ISOLATION AND CHARACTERIZATION OF DELETION

MUTANTS OF BACTERIOPHAGE LAMBDA

Thesis by

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ABSTRACT

A method for the isolation of deletion mutants of lambda is re-ported utilizing thermal inactivation as a selective enrichment tech-nique. Thermal inactivation of lambda is shown to depend upon the amount of DNA in the phage, the temperature of inactivation and the ionic make-up of the medium. A phenotypically heat resistant, less dense class of phage is reported which, upon growth, returns to the density and heat characteristics of wild type phage.

The several hundred deletion mutants isolated have many properties in common with the lambda b2 mutant. All mutants are of lambda immun-ity and host restriction type. In these mutants, the amount of DNA deleted, calculated from their density, varies from 10 to 30%. All of the mutants like b2 form abortive lysogens. Preliminary experiments indicate that the mutants retain the recombination function of lambda. Some of the deletion mutants show reduced recombination for the region deleted, the middle segment of the genome.

However, a number of the deletion mutants exhibit abnormally high recombination for the middle segment of the genome and in one of these mutants, lambda b130, a physical discontinuity in the DNA of the phage occurs at or near the locus which manifests high recombination. This discontinuity resembles in structure the cohesive ends of lambda DNA.

In an attempt to explain the properties and bizarre structure of the chromosome of b130, a model for lambda integration and excision is proposed. As a result of this model, the high recombination of certain lambda deletion mutants is thought to be due to the inclusion of a

small region of host DNA into the middle segment of the DNA of these

mutants. It is proposed that this included region resembles closely

the cohesive ends of lambda leading to the production of a new set

of cohesive ends and localized high recombination. The model intro-

duces several new notions concerning the integration of lambda into

the chromosome which are experimentally testable.

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GENERAL INTRODUCTION

Of the more complex bacteriophage known, lambda and T4 have

been studied in the greatest detail with respect to genetics, phy-

siology and morphology. These studies have been greatly aided by the

isolation of conditional lethal mutants (Epstein, et. al., 1963)

which behave normally under one set of conditions but are defective

under another set of conditions.

Approximately 70 genes have been identified in T4, and 30 genes

have been found in lambda. However, the genetic maps of both phage

contain large gaps. These gaps may not contain functional genes or,

alternatively, these gaps could reflect the presence of non-essential

genes which are not required for basic viral growth but perform non-

vital functions, such as in recombination. Some presumed non-essen-

tial phage genes could also provide information already contained in

the host and thus duplicated by the phage. On the assumption that an

average gene length is 1000 nucleotide base pairs of the phage DNA,

T4 should contain about 200 genes and lambda should contain about 50

genes. It is reasonable to assume that a majority of the essential

genes of both phage types have been identified suggesting the presence

of many non-essential genes in each phage.

A genetic map of lambda is presented in Figure 1. The function

of the various genes has largely been determined or inferred. Genes

A, B, W, C, V, D, E and F are concerned with the conversion of intra-

cellular, non-infective phage DNA into infective phage DNA and the

production of the head proteins (Siminovitch, personal communication)

