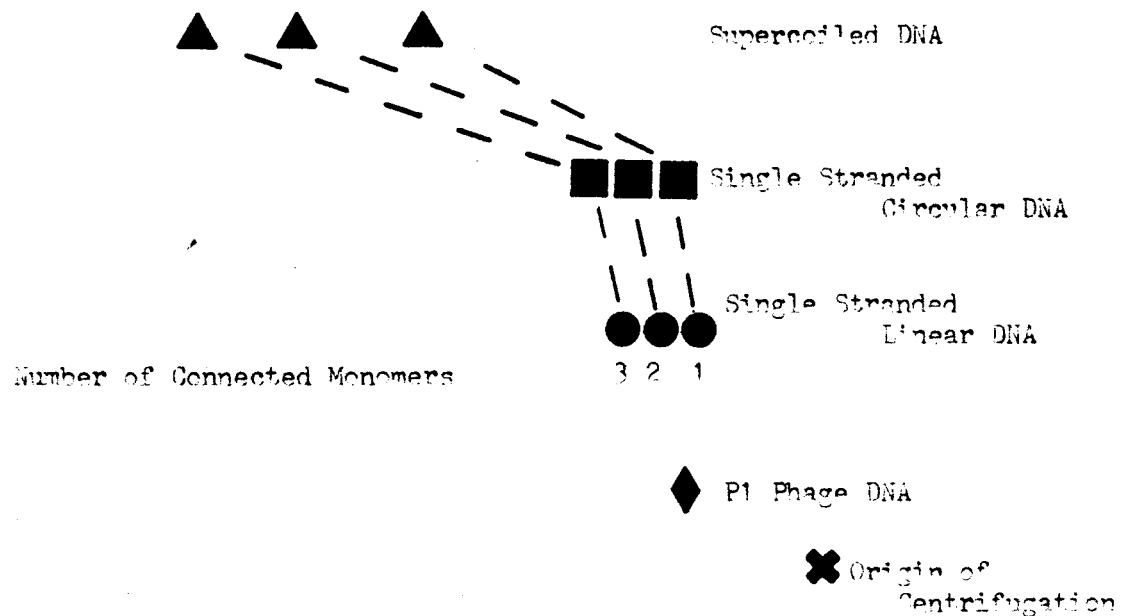
Burgi and Hershey (8) developed sucrose gradient sedimentation as a technique for the comparison of the relative sedimentation rates of different DNAs. They demonstrated that in linear sucrose gradients at constant centrifugal force, the sedimentation rate is a constant. Thus $S_1/S_2 = D_1/D_2$, where D is the distance sedimented

Figure 3. Relative sedimentation distances of P1 chromosomal forms in neutral and alkaline linear sucrose gradients. To aid in the interpretation of isokinetic sedimentation gradients, a theoretical sedimentation scale was constructed. The scale depicts the distances sedimented by linear, circular, and supercoiled P1 chromosomal forms in alkaline and neutral linear sucrose gradients. In all the isokinetic sedimentation experiments bacteriophage P1 chromosomes were used as the sedimentation standard. The theoretical sedimentation points were calculated through the use of two formulas. The first, $\frac{S_1}{S_2} = \frac{d_1}{d_2}$ (54,56), is theoretically valid for any two macromolecules in an identical linear sucrose gradient. The second, $\frac{S_1}{S_2} = \left[\frac{M_1}{M_2} \right]^{\text{coef.}}$ (44,54,61), is an empirical formula valid for a particular chromosomal configuration with the coefficient dependent on the molecular form of the DNA under study. The scale expands linearly from the origin for increasing distances sedimented by the P1 phage DNA standard. Sedimentation is from right to left.

NEUTRAL SEDIMENTATION



ALKALINE SEDIMENTATION



by the appropriate DNA molecule (56). Studier (54) has shown that $S_1/S_2 = (M_1/M_2)^{\text{coef.}}$ (where M_1 and M_2 represent the molecular weight of two different DNA molecules sedimented under identical conditions) (44,61). It can, therefore, be stated that $S_1/S_2 = D_1/D_2 = (M_1/M_2)^{\text{coef.}}$ (56). Kavenoff has shown that these relationships are accurate to at least molecular weights of 10^9 (32).

Relative sedimentation coefficients have been determined for the different chromosomal forms of DNA (12). Assigning duplex linear DNA a relative coefficient of 1.00 in a neutral sucrose gradient, open circular DNA will have a relative sedimentation coefficient of 1.14, and supercoils will have a relative sedimentation coefficient of 1.8 under identical conditions (12,29,62).

If the same conditions of sedimentation are used at an alkaline pH, linear single strand DNA has a relative sedimentation coefficient of 1.14, circular single strand DNA has a relative sedimentation coefficient of 1.28, and supercoiled DNA has a relative sedimentation coefficient of 3-4 (12). For a particular DNA molecular form empirical formulas relating S (Svedburg units or sedimentation coefficient) and molecular mass (M) have been obtained. These formulas take the form $S = b_s + k_s M_s^a$ (19) where b_s, k_s and a are constants. The value of the constants (b_s, k_s and a) has been determined by experimentation and applied to the various empirical formulations. These empirical formulas are $S_{20w}^0 = 2.8 + 0.00834M^{0.479}$ (12,26) for linear duplex DNA; $S_{20w}^0 = 2.7 + 0.1759M^{0.445}$ for open circle DNA; and $S_{20w}^0 = 7.44 + 0.00243M^{0.58}$ for supercoils (12,26); for linear denatured DNA,

$S_{20w}^0 = 0.0528M^{0.40}$ (54). This value was multiplied by a factor of 1.14 for circular molecules (10) and by 3-4 for supercoils in an alkaline gradient (12).

To identify Pl chromosomes among the sedimented and fractionated DNAs, nucleic acid filter hybridizations have been employed. Under the appropriate conditions of ionic strength, pH, and temperature, single DNA strands will anneal to complementary DNA strands, thereby reforming duplex DNA with very little aggregation of genetically heterologous DNA. With the Denhardt method (14) an excess of nonradioactive single strand probe DNA is attached to nitrocellulose membrane filters, and then the remaining DNA binding sites on the membrane filters are blocked. When a mixture of single strand DNA is annealed with such "DNA filters", the filter only binds complementary "target" sequences from the mixture. The binding can be quantitated by measuring the fraction of the total input radioactive DNA mixture bound to the filter. This method allows determination of the DNA species in any particular portion of a sedimentation gradient. As long as the probe DNA is in high molar excess and annealing goes to completion, the Denhardt method permits the simplest genetic identification of the sedimented and fractionated DNAs.

Ethidium bromide intercalates between the bases of DNA in the Watson-Crick configuration partially untwisting the helix and, thus, decreasing its density. The amount of ethidium bromide intercalation is least for supercoiled form I. The covalently closed structure of form I limits the untwisting of the Watson Crick helix which

is necessary for EB intercalation. Relaxed forms II and III bind more ethidium bromide (3). Thus, there is a differential decrease in density which allows a separation of supercoils and relaxed DNAs by isopycnic fractionation in the presence of EB (3). A modified CsCl-EB isopycnic fractionation method was used in this research to isolate P1 and P1dlac supercoils and study their accumulation during the lytic cycle.

MATERIALS AND METHODS

Bacterial Strains:

Four bacterial strains were used in the course of these investigations.

1. E. coli K-12 SC1800 F⁻ proAB-lac deletion XIII argI xyl
mal strA his metB
2. E. coli K-12 SC1801 is SC1800 lysogenic for Plcl. 100ts.
3. E. coli K-12 HfrH proAB-lac deletion XIII (Plcl. 100dlac)
4. E. coli K-12 HfrH proAB-lac deletion XIII (Pl).

The Pl and Pl_{dlac} prophages have heat labile cl repressor functions controlling lytic cycle induction (52). The lytic cycle was induced by shifting the incubation temperature of the lysogenic bacterial cultures from 32° to 40°C.

Media:

TY broth was the medium used to grow the bacteria in this study. TY broth consisted of 10g/l tryptone (Difco) (19). The pH of the medium was adjusted to pH 7.0-7.4 using 1.0 M NaOH (Sigma) or 1.0 M HCl (Fisher).

When phage titrations were performed, LB agar was used. LB agar was made with 10 g/l tryptone, 5 g/l NaCl, 5 g/l yeast extract, and 10 g/l agar (Difco). LB broth was made in the same manner except agar was not added to the medium. The media were made pH 7.2-7.4 (52). Soft nutrient agar was the same as LB agar except the agar concentration was 0.5%.

Bacterial Growth and Harvesting:

Bacteria were grown in a constant temperature shaker (Lab-Lines Instruments, Inc., Model 3595) at 32°C. When lytic cycle induction was desired, the bacterial cultures were shifted to 40°C in a gyrotory water bath shaker (New Brunswick Scientific Co., Inc., Model G79). Bacterial growth and/or phage lysis was monitored on a photoelectric colorimeter (Klett Mfg. Co., Model 900-3).

Bacteria were grown in exponential phase in TY broth to titers of 2×10^8 bacteria/ml before harvesting or lytic cycle induction.

Bacterial growth was stopped by chilling the cultures at 0°C and adding NaCN (Mallinckrodt) to a final concentration of 0.05 M. The NaCN served to inhibit energy requiring metabolic processes of the bacteria, in particular further DNA replication. The cultures were then centrifuged at 10,000 x g for 10 min at 4°C in an IEC A-147 angle head rotor. The pellets were then well drained and prepared for protoplast formation. A wash step was omitted, since the time involved was found to promote premature lysis.

When phage titrations were performed, 5×10^7 SC1800 bacteria were grown in LB broth to exponential phase, embedded with the appropriate phage in soft nutrient agar on LB agar plates.

Bacterial Lysis:

Bacterial protoplast formation and lysis were performed by combining two lysis techniques (46,58). This procedure was necessitated by a possible failure of Fl and Pldlac LCF to separate from membrane complexes, under the very same conditions found successful with

other lysogenic systems. It was suspected that P1 modification of membranes, mediated perhaps by P1_{ban} protein (13), renders the complex relatively insensitive to pronase-detergent treatment at a pH in the neutral or mildly alkaline ranges. (see discussion).

Soln. A

0.25 M TRIS (tris (hydroxymethyl) aminomethane HCl) (Sigma) pH 8

Soln. B

0.25 M TRIS pH 8

5.0 mg/ml lysozyme (Sigma) (added to soln. B on the day of the experiment.

Soln. C

0.25 M TRIS pH 8

0.25 EDTA (ethylenediamine tetraacetic acid) (Sigma).

Soln. D

0.06M TRIS

0.06M EDTA

10% sodium lauroyl sarcosinate-30 (sarcosyl) (Chemical Additives Co.)

After harvesting, 10^9 bacteria were resuspended in 0.3 ml of soln. A and 0.1 ml soln. B. The bacteria were left at 0°C for 5 min and then 0.1 ml of soln. C was added to the bacterial suspension. After another 5 min at 0°C, 0.5 ml of soln. D (containing 10% sarcosyl) was added to the protoplasmic suspension to initiate lysis. When the protoplast suspension had cleared, 1.0 mg (0.1 ml) self digested protease (Sigma) (46) in 0.25 M TRIS-HCl (pH 8) was added to the lysate. The lysate was then dialyzed overnight at 4°C in 0.01 M EDTA and 0.01

M TRIS (pH 8). In the morning the lysate was made 1.0% in SDS (soln. D, with sodium dodecyl sulfate (SDS) (Matheson) as the detergent) and was placed on a 5-20% (w/v) linear sucrose gradient for sedimentation.

When a batch preparation of plasmids was desired, 4×10^{10} bacteria were grown and harvested as described above. The bacteria were resuspended in 5.3 ml soln. A containing 25% (w/v) sucrose (Fisher) and 0.7 ml soln. B. After 5 min at 0°C , 1.5 ml soln. C. was added to the suspension. The suspension was maintained at 0°C for 5 min and then 7.5 ml of soln. C, containing Triton X-100 (31) (Sigma) as the detergent and 200 $\mu\text{g}/\text{ml}$ ethidium bromide (EB) (Mallinckrodt), was added to the protoplasts. Before the addition of soln. D, the protoplast suspension was transferred to the dark to prevent photodynamic damage to DNA-EB complexes (3). After lysis had occurred, the lysate was immediately centrifuged.

Radioactive Labeling:

Radioactive labeling of bacterial cultures was achieved by adding 0.05 mmole ^3H -thymidine (ICN) (specific activity 60-90 Ci/mmole) to 5.0 ml bacterial cultures. In experiments in which it was desired to competitively inhibit ^3H -thymidine incorporation, 2.3 mmoles cold thymidine (Sigma) per ml of culture was added at the appropriate time (pulse-chase).

When radioactive P1 bacteriophage was desired, the lysogenic bacteria were induced at 40°C , and labeled with 0.05 mmole ^3H -thymidine or 1.0×10^{-5} mg H^{32}PO_4 (specific activity 60-90 Ci/mg) (Amersham/Searle). After bacteriophage production was allowed to go to

completion the bacterial cellular debris was removed by centrifugation (4°C , $10,000 \times g$, 10 min. IEC A147 rotor) and the supernatant was prepared for bacteriophage harvest.

LCF Sedimentation:

After bacterial lysis, 0.2 ml aliquots of the lysate were gently layered on a neutral and/or alkaline 5-20% (w/v) sucrose gradient containing 1.0 ml of a 60% sucrose solution on the bottom of the gradient. The neutral gradients (12 ml) contained 10^{-2} M TRIS pH 7.4, 1.0 M NaCl, and 10^{-3} M EDTA. The alkaline gradients were identical to the neutral gradients except that they were 0.1 M NaOH and pH 12. For the alkaline gradient centrifugation the bacterial lysates were made 0.1 M NaOH before layering. The gradients were then centrifuged at $200,000 \times g$ (neutral) or $240,000 \times g$ (alkaline) at 15°C for the desired time in an IEC swinging bucket rotor SB-283. After centrifugation, fractions were collected from the bottom by puncturing the polyallomer centrifuge tube (IEC) with a 19 gauge needle attached to plastic tubing leading to the collection vessels. Representative 5-20% (w/v) neutral and alkaline sucrose gradients were tested for linearity using an Abbe optical refractometer.

In the batch preparation of plasmids, the 15 ml bacterial lysate (containing Triton X-100 and EB) was prepared in a 30 ml centrifuge tube. The lysate was underlaid with an equal volume of CsCl (density 1.625) containing 100 $\mu\text{g/ml}$ ethidium bromide. The centrifugation proceeded at 4°C and $100,000 \times g$ for 14-20 h, in an angle head rotor (IEC A211). The scale of the procedure could be doubled by

changing the density of the CsCl to 1.61 gm/ml and using a 50 ml centrifuge tube. The CsCl density was an important factor in this isolation procedure. If the density was too low the plasmid band was too close to the pellet, and if the density was too high the pellet was less compact and tended to float free from the bottom of the centrifuge tube.

After centrifugation the pellets were removed with a long 12 gauge needle and syringe and the rest of the gradient was collected in fractions from the bottom of the tube through a 12 gauge needle, and syringe and dialysed in 0.1 M TRIS (pH 7.2) and 10^{-3} EDTA. When desirable, the EB could be removed from the DNA by multiple extractions with isopropanol (Fisher), prior to dialysis.

When ^3H -thymidine labeling was desired for the batch preparation, a parallel 10 ml culture (2×10^9 bacteria) was labeled (0.6 μCi ^3H -thymidine) and added to the main culture, after both bacterial cultures had been harvested by preparative centrifugation.

Phage Preparation:

Virions were produced by growing the appropriate lysogenic bacterial strain to exponential phase (2×10^8 bacteria/ml) and thermally (40°C) inducing phage production in the presence of 10^{-2} M MgCl_2 (Sigma). After the lytic cycle was completed, as indicated by bacterial lysis, the debris was removed by centrifugation (4°C , 10,000 x g, 10 min, IEC A147 rotor) and the supernatants were made 0.5 M in NaCl and 10% (w/v) in polyethylene glycol (PEG 6000, Sigma). The

PEG-supernatant was incubated overnight at 4°C. After 18-24 h the bacteriophage-PEG precipitate (67) was collected by centrifugation at 10,000 x g and 4°C for 20 min in IEC rotor A 147. The supernatant was discarded and the PEG-phage pellet was resuspended in 4.0 ml of 0.1 M TRIS, 10^{-2} M $MgCl_2$. Virions were then banded in a CsCl step gradient using IEC rotor SB-283. The step gradient contained CsCl at densities of 1.3 g/ml, 1.5 g/ml and 1.7 g/ml in phage buffer (0.01 M $MgCl_2$, 0.1 M TRIS pH 8, and 0.01 M NaCl). A 200,000 x g, 4°C and two hour centrifugation was employed. The phage bands were collected through a needle and syringe and dialized overnight at 4°C in phage buffer.

For sedimentation studies the phage DNA was made 1-2% (w/v) in SDS and 10^{-2} M in EDTA at 65°C to disrupt the phage capsid; then gently treated with cold redistilled phenol. The aqueous phase was dialysed in 0.01 M TRIS (pH 7.2) and 10^{-3} M EDTA. The bacteriophage DNA (0.05 ml) was then gently layered onto sedimentation gradients or refrigerated for future studies.

Superinfection with Bacteriophage P1:

Plasmid lysogens were grown to exponential phase in TY medium, centrifuged and washed once in fresh medium. The cultures were then resuspended in 2 ml of TY medium with 10^{-3} M $CaCl_2$ and infected with freshly prepared P1 virions at m. o. i. of 2.5. After 20 minutes at room temperature, the cultures were washed in 20 ml of TY, centrifuged and resuspended in medium at a titer of 2.5×10^7 cells/ml. The bacterial cells were grown to exponential phase and a titer of

of $2-4 \times 10^8$ cells/ml, before the experiments proceeded.

Phage Titration:

Phage titrations were performed by mixing 0.1 ml CaCl_2 (0.1 M) (Sigma), 2.5 ml soft nutrient agar (46°C), 0.2 ml indicator bacterial strain, E. coli SCL800 (4×10^7 bacteria growing in the exponential phase), 0.1 ml of the appropriate phage dilution and pouring the mixture onto LB plates. After overnight incubation at 40°C , the phage plaques were counted.

DNA Filter Hybridization:

DNA was prepared through cold phenol extraction of bacterial lysates or virions. The method of Denhardt (14) was used for the detection of complementary DNA on nitrocellulose filters.

Double strand DNA was denatured by heating to 100°C for 10 minutes in 6 x SSC (SSC contains 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) quickly cooled and extensively vortexed. The DNA concentration was $0.5 \mu\text{g/ml}$ as determined by a Beckman DB spectrophotometer (260 nm) (1.0 O-D. unit is equivalent to $50 \mu\text{g/ml}$ DNA). The DNA solution (10 ml) was passed slowly through a Millipore HAWP 25mm filter (.45 mm pore size) prewashed with 6 x SSC. The filters were then dried overnight at 65°C and placed in a vacuum oven at 80°C and 76 mm Hg for two hours.

The filters were incubated in a solution containing 0.02% Ficoll (Pharmacia), 0.02% polyvinylpyrrolidone (Sigma) and 0.02% bovine albumin (GIBCO, fraction V) in 3 x SSC overnight at 65°C , to mask non-specific DNA binding sites. This preincubation mixture was removed before hybridization was begun.

The labeled DNA to be annealed was brought up to 6 x SSC; heat denatured, sheared by vortexing; and incubated with the appropriate DNA filter overnight at 65°C. In the morning the filters were washed in SSC until the wash was free of radioactive counts. The filter was then dried and counted in a liquid scintillation counter.

In some experiments, P1 DNA hybridization filters were incubated in the presence of purified SC1800 DNA (1.0 µg) to competitively inhibit any ³H E. coli DNA from attaching to the P1 DNA filters. Similarly, the P1dlac DNA hybridization filters were incubated in the presence of E. coli (1.0 µg) and P1 phage DNA (1.0 µg) to competitively inhibit radioactivity labeled E. coli or P1 DNA from binding to the filter. These methods of hybridization allowed very selective binding of the labeled P1 DNA on the P1 filters and very selective binding of the lac gene portion of the P1dlac to the P1dlac filters.

Liquid Scintillation Counting:

DNA was precipitated with 5% (w/v) cold trichloroacetic acid (TCA) (Sigma) (22.23). Unlabeled calf thymus DNA (200 µg/sample) (Sigma) was added as a carrier for the H³ labeled DNAs. Precipitates were collected on 0.45 µm pore size Millipore filters and washed four times with cold 95% (v/v) ethanol. The filters were then dried at 65°C, placed in liquid scintillation vials (Beckman) and covered with 10ml of scintillation fluid Toluene Scintillar - Mallinckrodt, with 4.2 mg/ml, 1.1, 5-diphenyloxazole (PPO) (Sigma) and 0.53 mg/ml, 1.1, 4-bis-2 - 5 - phenyloxazolyl - benzene (POPOP)

(Packard) . The radioactive isotope was counted in a Nuclear Chicago Unilux II or a Searle Mark II liquid scintillation counter.

Phenol Extraction of DNA:

In some cases the DNAs were phenol extracted. The lysates were made 0.2M in sodium acetate (Sigma) and extracted two times with cold redistilled phenol, saturated with 0.1 M Tris pH 7.4. The aqueous (DNA) phase was then dialyzed in an appropriate buffer.

RESULTS

Batch Preparation of Plasmids;

Simple "cleared lysate" methods for the isolation of supercoiled plasmids have been developed. They are effective for plasmids with masses of less than 10^6 daltons. In these methods (11,21,31,66), protoplasts are formed and gently lysed by detergent. Debris including host DNA is pelleted by centrifugation, while the supercoiled plasmids remain in the supernatant. They can then be concentrated and analyzed by several techniques (9,33,50,65). However, the cleared lysate methods are not adequate for supercoiled plasmids as large as P1, since such large supercoils become entrapped in the pelleting debris. Methods have been developed for the isolation of large plasmids (40,53,65), but they are tedious and were not considered feasible for the numerous supercoil isolations that were anticipated in the course of these studies. Therefore, a simpler large plasmid isolation method was sought and successfully developed. In this method a lysate of bacterial protoplasts is gently prepared and the chromosomes are sedimented into a CsCl-EB pad. After an overnight centrifugation, DNAs are visualized by the fluorescence of EB excited by UV light. Figure 4 illustrates how DNAs are visualized at the end of this procedure. Chromosomes in the right tube were from E. coli K12 (P1d1ac), while those in the left tube were from its lac⁻ segregant. The band near the top of the tube was merely EB concentrated at the top of the CsCl zone. The lint like debris pre-

Figure 4. Batch fractionation of plasmids from E. coli K 12 (Pldlac) and a lac⁻ segregant. Chromosomes from 4×10^{10} protoplasts were sedimented into a CsCl-EB pad. The 30 ml centrifuge tube initially contained equal volumes of lysate and CsCl-EB. After 18 h of centrifugation (4°C, 100,000 x g) in an IEC A211 angle head rotor, chromosomes were visualized by UV stimulated fluorescence.

To achieve DNA detection by isotopic labeling, parallel bacterial cultures (2×10^9 bacteria) were labeled with ³H-thymidine (0.6 μ Ci) and added to the appropriate main culture before protoplast formation was initiated.



E. coli K12(lac⁻)
Control

E. coli K12(P1dlac)

Interface CsCl and lysate

Relaxed DNA

Supercoiled DNA

Pellet

sent below this zone was not a common feature of our gradients, and was a broth contaminate. The distribution of ^3H -thymidine labeled DNA in these gradients is shown in Figure 5. The pellets (which were withdrawn prior to drop collection) contained 96% of the DNA of the Pldlac lysogen and 98% of the lac⁻ segregant. The denser, furthest sedimenting, peaks contained the supercoils, while the less dense peaks contained relaxed DNA. These identifications have been confirmed by electron microscopy (6).

This method owes its utility to the fact that host DNA sediments through the CsCl-EB zone in which free DNA is buoyant. The requisite high density must be conferred by an aggregation of the host DNA with dense bacterial debris, in particular the Cs⁺ salts of ribosomal RNA. Thus with the great excess of host DNA pelleted, the banded free DNA is easily visualized in the centrifuge tube.

Both the Pldlac lysogen and its lac⁻ segregant contained a 62×10^6 dalton plasmid (6), which is identical in size to the F⁺ plasmid (50). The Pldlac lysogen was initially produced in a Hfr Hayes line in which F was integrated in the host chromosome. The integrated state is known to be unstable, as F⁺ substrains rapidly segregate out F plasmids (50). The Pldlac line had evidently reverted to the F⁺ state, with the consequence that the F⁺ plasmid was present in both the Pldlac lysogen and its lac⁻ segregant.

When chromosomes from a plasmid free strain, such as SC1800 F⁻ were sedimented, a DNA band was not present at the supercoil position. Frequently, there was so little breakage of host DNA from the dense aggregate, that even buoyant relaxed DNAs could not be detected by fluorescence (Figure 6).

Figure 5. Distribution of radioactive DNA from the batch fractionated protoplasts shown in Figure 4. The pellets from the E. coli K 12 (P1dlac) (●) and the lac⁻ segregant (Δ) were removed with a syringe. The gradients were then collected in fractions from the bottom of the tube, and TCA precipitated radioactivity was determined. Sedimentation was from right to left. Pellets contained 98% and 96% of the control and E. coli K 12 (P1dlac) gradients respectively.

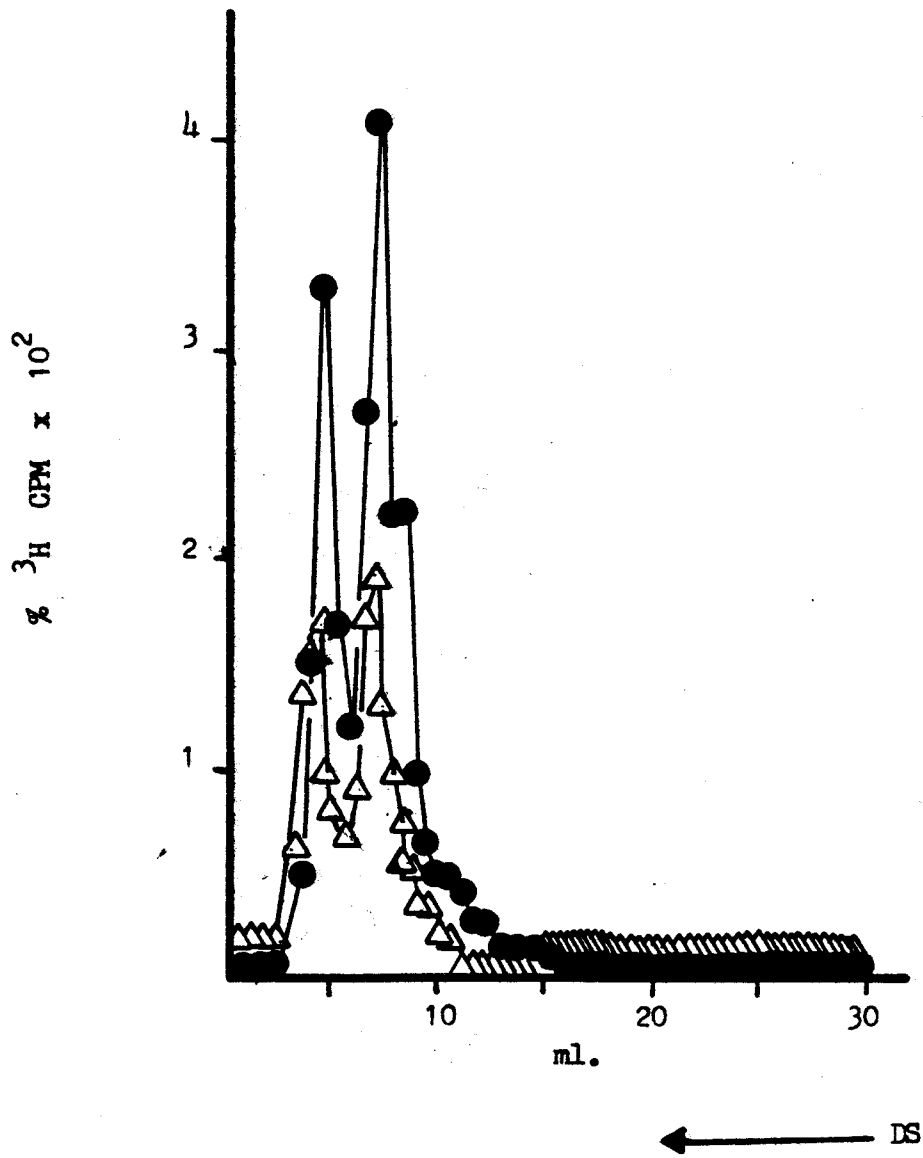
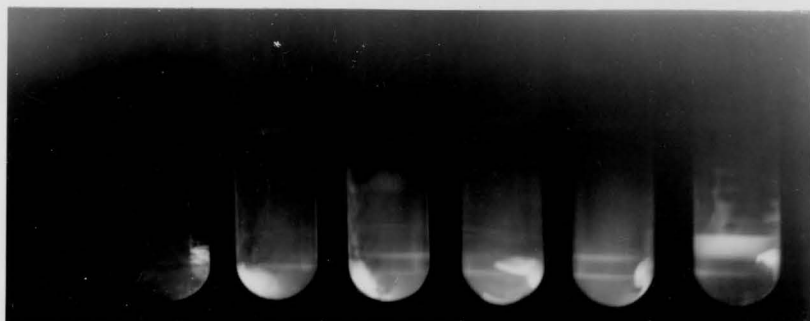


Figure 6. Batch fractionation of E. coli SC1801 (P1) by sedimentation into CsCl-EB pad. Cultures of E. coli SC1801 (P1) 4×10^{10} bacteria) were induced for the time indicated at 40°C. E. coli SC1800 was used as a non-lysogenic parallel control. The cultures were prepared for the batch CsCl-EB fractionation method and centrifuged for 18 hours, at 100,000 x g, 4°C in an IEC A211 angle head rotor. The chromosomes are visualized by UV excited fluorescence.

Min. at 40°C	<u>E. coli</u> SC1800			<u>E. coli</u> SC1801(P1)		
	32	0	8	16	24	32



Relaxed DNA
Supercoiled DNA

Strains with a single 6×10^7 dalton plasmid such as SC1801 F⁻ (P1) have typically yielded a supercoil band containing five to eight μg of DNA, while $10 \mu\text{g}$ $\left[(6 \times 10^7 \text{ MW} \times 2 \text{ plasmids/cell} \times 5 \times 10^{10} \text{ cells}) \div (6 \times 10^{23} \text{ gm}) \right]$ were theoretically expected.

The experiment shown in Figure 6 was used to qualitatively assay for the presence of supercoils during the lytic cycle. At eight min after induction, the supercoils had almost disappeared, relative to the uninduced control. There was a subsequent accumulation of supercoils at later times. The abundant band of relaxed DNA (least rapidly sedimenting) present in the 32 minute sample was partially attributed to sheared DNA from bacteria undergoing premature lysis. These results demonstrate that P1 supercoils are a normal lytic cycle intermediate.

Isotopic Labeling:

The sedimentation banding method, though supplying information on supercoil accumulation, did not provide any information regarding the size of the supercoils or relaxed P1 chromosomes, or the kinetics of their formation. For kinetic studies, isotopic labeling of chromosomes was essential. The incorporation of ^3H -thymidine into induced bacteria was accordingly examined.

Cultures of 10^9 lysogenic SC1801 (P1) were exposed to ^3H -thymidine ($0.6 \mu\text{Ci}$) for two min, at selected times in the lytic cycle, and then harvested. If P1 DNA is replicated at the same rate as E. coli DNA (45 min/generation), the two min corresponds to about two rounds of replication of the P1 genome. After the bacteria were

harvested and the DNA purified, DNA hybridizations were performed against P1 and E. coli DNA filters, to assess the genotype of the pulse labeled DNA. Hybridization controls with pure P1 and E. coli DNA established that about 15% of the input DNA was bound to the DNA target filters, for both P1 and E. coli DNA. Results are shown in Table 1.

E. coli DNA synthesis did continue throughout the interval assayed, but at a decreasing rate. A resolution of this DNA synthesis into components for net chromosomal replication and repair synthesis has not been attempted. Synthesis of P1 DNA was initiated by five minutes after lytic cycle induction, increased in rate until twenty minutes, and then diminished slightly. Even at five minutes, P1 DNA synthesis was about three times as rapid as E. coli DNA synthesis.

The preferential P1 DNA synthesis, combined with the great difference in E. coli and P1 genome sizes, suggests that P1 LCF in linear sucrose gradients might be tentatively identified without resorting to tedious DNA filter hybridizations. Host genomes should be rapidly pelleted under conditions in which pulse labeled P1 LCF were still sedimenting through the sucrose gradient.

Sedimentation Controls:

A lysozyme EDTA-detergent lysis procedure, was used to prepare cellular chromosomes. Lysis did occur as evidenced by visual clearing of the bacterial harvest and the high viscosity of the lysate. The lysates were then treated with pronase and SDS in an attempt to

<u>E. coli</u> Strain	Minutes at 40°C	% of total cpm bound		<u>% Pl Filter</u> <u>% <u>E. coli</u> filter</u>
		Pl filter	<u>E. coli</u> filter	
Pl DNA	-	15	2	7.5
SC1800	-	1.6	16	.1
SC1801(Pl)	0	2.2	3.9	.56
SC1801(Pl)	5	4.1	1.6	2.6
SC1801(Pl)	10	5.4	2.7	2.0
SC1801(Pl)	15	7.3	2.7	2.7
SC1801(Pl)	20	16	5.8	2.7
SC1801(Pl)	25	11	1.8	5.9
SC1801(Pl)	35	5.9	.8	7.4

Table 1. Incorporation of ^3H -thymidine into Pl and E. coli chromosomes during the lytic cycle. SC1801(Pl) cultures were induced at 40°C for the times indicated. Each 5 ml(10^9 cells) culture was ^3H -thymidine (0.6 μCi) labeled during the last two minutes of growth. The DNA was purified and Denhardt DNA filter hybridizations (14) were performed to identify the newly synthesized DNA. Results are expressed as the percent of total input TCA precipitable radioactivity annealed to the target DNA.

generate LCF free from proteins - a procedure which has been shown to be effective for several phage infection systems (46,58,65). The activity of the pronase-SDS under the conditions employed in these experiments was demonstrated by inactivation of a site specific endonuclease (Sal I) which was added to the lysate during pronase digestion. However, the pronase - SDS treatment did not destroy all cell proteins. Activity of DNA bound endogenous nucleases could be demonstrated upon the addition of Mg^{2+} .

Centrifugation conditions were chosen to provide for the resolution of relaxed and supercoiled monomers, while not pelleting 4G (genome unit) supercoils. Bacteriophage P1 DNA was used as the experimental standard, and positions of supercoils were calculated using the RSD (relative sedimentation distances) scale (Figure 3). With these chosen conditions, it was found that greater than 95% of the labeled E. coli DNA sedimented into the bottom of the centrifuge tube.

If the majority of the labeled E. coli DNA had not pelleted, tedious annealing experiments against P1 DNA filters would have been necessary to identify the P1 DNA. Since most of the labeled E. coli DNA did pellet, the gradient pattern could be tentatively attributed to P1 LCF. Therefore, only critical sedimentation fractions needed to be identified by DNA annealing experiments.

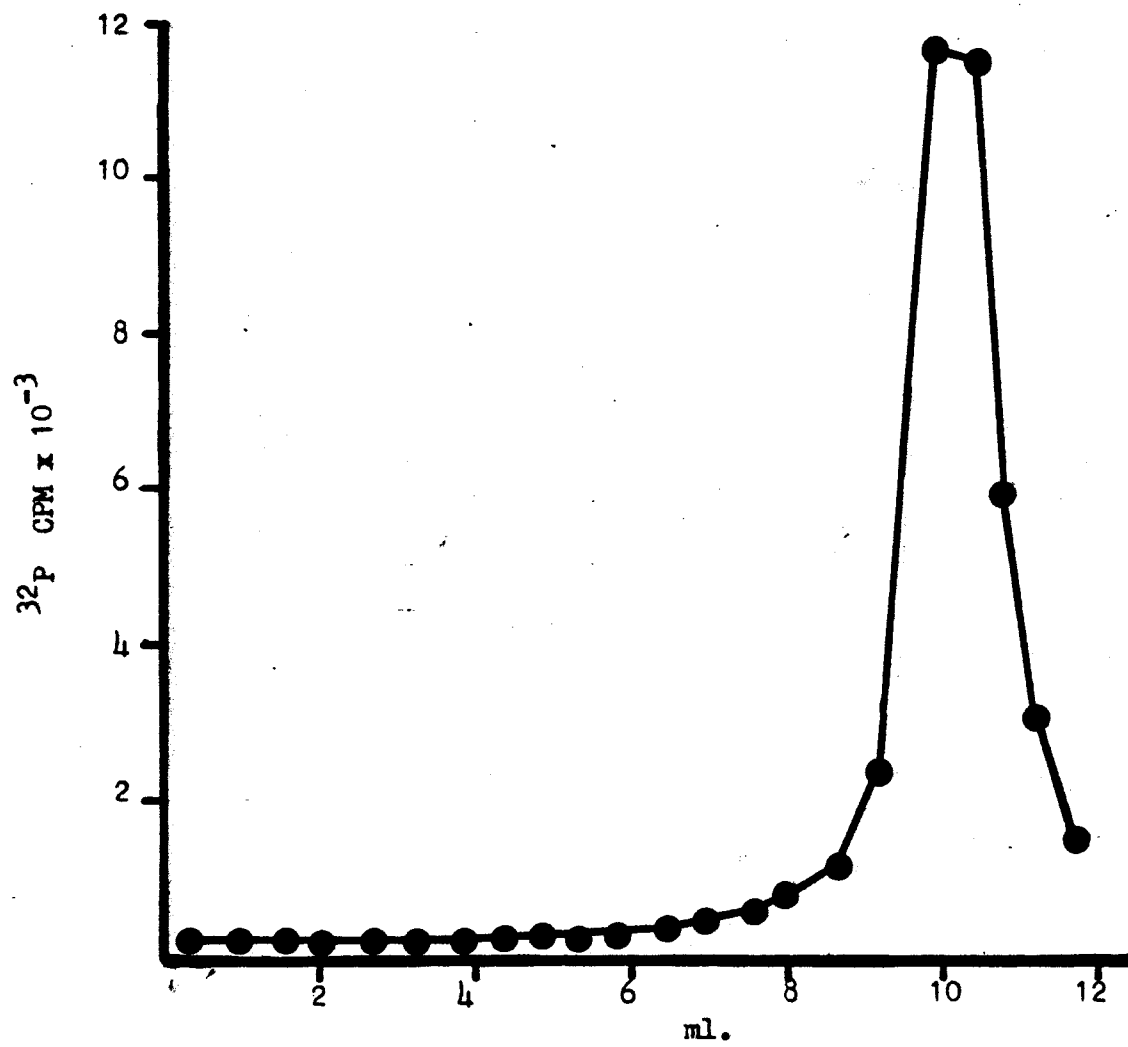
It was essential to show that chromosomal aggregation did not occur during sedimentation. For this purpose, ^{32}P labeled phage P1 chromosomes were compared as internal and external standards. Phenol extracted P1 phage DNA labeled with ^{32}P was centrifuged in two 5-20%

alkaline sucrose gradients, for 1.6 hours, at 240,000 x g and 15° C. The control contained ^{32}P -P1 chromosomes. The second gradient was representative of the most exacting conditions encountered in these studies. The problem of DNA aggregation is the worst when large concatemers are present at higher DNA concentrations. As is shown below, maximal concatemer accumulation occurs around 26 minutes into the lytic cycle. Lysate volumes were those used in all the experiments in this study. The bacterial lysate (*E. coli* K12 (P1), 10^9 bacteria) was mixed with ^{32}P -P1 phage DNA and centrifuged in parallel to the control.

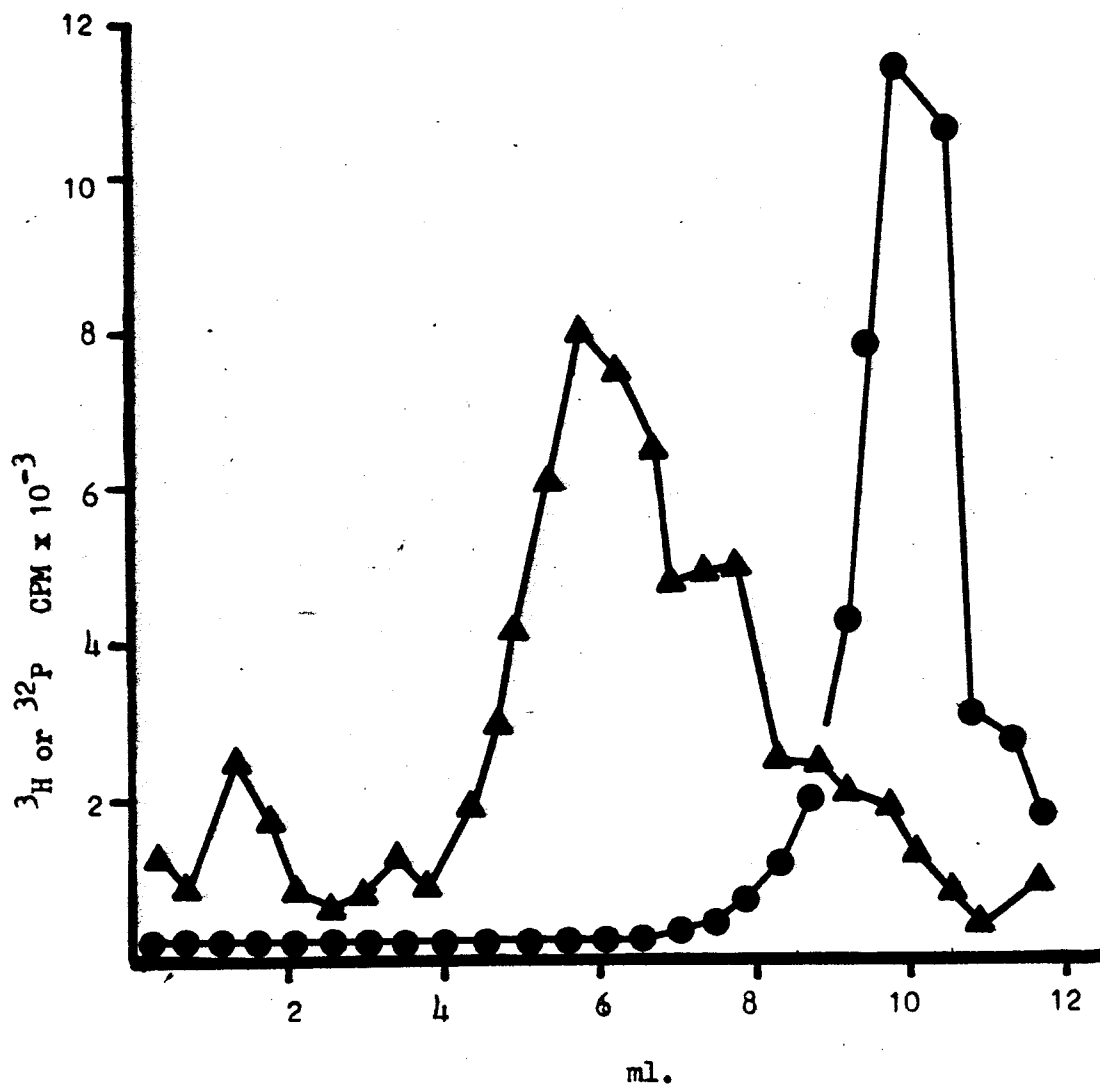
The ^{32}P -P1 phage DNA in the control (Figure 7) and experimental (Figure 8) sucrose gradients sedimented at the same rate, despite the presence of rapidly sedimenting LCF in the experimental tubes. Therefore, DNA aggregation does not occur with the small quantity (0.2 ml) of bacterial lysate employed, in the following analytical fractionations.

It would have been ideal to continue the use of ^{32}P -P1 chromosomes as an internal standard, however the 2 week halflife of ^{32}P renders the ^{32}P -P1 chromosomes too unstable for routine use. The ^{32}P beta decay produces primary single strand and some secondary double strand DNA breaks. Such damaged DNA cannot be used as a sedimentation standard. Also, the high energy of the ^{32}P beta radiation discouraged its routine use in the laboratory. Tritium has a 12 year halflife, and a lower energy beta decay. Therefore, ^3H -thymidine labeled chromosomes were used as an external standard in most of the following experiments because of their greater stability (44).

Figure 7. Sedimentation of ^{32}P labeled P1 phage DNA. Purified ^{32}P -P1 phage DNA was centrifuged at $240,000 \times g$, 15°C for 1.6 hours through a 5-20% (w/v) linear alkaline sucrose gradient in an IEC SB283 rotor. Sedimentation was from right to left. Fractions were collected from the bottom, and TCA precipitated in preparation for radioactive determinations.



← DS



Pl Lytic Cycle:

A set of sedimentation experiments was performed to characterize LCF which contained newly synthesized DNA. Pl lysogens were induced and labeled with ^3H -thymidine for the last min prior to harvesting. Harvesting times from 6 to 26 min were chosen. Lysates of the bacteria (10^9) were prepared and sedimented through neutral and alkaline linear sucrose gradients (Figure 9 and 10).

In the neutral gradients a large portion (85-90%) of the newly synthesized DNA pelleted. The pelleting of the DNA has two interpretations. One is that the replicative forms are high G LCF, such as firewheels (Figure 2E). It is also possible (but unlikely) that the pronase-SDS treatment failed to free chromosomes of proteins which caused aggregation of small LCF. Radioactive peaks present in the gradients were pooled and annealed against Pl DNA hybridization filters. It was confirmed that ^3H -Pl DNA did carry most of the radioactivity.

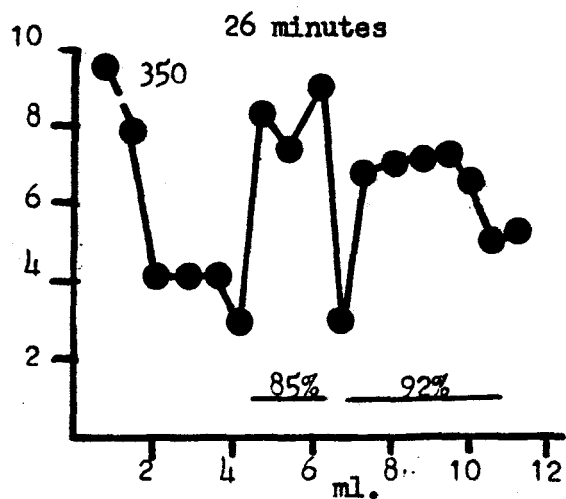
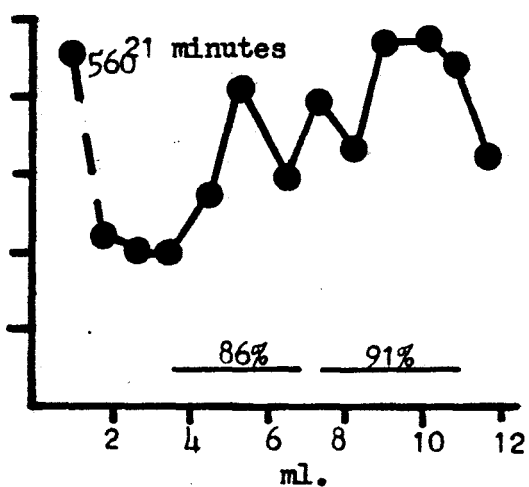
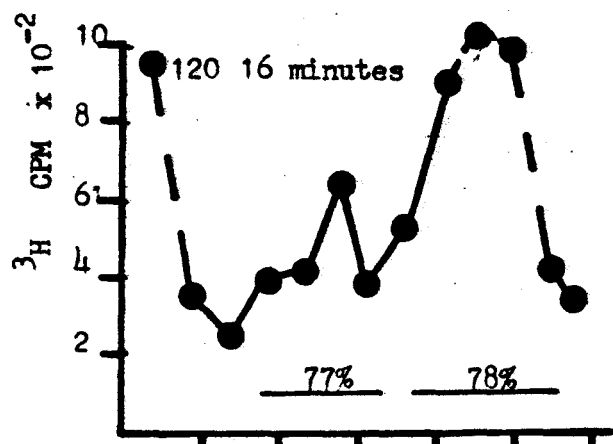
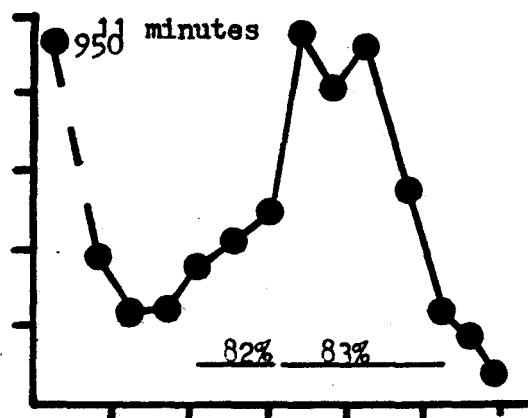
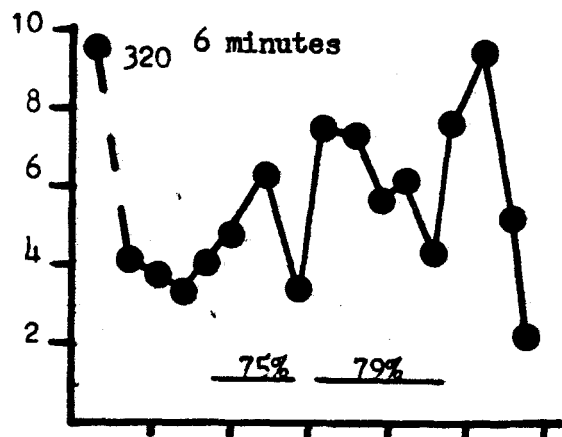
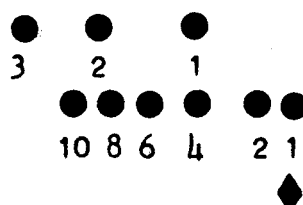
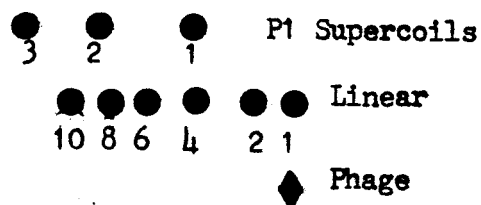
At 6 minutes there is resolved a slowly sedimenting peak (less than 1G), which might be Okazaki fragments (41). There is a broad peak in the relaxed 1-4 G range. Its leading edge might contain 1G supercoils. There is also a more rapidly sedimenting fraction whose peak is at 2G supercoils or 6-8 G relaxed DNAs. Throughout the interval assayed (up to 26 minutes) relaxed LCF less than 4G constitute a large portion of the unpelleted DNA. More rapidly sedimenting LCF, which could be supercoils, are also present at all the time periods studied.

More decisive data were obtained from the alkaline gradients

Figure 9. Sedimentation of newly synthesized chromosomes from E. coli SC1801(P1): native DNA form. Cultures of E. coli SC1801(P1) (10^9 bacteria) were induced at 40°C and grown for the times indicated. The bacteria were labeled with ^3H -thymidine (0.6 μCi) during the last minute of growth. Lysates were prepared and the chromosomes were centrifuged through a linear 5-20% (w/v) neutral sucrose gradient at $200,000 \times g$, and 15°C for 1.8 hours in an IEC SB283 rotor. Sedimentation was from right to left. Fractions were collected from the bottom, and TCA precipitated in preparation for radioactive determinations.

Above each Figure or set of Figures is located the Relative Sedimentation distance scale (RSD). Each set of points on the scale is labeled with the DNA species represented, and the molecular configuration of that DNA. The numbers on the RSD refer to the G number (number of unit genomes) represented by that particular point on the scale. The scale is adjusted for the type (neutral or alkaline) of sedimentation gradient being analyzed, and the conditions of centrifugation. In order to confirm the genetic identity of the radioactivity labeled DNA forming the peaks of the sedimentation profiles, aliquots of relevant fractions were pooled and hybridized against P1 and E. coli DNA filters (14). The hybridization efficiency of the DNA filters was about 30%. The percent of the radioactive DNA

that was P1 DNA is indicated above the bar delineating the pooled peaks on the sedimentation profile plots.



← DS

Figure 10. Sedimentation of newly synthesized chromosomes from E. coli SC1801(P1):denatured DNA form. Cultures of E. coli SC1801(P1) (10^9 bacteria) were induced at 40°C and grown for the time indicated. The bacteria were labeled with ^3H -thymidine (0.6 μCi) during the last minute of growth. Lysates were prepared and the chromosomes were centrifuged through a linear 5-20% (w/v) alkaline (denaturing) sucrose gradient at $240,000 \times g$, and 15°C for 1.8 hours in an IEC SB283 rotor. Sedimentation was from right to left. Fractions were collected from the bottom, and TCA precipitated in preparation for radioactive determinations.

Above each Figure or set of Figures is located the Relative Sedimentation Distance scale (RSD). Each set of points on the scale is labeled with the DNA species represented, and the molecular configuration of that DNA. The numbers on the RSD refer to the G number (number of unit genomes) represented by that particular point on the scale. The scale is adjusted for the type (neutral or alkaline) of sedimentation gradient being analyzed, and the conditions of centrifugation. In order to confirm the genetic identity of the radioactivity labeled DNA forming the peaks of the sedimentation profiles, aliquots of relevant fractions were pooled and hybridized against P1 and E. coli DNA filters (14). The hybridization efficiency of the

DNA filters was about 30%. The percent of the radioactive DNA that was P1 DNA is indicated above the bar delineating the pooled peaks on the sedimentation profile plots.

● ● ●
3 2 1

P1 Supercoils

● ● ●
3 2 1

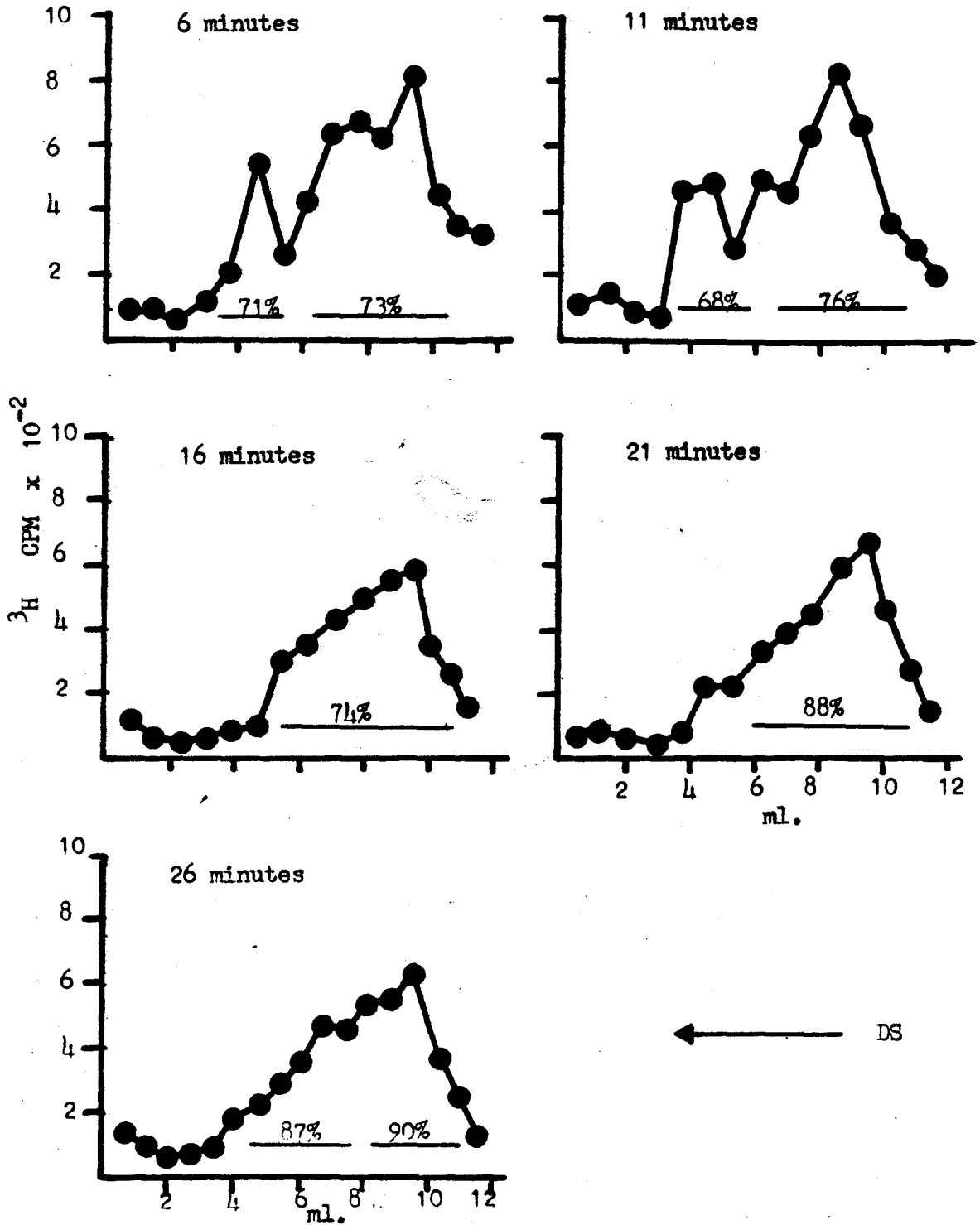
● ● ● ● ● ●
10 6 4 2 1

Linear

● ● ● ● ● ●
10 6 4 2 1

◆ Phage

◆



(Figure 10). These alkaline gradients contained few if any pelleted LCF strands. Hence the radioactivity in the gradients does represent all the LCF. Relaxed LCF are separated into constituent strands in alkali, so that constituent concatemer strands can be identified. There is also a better separation of supercoils and equivalent size single strands.

In the 6 and 11 minutes lysates, single strands in the 1-8 G range were present. This fact establishes that newly synthesized DNA is added to preexisting concatemers. Most of these strands must be constituents of the pelleting LCF prevalent in the neutral gradient.

The alkaline gradient also had a discrete more rapidly sedimenting LCF, peaking at the 2 G supercoil position. At later times the 1-8 G mass single strands were still present, but there appeared to be a decrease in the peaks attributable to 2 G supercoils. If supercoils larger than 5 G were present, they would have been pelleted in our gradients and could not have been detected.

The smaller than 1 G DNA seen in the neutral gradients was also present in the alkaline gradients. However, these DNAs constituted only a shoulder under the more prevalent higher G strands. The resolution of the small G DNA in the neutral gradients as a peak must have occurred because of the high percentage of large G DNA that pelleted.

It is noteworthy that single strands shorter than 1 G were not a predominant DNA species. This fact shows that the theta replicative forms (Figure 2C), which would yield 1 G and smaller strands

upon denaturation, cannot be the predominant P1 replicating form.

Pulse chase experiments were necessary to detect the molecular forms that newly synthesized DNA "passes through" as the lytic cycle progresses. To establish their feasibility ^3H -thymidine uptake and termination controls were first performed. P1 lysogenic cultures were induced for five minutes or fifteen minutes, and labeled with ^3H -thymidine for one minute. Excess cold thymidine was then added to competitively inhibit ^3H -thymidine uptake. Aliquots of the bacteria were then harvested, lysed and analysed. Figure 11 shows the results. Radioactive ^3H -thymidine uptake ceased shortly after the addition of the cold thymidine chase. The TCA precipitable counts remained at a constant level throughout the remaining time of the experiment. Therefore, it was feasible to limit the period of ^3H incorporation into DNA, and perform pulse-chase experiments.

E. coli SC1801 (P1) was grown at 40°C , and pulsed for one min with ^3H -thymidine and cold thymidine chased. Aliquots of the bacteria were harvested at appropriate times, lysates were prepared, and chromosomes centrifuged in linear 5-20% neutral and alkaline sucrose gradients.

Examination of the neutral gradients (Figure 12) showed that a majority of the LCF pelleted at 6 and 11 min. For the later sample intervals, the ^3H -cpm scale was changed to better represent the data. The pelleting demonstrates that DNA which enters concatemers remains in concatemers for an extended period. In the early time intervals there are peaks of 1-2 G supercoils and relaxed DNA

Figure 11. Competitive inhibition of ^3H -thymidine uptake by excess cold thymidine in E. coli SC1801(P1). Cultures of E. coli SC1801(P1) (10^9 bacteria) were induced at 40°C , labeled with ^3H -thymidine ($0.6\ \mu\text{Ci}$) for 1 minute at 4 min (●) or 15 min (▲). Cold thymidine (11.5 mmoles) was then added to each culture. Aliquots of each culture were collected, lysed, and TCA precipitated in preparation for radioactive determinations.

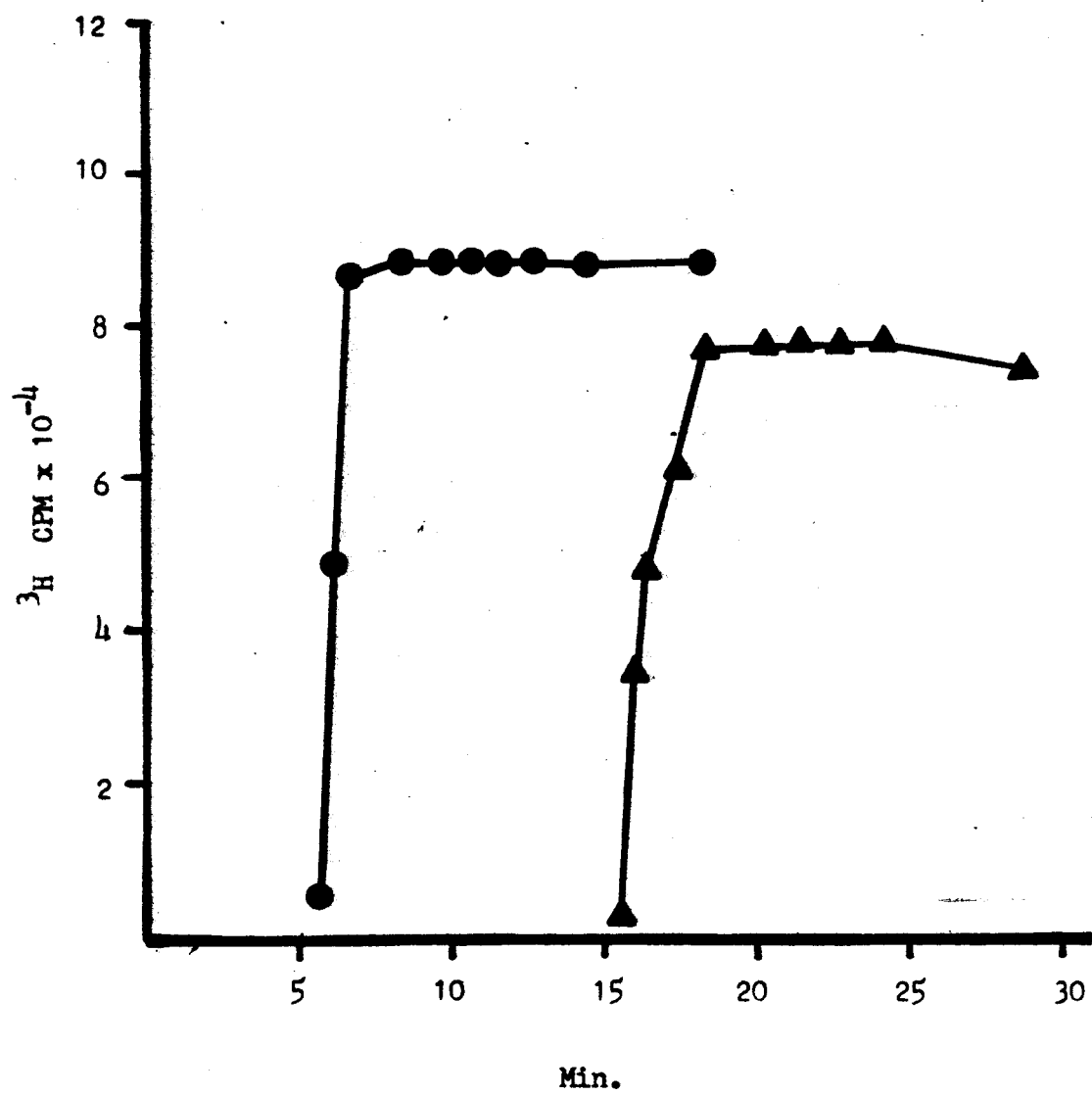
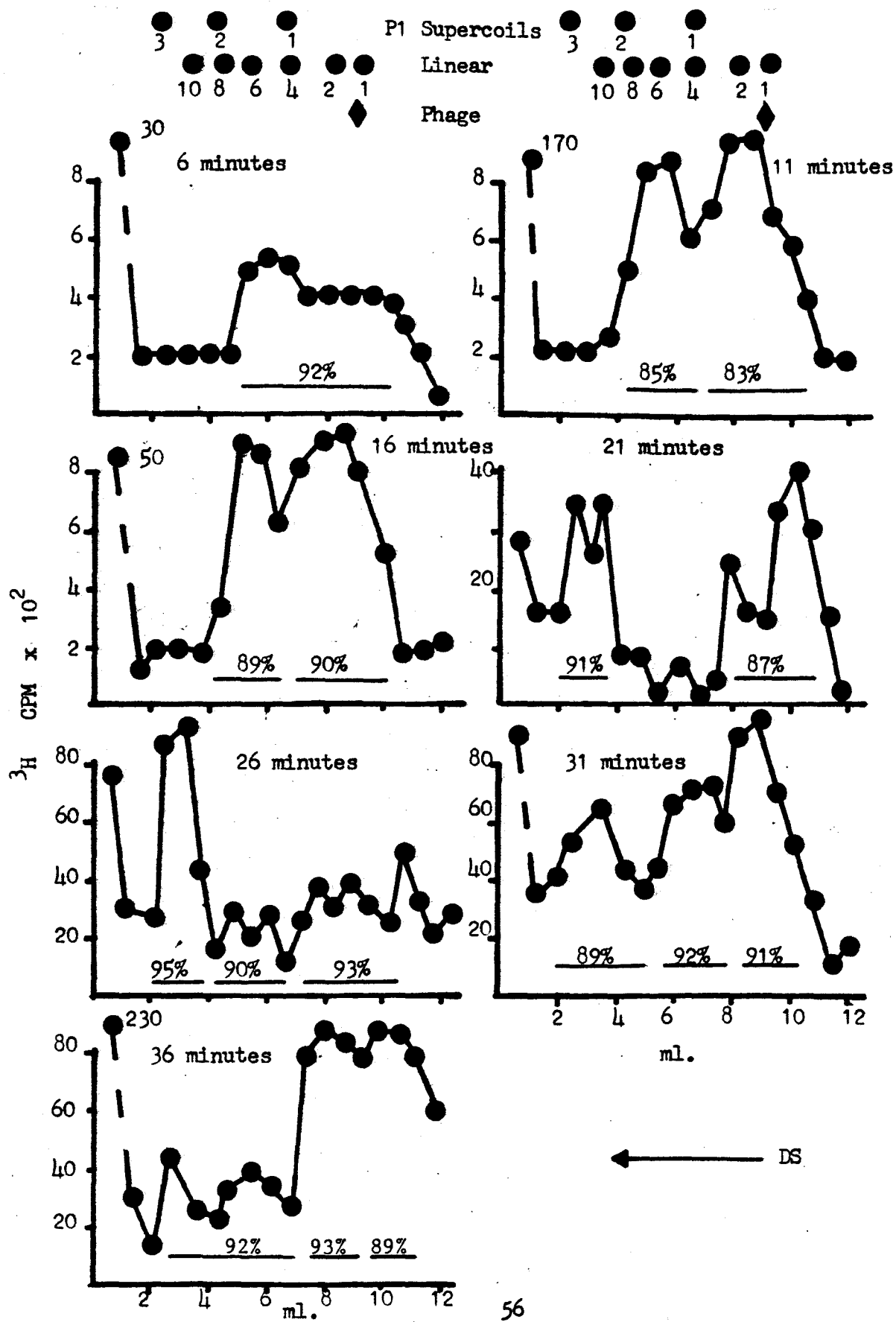


Figure 12. Generation of P1 lytic cycle chromosomal forms from newly synthesized DNA: native DNA forms. E. coli SC1801(P1) (10^9 bacteria) were induced at 40°C for the time indicated. Each culture was labeled for one min with ^3H -thymidine ($0.6\ \mu\text{Ci}$). Cold thymidine ($11.5\ \text{mmoles}$) was added to the cultures at the end of the labeling period to competitively inhibit further ^3H -thymidine incorporation (Figure 11). After the growth period, bacterial lysates were prepared, and the chromosomes were centrifuged through a linear 5-20%(w/v) neutral sucrose gradient at $200,000 \times g$ and 15°C for 1.8 hours in an IEC SB283 rotor. Sedimentation was from right to left. Fractions were collected from the bottom, and TCA precipitated in preparation for radioactive determinations. The ^3H -thymidine pulse was from 4 to 5 min of the lytic cycle for the 6, 11, 16 and 21 min samples and from 15 to 16 min of the lytic cycle for the 21, 26, 31 and 36 min samples. Only one 21 min sample is shown, since both 21 min sedimentation profiles are similar.

Above each Figure or set of Figures is located the Relative Sedimentation Distance scale (RSD). Each set of points on the scale is labeled with the DNA species represented, and the molecular configuration of that DNA. The numbers on the RSD refer to the G number (number of unit genomes) represented by that

particular point on the scale. The scale is adjusted for the type (neutral or alkaline) of sedimentation gradient being analyzed, and the conditions of centrifugation.

In order to confirm the genetic identity of the radioactivity labeled DNA forming the peaks of the sedimentation profiles, aliquots of relevant fractions were pooled and hybridized against Phi and E. coli DNA filters (14). The hybridization efficiency of the DNA filters was about 30%. The percent of the radioactive DNA that was Phi DNA is indicated above the bar delineating the pooled peaks on the sedimentation profile plots.



in the 1-4 G range. At 21 min and later more LCF are present in the gradients and a peak of 2-3 G supercoils is evident. Beginning at 26 min there is a progressive decrease in the LCF sedimentation rate. By 36 min the majority of the unpelleted radioactive DNA has the sedimentation positions of PlB and PlS virion chromosomes.

The alkaline sucrose gradients (Figure 13) indicate the presence of concatemer strands in the 2-10 G range, from at least 11 min onward. From 11 min until 26 min there is a progressive increase in the LCF sedimentation rate. However after 26 min, a progressive decrease in LCF sedimentation rate is apparent.

The 36 minute alkaline sample provided some further information. Despite the fact that most of the radioactivity within the neutral gradient was in the form of mature PlS and PlB chromosomes, the peak of radioactivity in the alkaline gradient was at the position of an 8 G single strands. Mature 1 G strands which must have been present were under the shoulder of this peak. Therefore, in the 36 minute sample most of the Pl radioactivity in the neutral gradient had in fact pelleted in very massive LCF. These LCF could have been as massive as 5 G supercoils or much more massive relaxed chromosomal forms.

The genetic identity of the radioactive peaks was confirmed by DNA hybridization experiments. Sedimentation fractions corresponding to radioactive peaks were pooled and hybridized against Pl and E. coli DNA filters. The hybridizations established that greater than 70% of the ^3H -thymidine was in Pl DNA.

Figure 13. Generation of P1 lytic cycle chromosomal forms from newly synthesized DNA;denatured DNA forms. E. coli SC1801(P1) (10^9 bacteria) were induced at 40°C for the time indicated. Each culture was labeled for one min with ^3H -thymidine (0.6 μCi). Cold thymidine (11.5 mmoles) was added to the cultures at the end of the labeling period to competitively inhibit further ^3H -thymidine incorporation (Figure 11). After the bacterial harvesting bacterial lysates were prepared, and the chromosomes were centrifuged through a linear 5-20% (w/v) alkaline sucrose gradient at $240,000 \times g$ and 15°C for 1.8 hours in an IEC SB283 rotor. Sedimentation was from right to left. Fractions were collected from the bottom, and TCA precipitated in preparation for radioactive determinations.

The ^3H -thymidine pulse was from 4 to 5 min of the lytic cycle for the 6, 11, 16 and 21 min samples and from 15 to 16 min of the lytic cycle for the 21, 26, 31 and 36 min samples. Only one 21 min sample is shown, since both 21 min sedimentation profiles are similar.

Above each Figure or set of Figures is located the Relative Sedimentation Distance scale (RSD). Each set of points on the scale is labeled with the DNA species represented, and the molecular configuration of that DNA. The numbers on the RSD refer to the G number (number of unit genomes) represented by that

particular point on the scale. The scale is adjusted for the type (neutral or alkaline) sedimentation gradient being analyzed, and the conditions of centrifugation.

In order to confirm the genetic identity of the radioactivity labeled DNA forming the peaks of the sedimentation profiles, aliquots of relevant fractions were pooled and hybridized against P1 and E. coli DNA filters (14). The hybridization efficiency of the DNA filters was about 30%. The percent of the radioactive DNA that was P1 DNA is indicated above the bar delineating the pooled peaks on the sedimentation profile plots.

60

P1 Supercoils

3 2 1

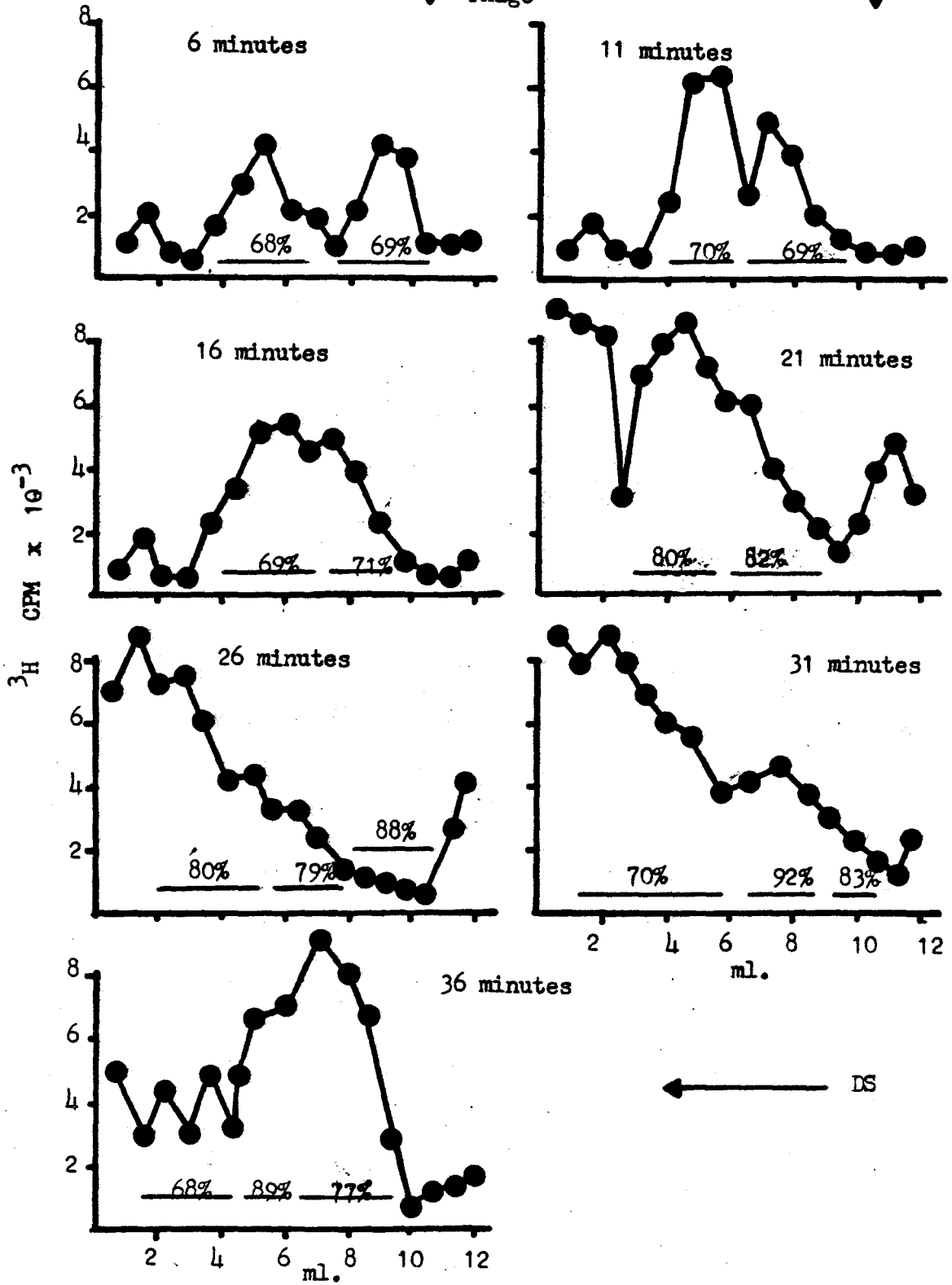
3 2 1

Linear

10 6 4 2 1

10 6 4 2 1

Phage



In the pulse-chase experiments two protocols were used because of technical limitations. For the 6, 11, 15 and 21 minute samples, the bacteria were pulsed with ^3H -thymidine at 4 to 5 min of the lytic cycle. The 21, 26, 31 and 36 min bacterial cultures were labeled with ^3H -thymidine from 15 to 16 min and then chased with excess cold thymidine. The two experiments overlap at the 21 minute time period. Sedimentation patterns of these overlapped samples were essentially identical, and only the 21 minute harvest pattern from the 15 min pulse chase experiment is shown. The identity of the patterns indicates the following: it takes less than five minutes (15 minutes pulse to 21 minute harvest) for newly synthesized DNA to become part of lytic cycle chromosomal forms as large as those whose synthesis was initiated at five minutes. This fact supports the earlier conclusion that newly synthesized DNA is incorporated into large pre-existing LCF.

Pl_dlac Lytic Cycle:

A Pl_dlac lysogen was analyzed in the same manner as the Pl lysogen. Because the Pl_dlac chromosome is not intact in virion populations, lytic cycles could not have been initiated by infections with virions from Pl_dlac lysogens. It was for this reason that all the experiments were initiated by induction of lysogenic populations, rather than virion infections.

Pl_dlac lysogenic cultures were grown for various periods of time at 40°C . Aliquots were labeled with ^3H -thymidine during their last minute of growth and prepared for analysis by sucrose gradient

sedimentation. The results from the neutral gradients are shown in Figure 14. The Pldlac plasmid has twice the molecular mass of the P1 plasmid. Therefore the RSD scale has been corrected for the increased mass.

The neutral gradients show pelleting of the majority of the LCF, as was seen in previous experiments. At 6 min there is a slowly sedimenting peak of 1 G relaxed chromosomes. Newly synthesized DNA was also present in the less than 1 G portion of the gradient. Larger relaxed forms are present at later sample times. In the 21 and 26 min samples, some PlB chromosomes are present.

The alkaline sucrose gradients (Figure 15) showed that there was newly synthesized DNA in the 1 to 10 G single strand range, at all the time periods studied. The most rapidly sedimenting LCF were represented at six minutes by a shoulder at the 1 G supercoil location or at the greater than 10 G single strand range. At 21 minutes and 26 minutes, single stranded virion DNA was probably present.

The fact that the alkaline gradient revealed large relaxed strands again implies that these chromosomes were present in the neutral gradient but had pelleted. Therefore, in the Pldlac lytic cycle, newly synthesized DNA is incorporated into large concatemers. The less than 1 G DNA in the neutral gradients constitutes only a small portion of the radioactivity observed in the alkaline gradients. These small DNA fragments may be Okazaki fragments (41).

Pulse-chase experiments were done with the Pldlac lysogen to

Figure 14. Sedimentation of newly synthesized chromosomes from E. coli K12 (Pldlac): native DNA form. Cultures of E. coli K12(Pldlac) (10^9 bacteria) were induced at 40°C and grown for the time indicated. The bacteria were labeled with ^3H -thymidine (0.6 μCi) during the last minute of growth. Lysates were prepared and the chromosomes were centrifuged through a linear 5-20% (w/v) neutral sucrose gradient at $200,000 \times g$, and 15°C for 1.8 hours in an IEC SB283 rotor. Sedimentation was from right to left. Fractions were collected from the bottom, and TCA precipitated in preparation for radioactive determinations.

Above each Figure or set of Figures is located the Relative Sedimentation Distance scale (RSD). Each set of points on the scale is labeled with the DNA species represented, and the molecular configuration of that DNA. The numbers on the RSD refer to the G number (number of unit genomes) represented by that particular point on the scale. The scale is adjusted for the type (neutral or alkaline) sedimentation gradient being analyzed, and the conditions centrifugation.

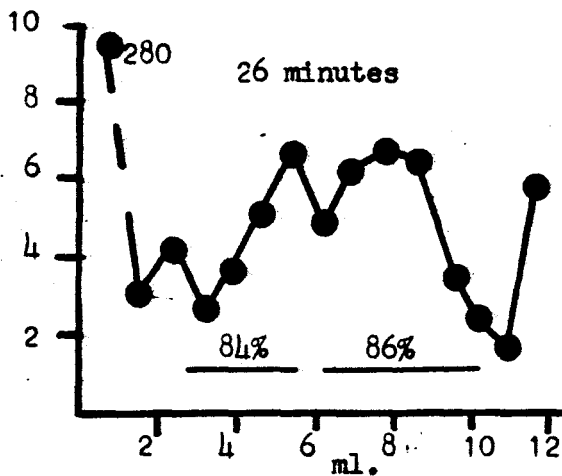
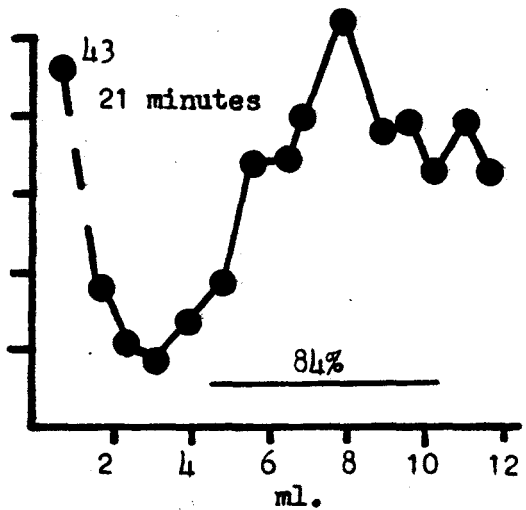
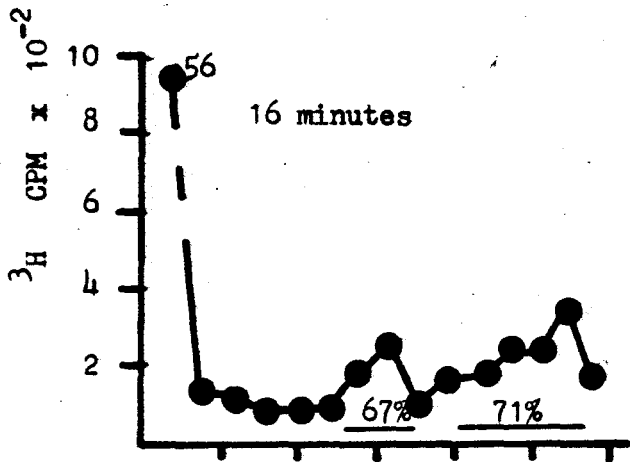
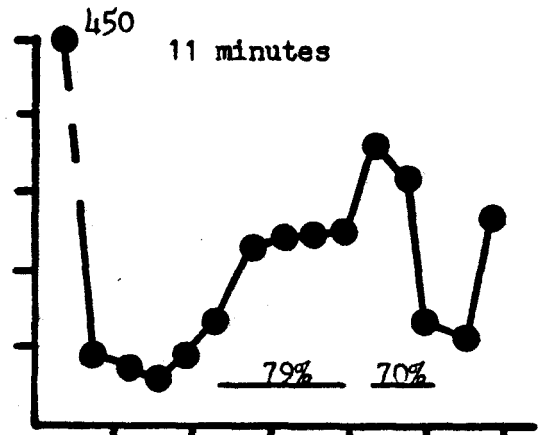
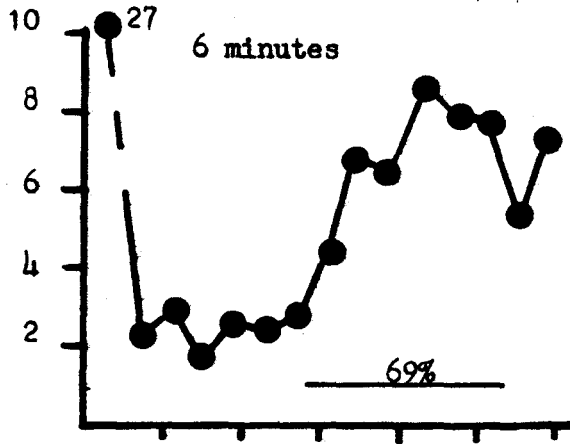
In order to confirm the genetic identity of the radioactivity labeled DNA forming the peaks of the sedimentation profiles, aliquots of relevant fractions were pooled and hybridized against P1 and E. coli DNA

filters (14). The hybridizations efficiency of the DNA filters was about 30%. The percent of the radioactive DNA that was P1 DNA is indicated above the bar delineating the pooled peaks on the sedimentation profile plots.

P1dlac Supercoils

● ● ● ● ● Linear
5 4 3 2 1
◆ Phage

● ● ● ● ●
5 4 3 2 1
◆



← DS

Figure 15. Sedimentation of newly synthesized chromosomes from *E. coli* K12(Pl~~dlac~~) denatured DNA form. Cultures of *E. coli* K12(Pl~~dlac~~) (10^9 bacteria) were induced at 40°C and grown for the time indicated. The bacteria were labeled with ^3H -thymidine (0.6 μCi) during the last minute of growth. Lysates were prepared and the chromosomes were centrifuged through a linear 5-20% (w/v) alkaline sucrose gradient at 240,000 x g, and 15°C for 1.8 hours in an IEC SB 283 rotor. Sedimentation was from right to left. Fractions were collected from the bottom, and TCA precipitated in preparation for radioactive determinations.

Above each Figure or set of Figures is located the Relative Sedimentation Distance Scale (RSD). Each set of points on the scale is labeled with the DNA species represented, and the molecular configuration of that DNA. The numbers on the RSD refer to the G number (number of unit genomes) represented by that particular point on the scale. The scale is adjusted for the type (neutral or alkaline) sedimentation gradient being analyzed, and the conditions of centrifugations.

In order to confirm the genetic identity of the radioactivity labeled DNA forming the peaks of the sedimentation profiles, aliquots of relevant fractions were pooled and hybridized against Pl and *E. coli* DNA filters (14). The hybridization efficiency of the DNA

filters was about 30%. The percent of the radioactive DNA that was P1 DNA is indicated above the bar delineating the pooled peaks on the sedimentation profile plots.

● 2 ● 1 P1dlac Supercoils

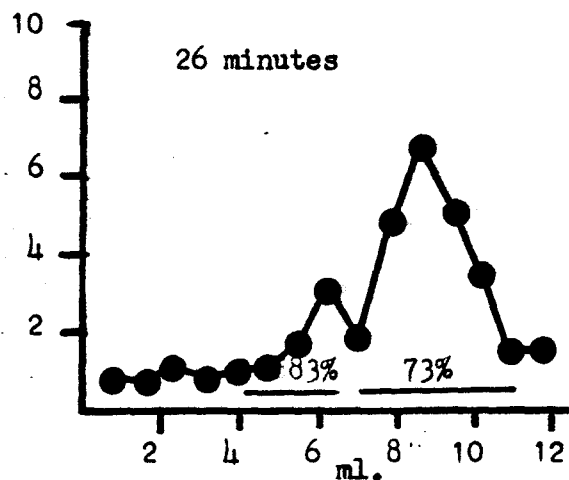
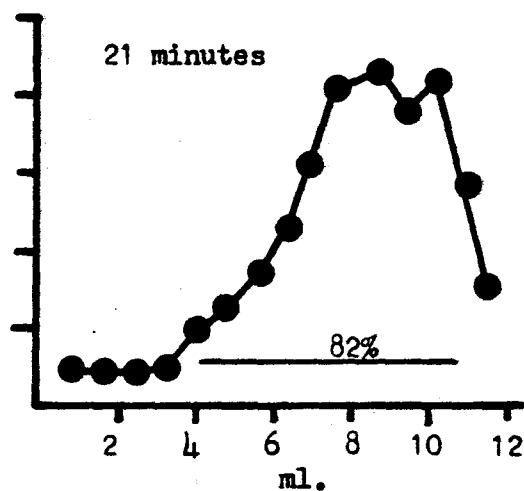
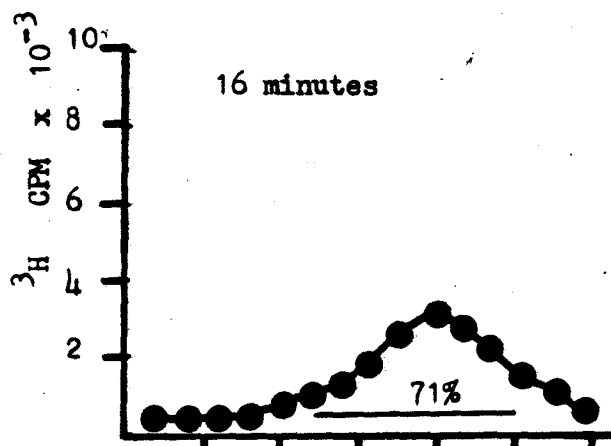
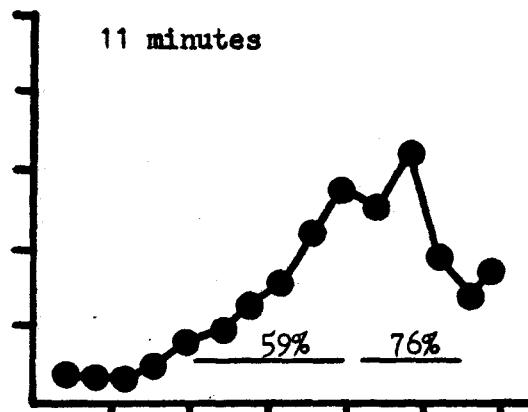
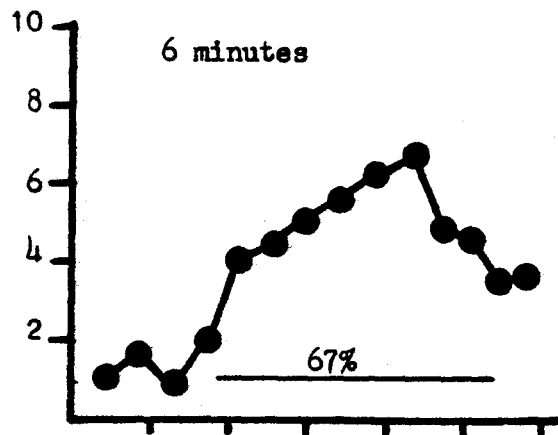
● ● ● ● ● ● Linear
10 8 6 4 2 1

◆ Phage

● 2 ● 1

● ● ● ● ● ●
10 8 6 4 2 1

◆



← DS

observe the chromosomal forms through which newly synthesized Pldlac DNA was passaged. The experimental conditions were the same as those used for the Pl pulse-chase experiments.

The neutral gradients (Figure 16) showed that the newly synthesized DNA has again pelleted. A minority was passaged into 1 to 4 G sized chromosomes in the early time periods. In the 31 and 36 min samples, virion chromosomes have begun to accumulate.

Alkaline sucrose gradients give a better assessment of chromosomal strand size. The alkaline pulse-chase gradients (Figure 17) demonstrated that newly synthesized DNA was processed into relaxed chromosomal forms in the 1-10 G size or larger. Since 1 G supercoils were not present in the neutral gradients, supercoils do not contribute to the fast shoulder. Each time period studied showed a similar sedimentation profile. In addition the 31 and 36 minute lysates showed virion chromosomes. The discrepancy between the size of the relaxed chromosomes in the neutral and alkaline gradients is again attributable to LCF pelleting in the neutral gradients. The disproportionate amount of radioactivity seen as DNA of less than 1 G in the neutral and as a minor radioactive component of the alkaline gradients argues that this radioactivity might be Okazaki fragments (41).

An experiment revealing the contribution of recombination to concatemer formation was performed. It relied on the difference between Pl and Pldlac sedimentation for equivalent G unit molecules. The Pldlac lysogen was superinfected with bacteriophage Pl, and the lytic cycle was induced. The bacteria were pulse labeled for one

Figure 16. Generation of Pldlac lytic cycle chromosomal forms from newly synthesized DNA:native DNA forms.

E. coli K12(Pldlac) (10^9 bacteria) were induced at 40°C for the time indicated. Each culture was labeled for one min with ^3H -thymidine (0.6 μCi). Cold thymidine (11.5 mmoles) was added to the cultures at the end of the labeling period to competitively inhibit further ^3H -thymidine incorporation (Figure 11). After the growth period, bacterial lysates were prepared, and the chromosomes were centrifuged through a linear 5-20% (w/v) neutral sucrose gradient at $200,000 \times g$ and 15°C for 1.8 hours in an IEC SB283 rotor. Sedimentation was from right to left. Fractions were collected from the bottom, and TCA precipitated in preparation for radioactive determinations.

The ^3H -thymidine pulse was from 4 to 5 min of the lytic cycle for the 6, 11, 16, and 21 min sample and from 15 to 16 min of the lytic cycle for the 21, 26, 31 and 36 min samples. Only one 21 min sample is shown, since both 21 min sedimentation profiles are similar.

Above each Figure or set of Figures is located the Relative Sedimentation Distance scale (RSD). Each set of points on the scale is labeled with the DNA species represented, and the molecular configuration of that DNA. The numbers on the RSD refer to the G number (number of unit genomes) represented by that particular

point on the scale. The scale is adjusted for the type (neutral or alkaline) sedimentation gradient being analyzed, and the conditions of centrifugation.

In order to confirm the genetic identity of the radioactivity labeled DNA forming the peaks of the sedimentation profiles, aliquots of relevant fractions were pooled and hybridized against P1 and E. coli DNA filters (14). The hybridization efficiency of the DNA filters was about 30%. The percent of the radioactive DNA that was P1 DNA is indicated above the bar delineating the pooled peaks on the sedimentation profile plots.

72

P1dlac Supercoils

Linear

Phage

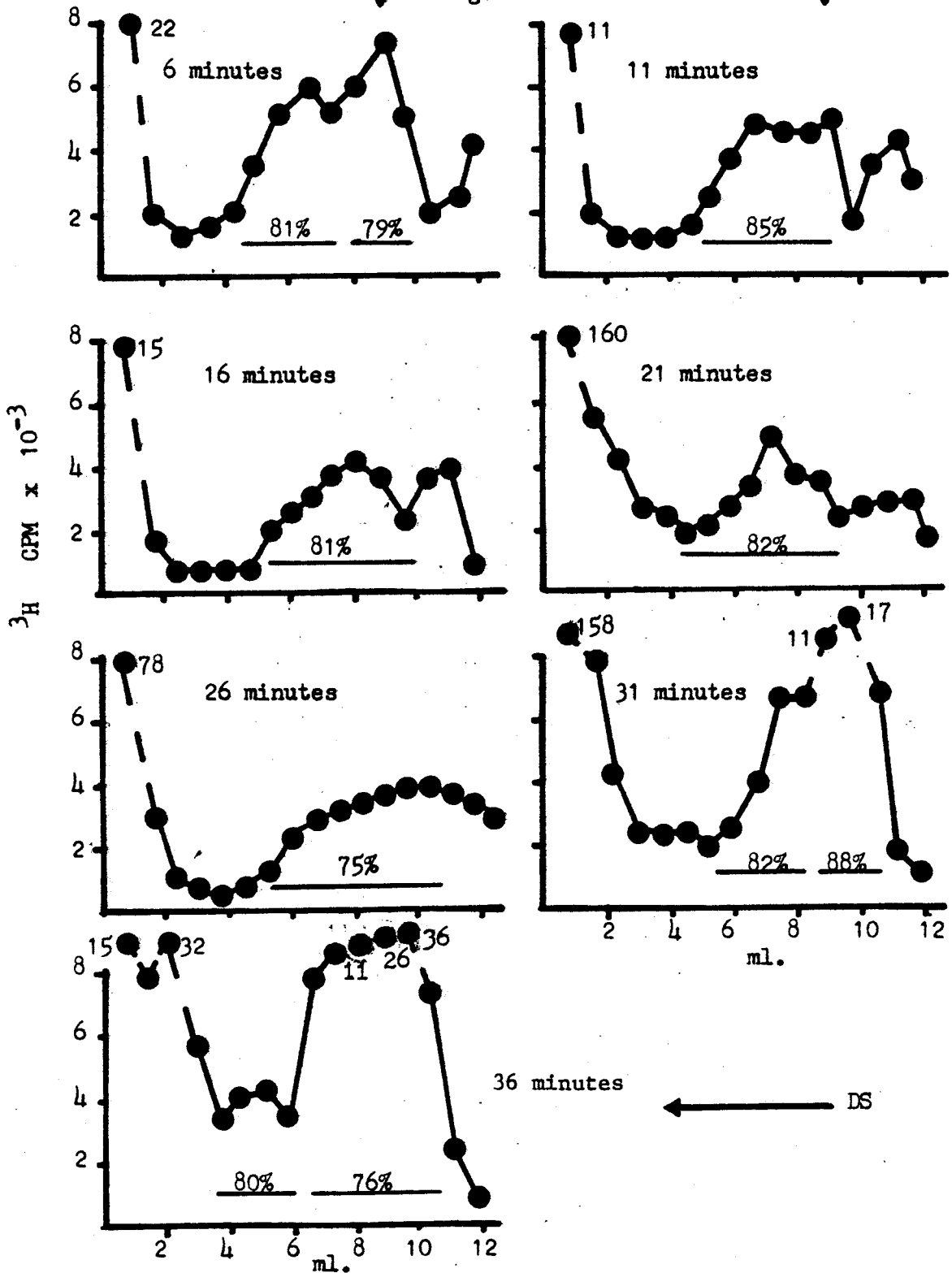
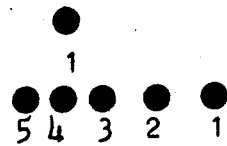
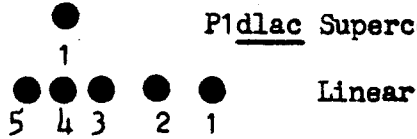


Figure 17. Generation of Pldlac lytic cycle chromosomal forms

from newly synthesized DNA: denatured DNA forms.

E. coli K12(Pldlac) (10^9 bacteria) were induced at 40°C for the time indicated. Each culture was labeled for one min with ^3H -thymidine (0.6 μCi). Cold thymidine (11.5 mmoles) was added to the cultures at the end of the labeling period to competitively inhibit further ^3H -thymidine incorporation (Figure 11). After the growth period, bacterial lysates were prepared, and the chromosomes were centrifuged through a linear 5-20% (w/v) alkaline sucrose gradient at $240,000 \times g$ and 15°C for 1.8 hours in an IEC SB283 rotor. Sedimentation was from right to left. Fractions were collected from the bottom, and TCA precipitated in preparation for radioactive determinations.

The ^3H -thymidine pulse was from 4 to 5 min of the lytic cycle for the 6, 11, 16 and 21 min samples and from 15 to 16 min of the lytic cycle for the 21, 26, 31 and 36 min samples. Only one 21 min sample is shown, since both 21 min sedimentation profiles are similar.

Above each Figure, or set of figures, is located the Relative Sedimentation Distance scale (RSD). Each set of points on the scale is labeled with the DNA species represented by that particular point on the scale. The scale is adjusted for the type (neutral or alkaline) sedimentation gradient being analyzed, and

the conditions of centrifugation.

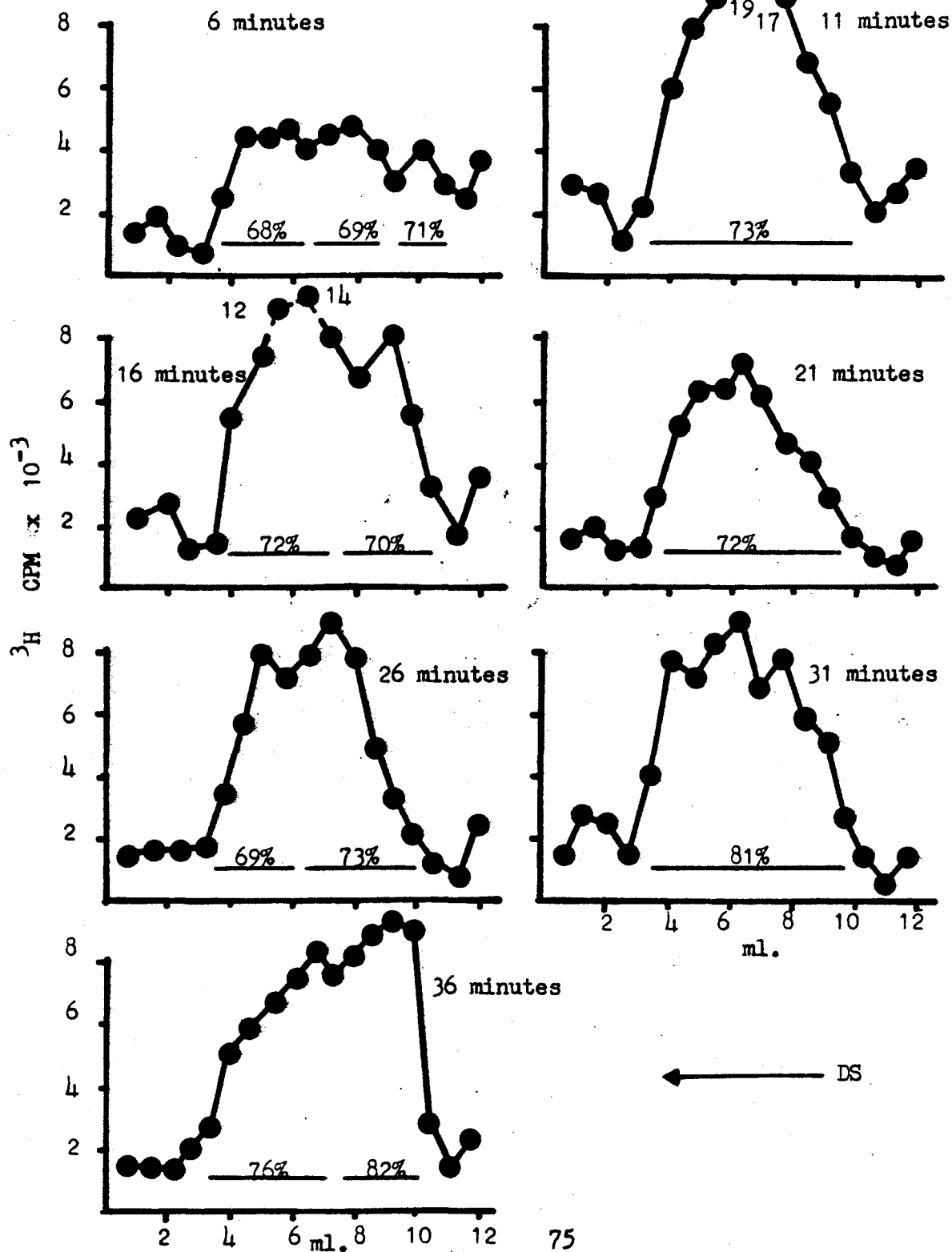
In order to confirm the genetic identity of the radioactivity labeled DNA forming the peaks of the sedimentation profiles, aliquots of relevant fractions were pooled and hybridized against P1 and E. coli DNA filters (14). The hybridization efficiency of the DNA filters was about 30%. The percent of the radioactive DNA that was P1 DNA is indicated above the bar delineating the pooled peaks on the sedimentation profile plots.

● 2 ● 1 P1dlac Supercoils ● 2 ● 1

●●●●●● Linear
10 8 6 4 2 1

◆ Phage

●●●●●● Linear
10 8 6 4 2 1



← DS

minute at fifteen minutes, and harvested twenty-six minutes after induction. This harvesting time was chosen as the time when the largest concatemers are present. As a control, a Pl infected Pl lysogen was run in parallel.

Sedimentation profiles of DNAs from superinfected Pl (Figure 18) and Pldlac (Figure 19) lysogens were similar. The individual fractions from the gradients were hybridized against DNA filters to establish genetic identities.

Combined Pl and Pldlac was detected by hybridization against Pl DNA filters. To selectively detect Pldlac, annealing of the radioactive Pl half of the Pldlac genome to Pldlac filters was blocked. This was achieved by adding excess cold Pl DNA to the hybridization samples (Table 2). Total Pl hybridizable counts for Pl and Pldlac lysogens are shown in Figures 20 and 21 respectively. Radioactivity of Pldlac alone is shown in Figure 22. Figure 23 is the distribution of Pl DNA alone, obtained by subtracting out the contribution of Pldlac from Figure 21.

Comparison of the Pldlac LCF distribution (Figure 22) with that of the Pl LCF (Figure 23) shows that the two sedimentation profiles are not superimposable. These plots establish that Pl LCF and Pldlac LCF do not frequently recombine. The two plots would have been superimposable if recombinant Pl and Pldlac predominated. These plots also show that LCF which could be 1-3 G Pl supercoils are present but that comparable Pldlac supercoils are not present; and the relaxed Pldlac strands are on the average shorter in G size than the Pl relaxed strands.

Figure 18. Sedimentation of P1 chromosomal forms during the lytic cycle of a P1 lysogen superinfected with P1 bacteriophage:denatured DNA forms. A culture of E. coli K12(P1) was superinfected with P1 bacteriophage. The superinfected lysogen was induced at 40°C, labeled with ³H-thymidine (0.6 µCi) at 14 to 15 min. Cold thymidine (11.5 mmoles) was added to the culture at the end of the labeling period to competitively inhibit further ³H-thymidine incorporation. After 26 min, bacterial lysates were prepared, and the chromosomes were centrifuged through a linear 5-20% (w/v) alkaline sucrose gradient at 240,000 x g and 15°C for 1.6 hours in an IEC SB283 rotor. Sedimentation was from right to left. Fractions were collected from the bottom, and TCA precipitated in preparation for radioactive determinations.

Above the Figure is located the Relative Sedimentation Distance scale (RSD). Each set of points on the scale is labeled with the DNA species represented, and the molecular configuration of that DNA. The numbers on the RSD refer to the G number (number of unit genomes) represented by that particular point on the scale. The scale is adjusted for the type (neutral or alkaline) sedimentation gradient being analyzed, and the conditions of centrifugation.

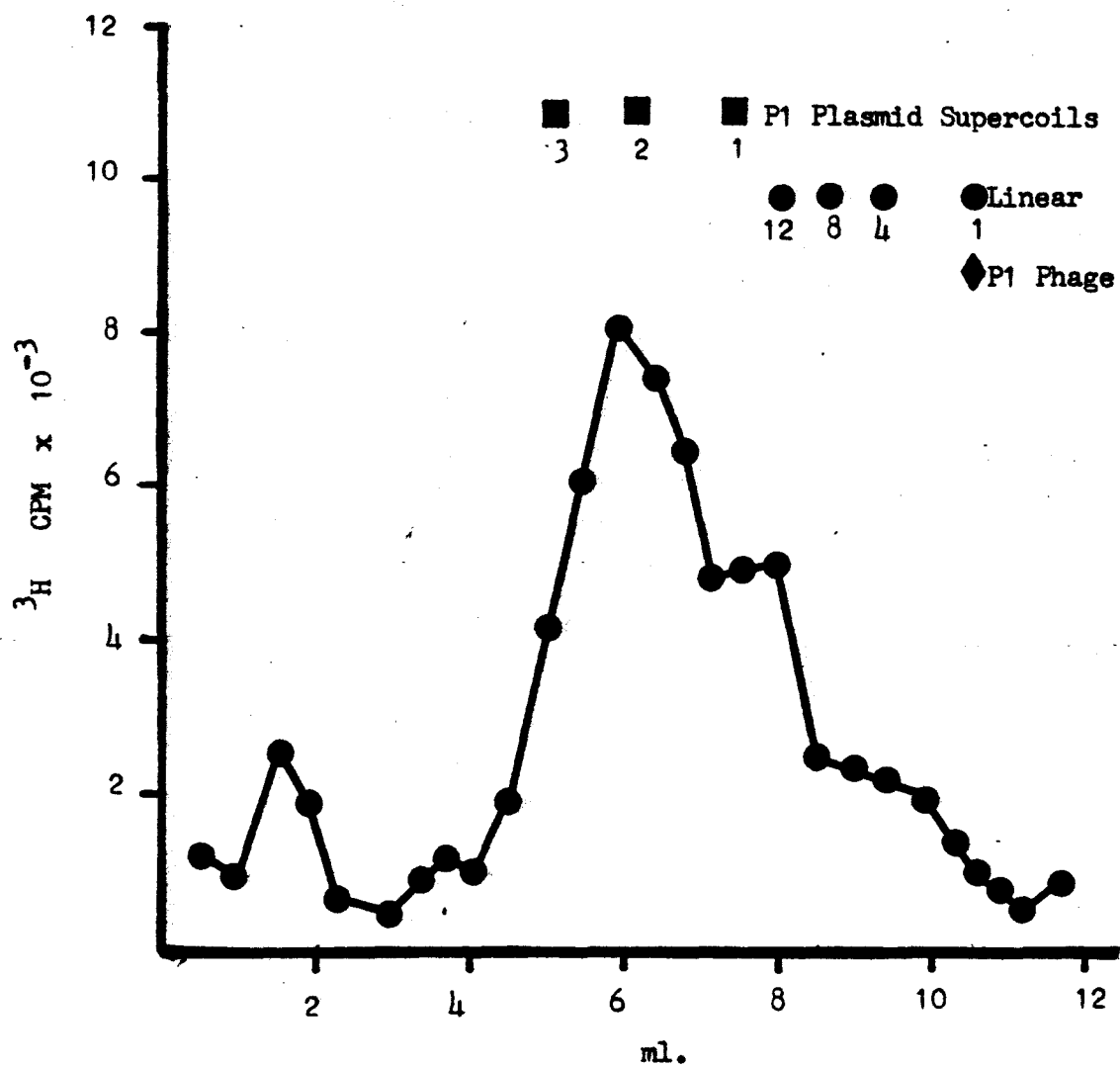
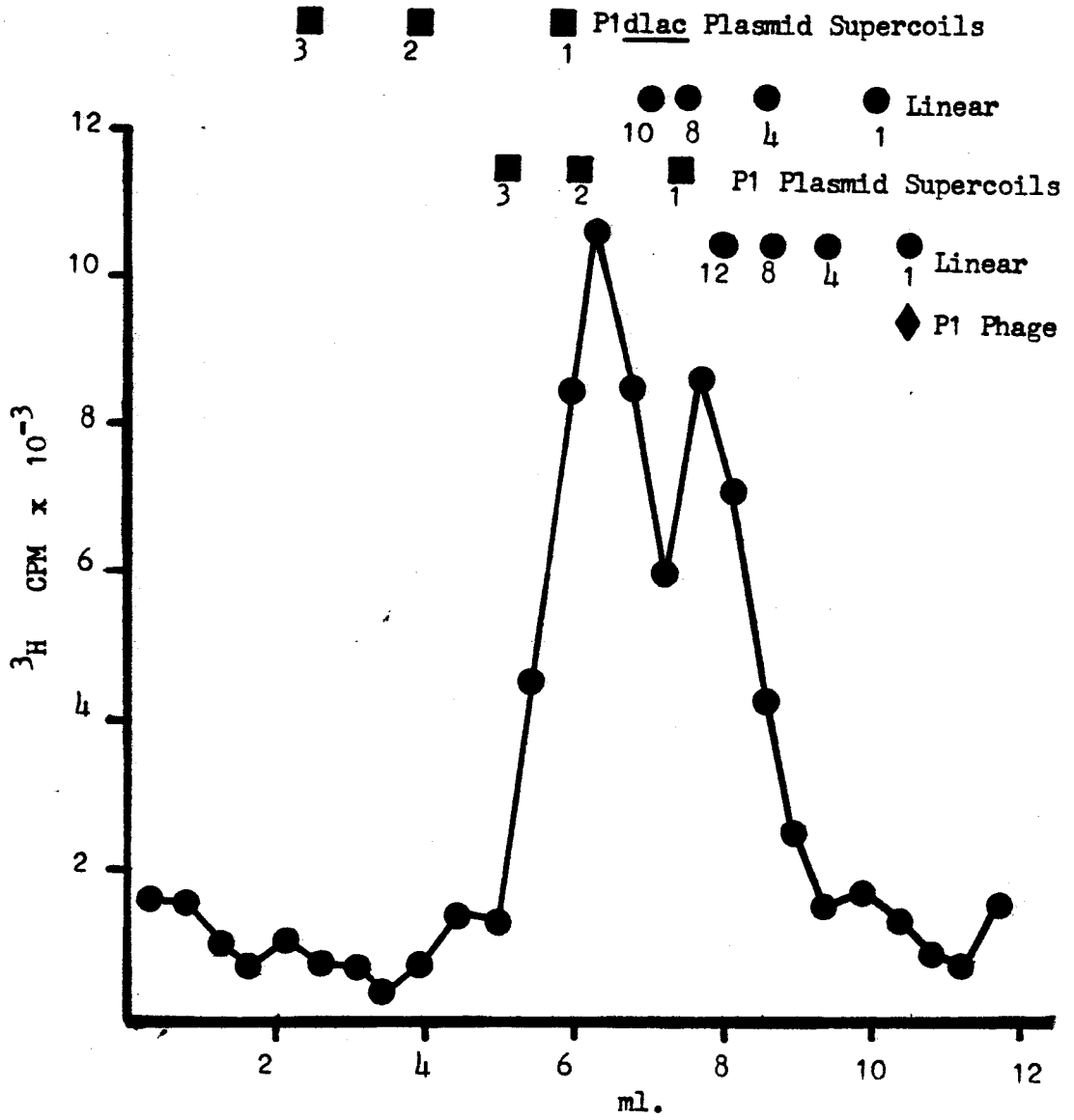


Figure 19. Sedimentation of P1 and P1dlac chromosomal forms during the lytic cycle of a P1dlac lysogen superinfected with P1 bacteriophage:denatured DNA forms. A culture of E. coli K12 (P1dlac) was superinfected with P1 bacteriophage. The superinfected lysogen was induced at 40°C, labeled with ³H-thymidine (0.6 μ Ci) at 14 to 15 min. Cold thymidine (11.5 mmoles) was added to the culture at the end of the labeling period to competitively inhibit further ³H-thymidine incorporation. After 26 min, bacterial lysates were prepared, and the chromosomes were centrifuged through a linear 5-20% (w/v) alkaline sucrose gradient at 240,000 x g and 15°C for 1.6 hours in an IEC SB283 rotor. Sedimentation was from right to left. Fractions were collected from the bottom, and TCA precipitated in preparation for radioactive determinations.

Above the Figure is located the Relative Sedimentation Distance scale (RSD). Each set of points on the scale is labeled with the DNA species represented, and the molecular configuration of that DNA. The numbers on the RSD refer to the G number (number of unit genomes) represented by that particular point on the scale. The scale is adjusted for the alkaline sedimentation gradient being analyzed, and the conditions of centrifugation.



← DS

Table 2. Percent of radioactive DNA annealed to Denhardt DNA hybridization filters (14). Purified ^3H -thymidine labeled P1 virion, Pldlac virion and E. coli SC1800 DNAs annealed to Denhardt DNA hybridization filters (17) in the presence of purified cold E. coli SC1800 (1.0 μg) and/or purified P1 bacteriophage DNA (1.0 μg) were determined. The concentrations of the purified probe DNAs were 0.03 $\mu\text{g}/\text{sample}$. The results were expressed as percent of total input radioactivity annealed to the target DNA.

Identity of ³ H-probe DNA	Identity of non-radioactive competitor DNA	DNA Hybridization Filters		
		Pl	<u>Pl</u> <u>dlac</u>	<u>E. coli</u>
Pl bacteriophage	<u>E. coli</u> (SC1800)	26.2%	23.3%	0.7%
	<u>E. coli</u> (SC1800)	0.9%	0.5%	0.2%
	and Pl bacteriophage			
<u>Pl</u> <u>dlac</u> bacteriophage	<u>E. coli</u> (SC1800)	25.2%	26.1%	0.7%
	<u>E. coli</u> (SC1800) and	0.9%	13.6%	0.4%
	Pl bacteriophage			
<u>E. coli</u> (SC1800)	<u>E. coli</u> (SC1800) and	0.3%	0.7%	1.6%
	Pl bacteriophage			
	Pl bacteriophage	0.5%	0.6%	25.6%

Figure 20. Total radioactive counts annealed to target P1 bacteriophage DNA hybridization filters: E. coli K12(P1). Aliquots of the ^3H -thymidine labeled E. coli K12(P1), superinfected with P1 bacteriophage, fractionation (Figure 18) were prepared for the Denhardt hybridization procedure (14). The hybridization reactions were done in the presence of purified E. coli SC1800 DNA (1.0 μg) to competitively inhibit nonspecific annealing of radioactive E. coli K12 DNA to the target DNA.

Above the Figure is located the Relative Sedimentation Distance scale (RSD). Each set of points on the scale is labeled with the DNA species represented, and the molecular configuration of that DNA. The numbers on the RSD refer to the G number (number of unit genomes) represented by that particular point on the scale. The scale is adjusted for the alkaline sedimentation gradient being analyzed, and the conditions of centrifugation.

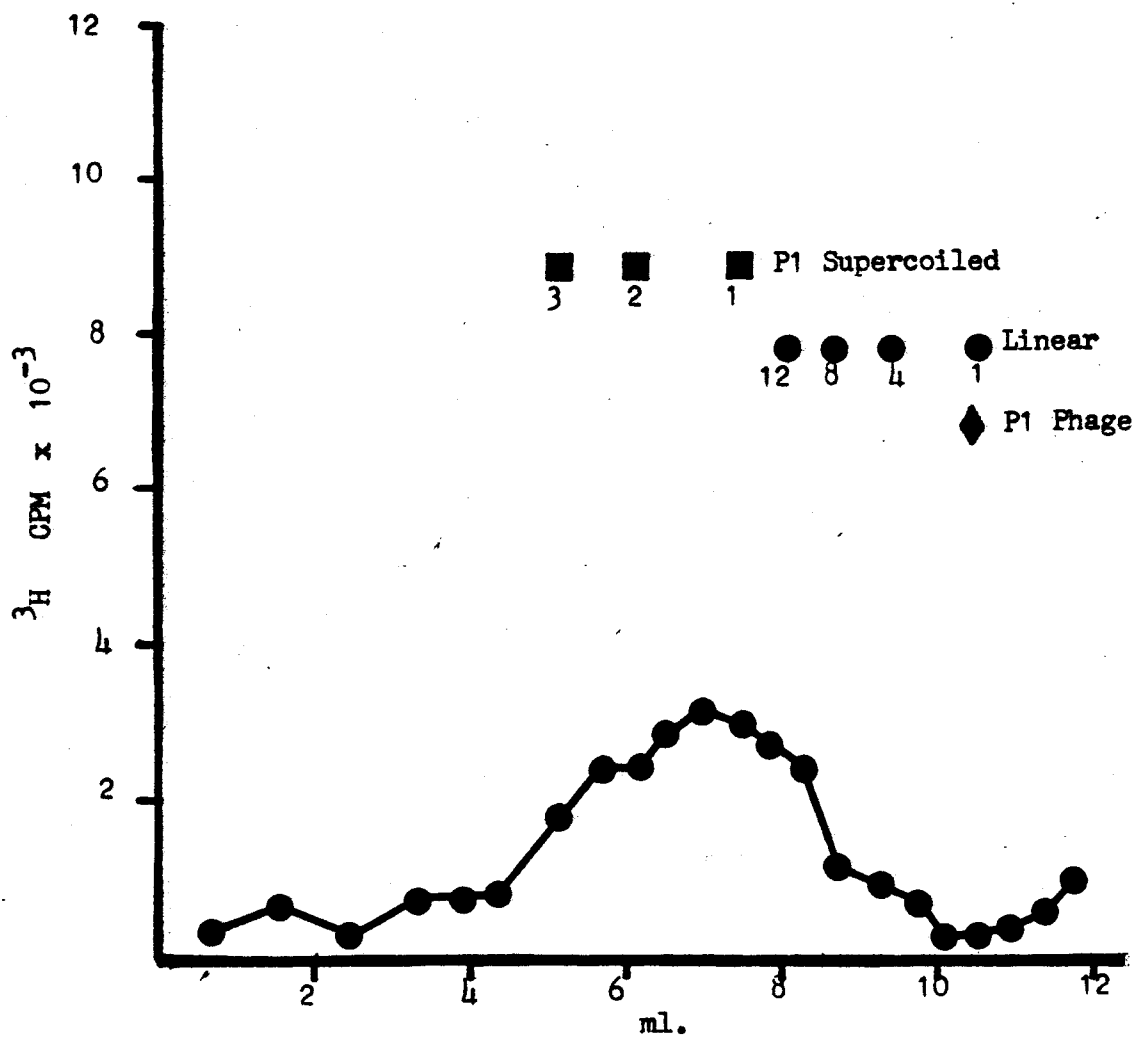


Figure 21. Total radioactive counts annealed to target P1 bacteriophage DNA hybridization filters: E. coli K12 (P1dlac). Aliquots of the ^3H -thymidine labeled E. coli K12(P1dlac), superinfected with P1 bacteriophage, fractionation (Figure 19) were prepared for the Denhardt hybridization procedure (14). The hybridization reactions were done in the presence of purified E. coli SC1800 DNA (1.0 μg) to competitively inhibit non-specific annealing of radioactive E. coli K12 DNA to the target DNA.

Above the Figure is located the Relative Sedimentation Distance scale (RSD). Each set of points on the scale is labeled with the DNA species represented, and the molecular configuration of that DNA. The numbers on the RSD refer to the G number (number of unit genomes) represented by that particular point on the scale. The scale is adjusted for the alkaline sedimentation gradient being analyzed, and the conditions of centrifugation.

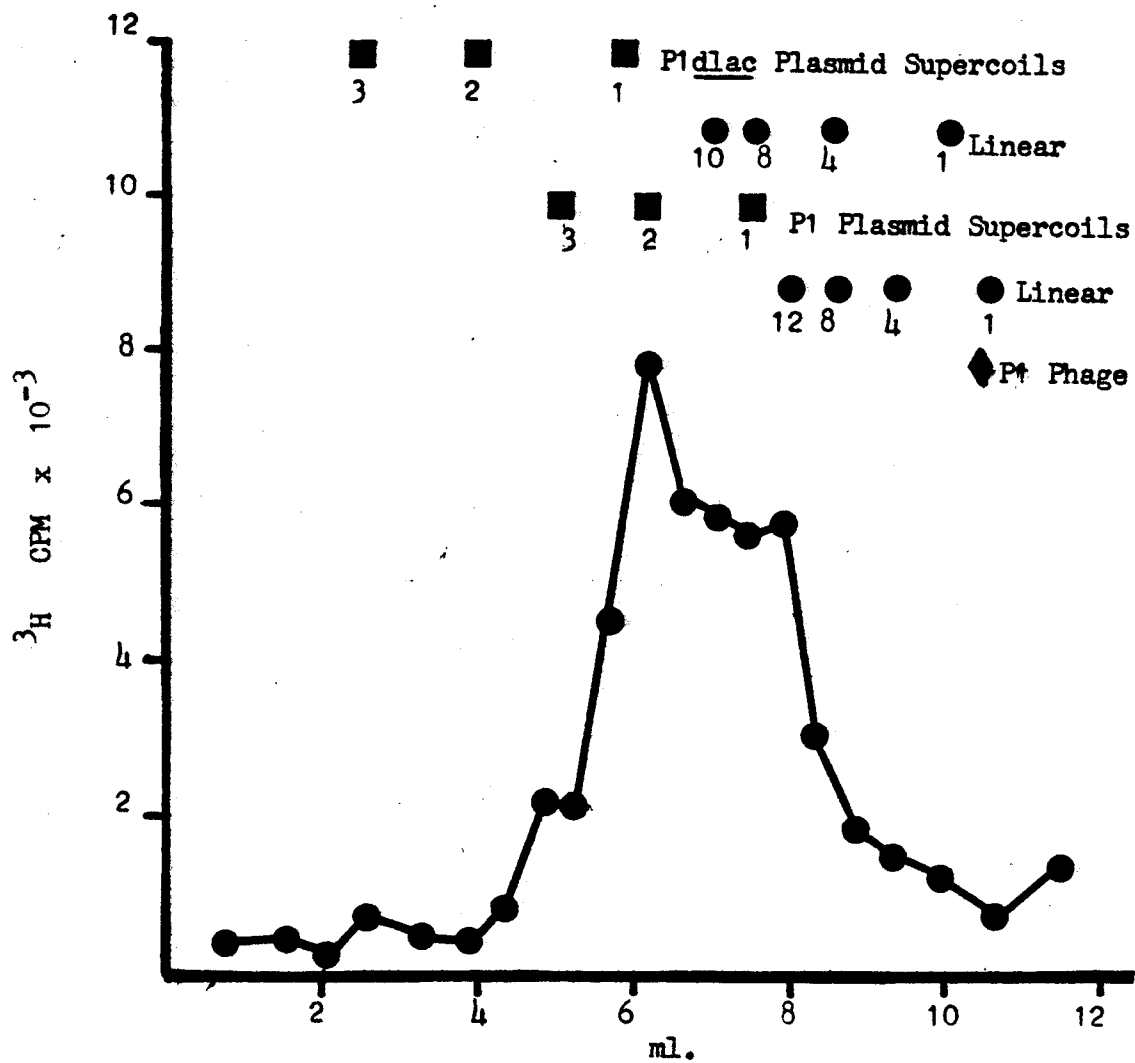


Figure 22. Location of the lac gene constituents of the E. coli K12(Pldlac) lysogen (superinfected with bacteriophage Pl) sedimentation gradients. Aliquots of the ^3H -thymidine labeled E. coli K12(Pldlac), superinfected with Pl bacteriophage, fractionation (Figure 19) were prepared for the Denhardt hybridization procedure (14). The hybridization reactions were done in the presence of purified E. coli SC1800 DNA (1.0 μg) and purified Pl bacteriophage DNA (1.0 μg) to competitively inhibit annealing of radioactive E. coli K12 DNA and radioactive Pl gene DNA to the target DNA.

Above the Figure is located the Relative Sedimentation Distance scale (RSD). Each set of points on the scale is labeled with the DNA species represented, and the molecular configuration of that DNA. The numbers on the RSD refer to the G number (number of unit genomes) represented by that particular point on the scale. The scale is adjusted for the alkaline sedimentation gradient being analyzed, and the conditions of centrifugation.

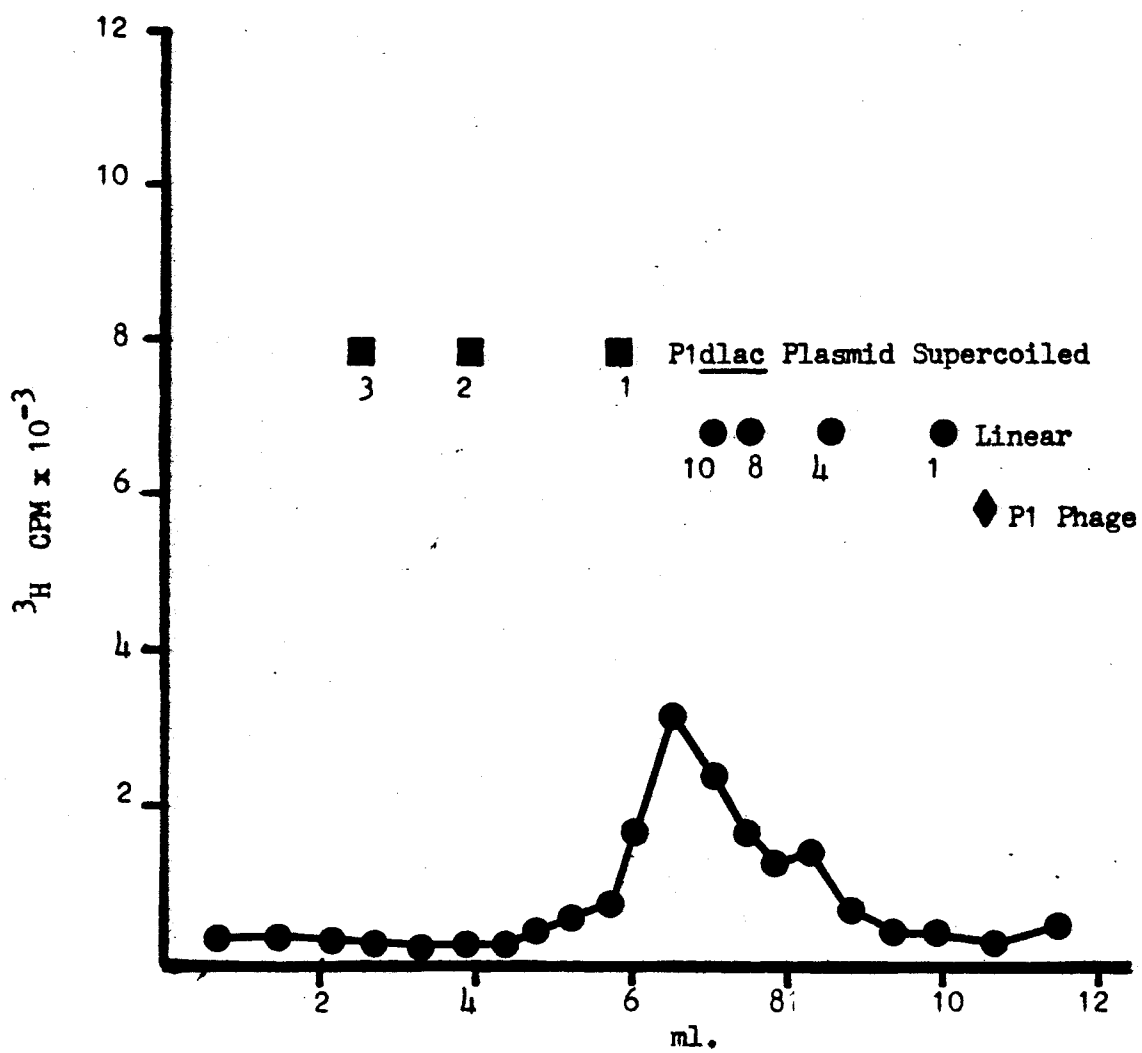
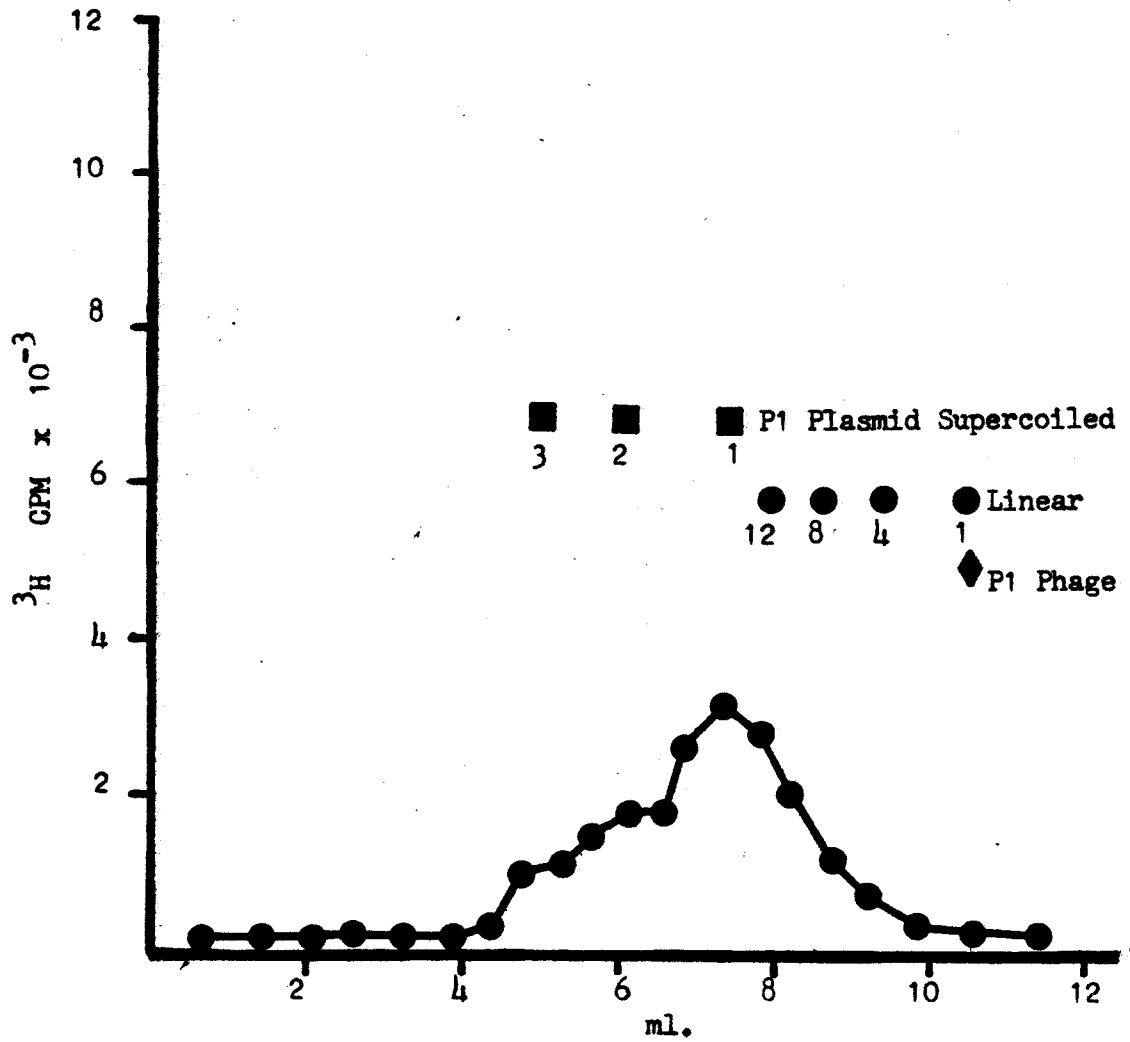


Figure 23. Location of the Φ 1 gene constituents of the E. coli K12 (Φ 1dlac) lysogen, superinfected with bacteriophage Φ 1, sedimentation gradient. The total radioactive counts annealing to Φ 1 bacteriophage DNA hybridization filters (14) were determined for the E. coli K12(Φ 1dlac), superinfected with Φ 1 bacteriophage, sedimentation gradient (Figures 19 and 21). The values obtained for the lac gene hybridization studies (Figure 22), from the same sedimentation gradient (Figure 19), were subtracted from the Φ 1 bacteriophage hybridization studies to determine the Φ 1 gene constituents of the sedimentation gradient (Figure 19).

Above the Figure is located the Relative Sedimentation Distance scale (RSD). Each set of points on the scale is labeled with the DNA species represented, and the molecular configuration of that DNA. The numbers on the RSD refer to the G number (number of unit genomes) represented by that particular point on the scale. The scale is adjusted for the alkaline sedimentation gradient being analyzed, and the conditions of centrifugation.



DISCUSSION

The primary purpose of this study was to characterize the lytic cycle chromosomal forms (LCF) of the Pl and Pl_{dlac} plasmids. An initial problem encountered was the isolation of Pl_{dlac} and Pl plasmids. Current procedures for the isolation of procaryotic plasmids are based upon variations of the cleared lysate method of Clewell and Helinski (11). The pelleting of host DNA with cell debris is a common feature of these plasmid preparations (11, 21). In this new method DNA shearing is avoided during the lysing procedure, and a dense aggregate of bacterial debris and DNA sediments through a 5.0M CsCl zone in which free DNA is buoyant. Entrapment of the plasmid in the pelleting host DNA is minimized because the pelleting occurs slowly over an 8-11 hour period. During this time there is a buoyant force driving any initially trapped plasmids through the mesh of pelleting host DNA.

Advantages of this procedure over previous methods are that: a minimum of manipulations are necessary; free plasmids are concentrated in bands; and larger plasmids can be isolated. However, the required cell debris aggregation does not occur for all bacterial species. While the procedure worked for Proteus mirabilis, Agrobacterium host DNA did not pellet (S. Farrand personal communication). The success of this procedure might be dependent on host membrane-chromosome attachments or the nature of the membrane itself.

This batch procedure was used to isolate the Pl_{dlac} and the Pl plasmid chromosomes. The Pl_{dlac} genome was found to be about twice as massive as the Pl genome (6). This difference has proven crucia-

lly useful in assessing the contribution of intramolecular recombination in concatemer formation.

Since the P1 chromosome is permuted (29), it was anticipated that the P1 lytic cycle intermediates would include concatemers. However, concatemers need not accumulate. They might only be generated as they are encapsidated. Through sedimentations in neutral and alkaline linear sucrose gradients, characterizations of the P1 and P1dlac LCF were accomplished. Pulse label experiments were used to detect the actual replicating forms (newly synthesized DNA). While pulse chase experiments allowed characterization of the fate of the synthesized DNA overtime.

Analysis of the data allowed the following specific conclusions to be drawn:

1. Most or all of the replicative forms of P1 and P1dlac are not linear monomers or theta forms (Figure 2C). Such replicative forms are comprised of base paired single strand components that do not exceed 1G. Such single strands would have been detected in large quantities in the alkaline sucrose gradients of the pulse chase and pulse harvest experiments. However, only a small fraction of the recovered radioactivity was present in that portion of the alkaline gradients representing less than 1G. This small fraction might merely be Okazaki fragments (41). With theta and monomeric linear replicative possibilities thus eliminated, the majority of the

replicative forms are probably rolling circles (Figure 2D) and/or firewheels (Figure 2E). Both of these possibilities are consistent with the observed multi-G single strands, detected through the analyses of newly synthesized DNA in alkaline sucrose sedimentation gradients.

2. Newly synthesized monomers are probably a recombination product. The pulse label experiments revealed that radioactive label is rapidly incorporated into monomers and concatemers regardless of the time of bacterial harvesting. But since monomers are not replicative forms, it can be deduced that monomers are quickly generated from concatemers by intramolecular recombination (Figure 2F).
3. Recombination is not a primary contributor to concatemer formation. If it were, P1 and Pldlac would have been united by recombination in the superinfection experiments, with the consequence that P1 and Pldlac genomes would form co-sedimenting complexes. However, discrete sedimentation profiles were observed for their respective LCF. In fact the sedimentation profiles of the individual LCFs were nearly the same as those of the non-superinfection experiments. These observations argue that P1 and Pldlac LCF do not undergo exten-

sive recombination. Hence, intermolecular recombination cannot be common enough to be the dominant concatemer forming process.

4. Most of the newly generated monomers are converted to replicative forms. While monomers are generated throughout the lytic cycle, most eventually become concatemer constituents. But intermolecular recombination as deduced above cannot be the major concatemer forming process. Hence, the monomers probably become concatemer constituents through a replicative process.
5. Since concatemers are produced in the Pldlac lysogens and they are not primarily generated by recombination, Pldlac must be proficient in concatemer formation. However, the superinfection experiments demonstrated that the Pldlac concatmers formed did not contain as many G units as those of Pl. This deficiency in large concatemer formation is cis dominant, as it is not alleviated by superinfecting Pl. It does not seem necessary to postulate a Pl gene defect hypothesis to explain the smaller average concatemer size. The smaller average Pldlac concatemer size could merely be a consequence of the size of the Pldlac genome. Since the Pldlac genome is two times the size of the Pl genome, its conca-

temers will have (roughly estimated) twice the susceptibility to intramolecular recombination as would Pl. Intramolecular recombination is directly related to the concentration of homologous gene segments within a concatemer. Therefore, once concatemers are formed, intramolecular recombination becomes a factor that has to be considered. Intramolecular recombination between the genomic units of the concatemers would generate smaller concatemers. Such concatemer size reduction is expected to proceed further for larger genome units.

6. At the onset of virion maturation both PlS and PlB chromosomes are evident. It is striking that these early PlB and PlS chromosomes are equally represented, since about 90% of the mature virions are PlB (59). There must be a switch from PlS to PlB maturation processes as the lytic cycle progresses. It can not yet be assessed whether PlS protein capsids are physiologically precursors of PlB, or whether PlS are a branch end-product of maturation.

Some of the reduction of concatemer size that accompanies virion maturation must be a direct consequence of the headful packaging system. It is expected however, that there is a second contributing factor. Studies of bacteriophage Pl chromosomes in-

dicates that three to four PlB virion chromosomes are precessionally cut from a single concatemer, and that one of three mature chromosomes has an end at map unit 92 (2). This fact suggests that some of the shortening of concatemers reflects a site specific nuclease action at map unit 92, which produces chromosome ends required by the headful packaging system.

These findings motivated the following scheme for lytic cycle chromosomal processes:

The initial lytic cycle replicative form is a rolling circle. It may further develop into a firewheel. Tails of the generated concatemers might develop forks, if replication is initiated within them (Figure 2B). Concatemers appear early in the lytic cycle (by 6 min) and eventually reach 15G units or more in size. The supercoil accumulation that begins after the initial supercoil decrease, seen at 8 min, results from intramolecular recombination within concatemers. The existence of a recombinational hotspot, which is responsible for the linearity of the bacteriophage (51), argues that generalized recombination is the minor contributor to this recombination of monomers. Late in the lytic cycle, long concatemers are probably split into shorter concatemers. Some of the concatemer breakdown is achieved by nuclease action at map unit 92 (2). Ends thus produced are used to initiate headful packaging. The final products are 80% PlB virions and 20% PlS virions (60). A further role of the site specific

nuclease can be speculated upon. If the "packing" nuclease has a higher affinity for supercoils than for relaxed DNA, it would effectively scavenge supercoils for the headful packaging system first. Supercoiled concatemers thus opened would suffice for filling P1B capsids. But, 61×10^6 dalton supercoiled monomers also cleaved in this manner could not fill P1B capsids. They could, however, fill the 24×10^6 dalton capacity of the P1S capsids. Such an early scavenging of supercoils could be responsible for the early maturation of P1S virions.

A continuation of this study would include the actual isolation of the replicative forms, as opposed to their mere detection as radioactive LCF. Chromosomes from sucrose gradients have generally been found to be unsuitable for electron microscopy because of contamination of the samples with cellular debris. LCF isolations have been achieved with other phage infected systems. However the P1-E. coli system poses special problems. The lambda-E. coli system is the next smallest phage system that has been characterized. The lambda bacteriophage chromosome is half the size of the P1 phage chromosome. Lambda LCF were isolated by isopycnic centrifugation techniques and found to be theta or low G rolling circle chromosomes. For P1 such isolations should ultimately be feasible. They will be complicated however, by the size of the P1 chromosomes and their higher sensitivity to shear breakage.

Bacteriophage T4 is twice the size of P1, and its LCF have been isolated. However, their isolation has been relatively simple, be-

cause T4 totally degrades the host genome (25). Hence infected host bacteria could be gently lysed and spread for electron microscopy (33) or autoradiography (9). In the Pl-E. coli system, E. coli DNA is not degraded. Hence, there is the extraordinary complication of separating large LCF from abundant E. coli DNA.

These complications could be minimized by isolating the very earliest LCF generated after induction of the lytic cycle. It may also prove feasible to utilize deletion mutants of Pl which have lost a large portion of the Pl genome but still retain normal replicative capacities. Their use would diminish the problem of chromosomal shear due to their reduced size.

There may be another novel problem in the Pl-E. coli system. LCF, harvested as early as 6 min after induction of the lytic cycle, were pelleted in the neutral gradient. This fact indicates that they sedimented at least as fast as 15G LCF relaxed forms. It seems unlikely that the LCF could have grown so large in such a time period, especially since it takes approximately 1 min for 1 round of replication to occur. Thus, it is suspected that the pronase-SDS, though proven effective in other phage systems (46,58,65), did not achieve a complete separation of LCF from bacterial components at pH 8 (though alkaline conditions allowed the desired separation). Pl directs the synthesis of a ban membrane protein (13) which functions during Pl chromosome replication. The Pl ban protein can act as a substitute for the E. coli dna B membrane protein. It is possible that it is the replicative complexes with ban protein that is resistant to pronase-SDS. Future experiments can be performed with

ban-mutants to determine if their replicative complexes will be pronase sensitive.

A comparison of LCF of P1 and other bacteriophages is present in Table 3. P1 is least similar to phage T7, but has some LCF characteristics in common with lambda and P22 phages. It is most like T4 phage in its replicative mode. However, P1 differs from T4 most significantly in that T4 has a very high rate of generalized recombination during the lytic cycle. It can be stated that the replicative form of P1 during the lytic cycle has characteristics of several other phage systems but its mode is not identical to any of them.

Table 3. Bacteriophage lytic cycle chromosomal forms. The lytic cycle chromosomal forms of several DNA bacteriophages are listed for comparison purposes. (ND-not detected) (4,7,17,18,20,24,25,28,29,49, 53,57,58,63,64)

Phage	Phage DNA	Replicative Form				Accumulate		generalized recombination
		branched monomers	Theta	rolling circle	firewheel	supercoils	concatemers	
P1	duplex, terminally redundant, permuted linear	ND	ND	throughout	possibly	near the end	throughout, 10-15G	low
P1 _{dlac}	duplex, linear	ND	ND	throughout	possibly	fewer than P1 near the end	throughout 6-10G	low
lambda	duplex, linear repetitious ends	ND	yes	yes	no	late	late 2-6G	high
T4	duplex, linear, terminally redundant, permuted	ND	ND	yes	yes	late	late 2-G	very high
T7	duplex, linear terminally redundant	early	ND	ND	ND	ND	yes	ND

P22	duplex, linear, terminally redundant, permuted	early	prob- ably early	late	ND	late	late less than 10 G	moderate
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SUMMARY

Temperate bacteriophage P1 was isolated by Bertani in 1951 from a lysogenic E. coli. The linear chromosomes of P1 phage have a molecular mass of 66×10^6 daltons, a 12% terminal repetition, and are genetically permuted. After injection into the host bacterium the phage DNA circularizes through recombination of its repetitious ends, forming a 61×10^6 dalton plasmid. Specialized lac⁺ transducing derivatives are sometimes generated during low frequency transduction. Previous studies have indicated that the P1dlac plasmid is larger than the virion chromosome (overlarge).

The purpose of this study was to characterize the lytic cycle replicative forms (LCF) of P1 and P1dlac. A novel and simple batch preparation for the isolation of large plasmids was first developed. A gently prepared crude lysate was sedimented into a CsCl-ethidium bromide pad. After overnight centrifugation bands of supercoiled and relaxed DNA could be visualized by UV excited fluorescence, and isolated for further purification. This methodology was used to demonstrate that P1 supercoils accumulate as the lytic cycle progresses, and to isolate the P1dlac plasmid. The overlarge character of the P1dlac plasmid was confirmed.

Neutral and alkaline linear sucrose gradients were used to characterize the LCF of Pl and Pldlac. ^3H -thymidine pulse harvest procedures were used to selectively label LCF with newly synthesized DNA. Pulse chase experiments were used to follow the molecular evolution of LCF during the lytic cycle.

Analysis of the sucrose gradient results revealed that linear monomers and theta forms are not the major LCF. Newly synthesized circular monomers are present during the lytic cycle and are probably derived from concatemers through intramolecular recombination. Most of these newly generated monomers are converted to replicative forms. Most replicative forms have concatemer components.

Chromosomes from bacteria in which Pl and Pldlac were jointly replicating were also analyzed by sucrose gradient sedimentations. Discrete sedimentation of Pl and Pldlac LCF occurred. This revealed that intermolecular recombination is not frequent enough to be the major contributor to concatemer formation.

A scheme for lytic cycle chromosomal processes based on these findings is proposed. The initial lytic cycle replicative form is a rolling circle form which may further develop into a firewheel replicative form. Concatemers appear early in the lytic cycle (by 6 min) and eventually reach 15 genome units or more in size. Tails

of the generated concatemers might develop forks, if replication is initiated within them. The supercoil accumulation that begins after the initial supercoil decrease, seen at 8 min., results from intramolecular recombination within concatemers. Late in the lytic cycle, long concatemers are probably split into shorter concatemers. Some of the concatemer breakdown is achieved by nuclease action at map unit 92. Ends thus produced are used to initiate headful packaging. The final products are 80% FLB virions and 20% FLS virions.

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Sept. 28, 1979
Date

Marvin Stodolsky
Director's Signature