Figure 1. A genetic map of lambda. The relative lengths of the left

arm (containing genes A through J), the middle segment (between J and

cI) and the right arm (cI through R) were determined from electron

microscopic measurements derived from experiments of R. Davis (person-

al communication). The relative locations and sizes of genes in the

two arms are based on genetic data of Parkinson (1967).
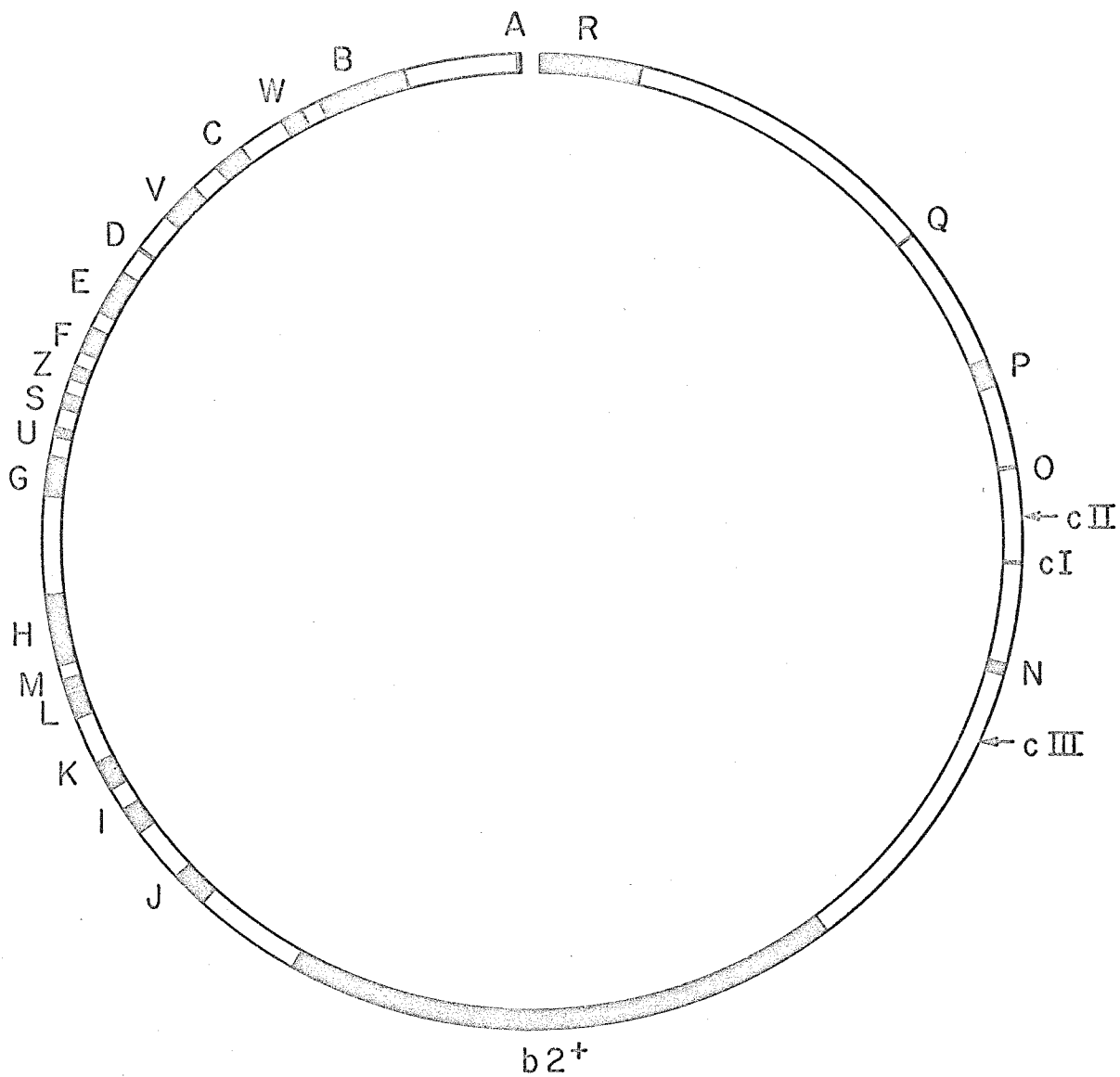
(Weigle, 1966)(Parkinson, 1967). Genes Z, S, U, G, H, M, L, K, I and J are concerned with the production of phage tails (Siminovitch, personal communication)(Weigle, 1966)(Parkinson, 1967). Thomas (1966) concluded that gene N is responsible for the induction of genes O and P which are concerned with DNA synthesis (Eisen, et. al., 1966). Gene Q is thought to be the inducer of the late genes located in the left arm (Dove, 1966) and gene R is responsible for the lambda endolysozyme (Campbell and Del Campillo-Campbell, 1963).

As can be seen from the map there are several large gaps. There is preliminary evidence for a gene between Q and R (Siminovitch, personal communication) and two genes may exist between cI and cII (Eisen, et. al., 1966). Zissler (1967) has evidence for a non-essential gene to the left of cIII and Radding, Szpirer and Thomas (1967) have located the exonuclease gene to the left of cIII. Thus, while in the region between genes J and cIII several genes have been identified, this region comprises almost 1/3 of the total genome indicating that numerous unidentified genes may be located in this interval.

Bacteriophage T4 has a relatively simple life cycle. After adsorption of the phage to a susceptible host the phage DNA is injected into the host. The DNA replicates and directs the formation of new phage particles. After a short time the cell lyses and 100-200 phage particles are released.
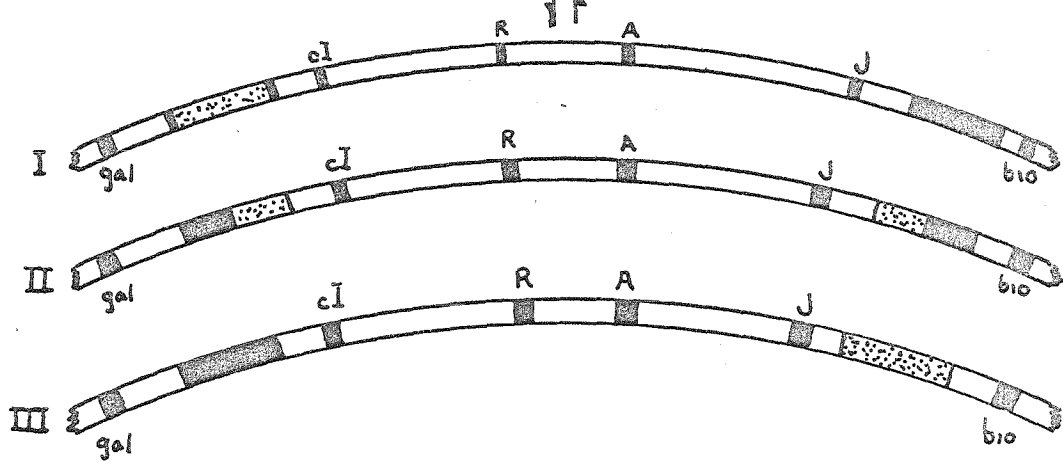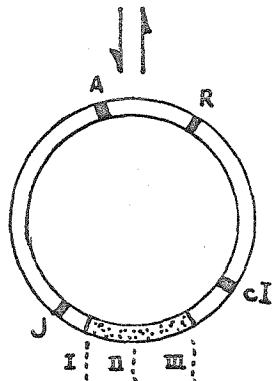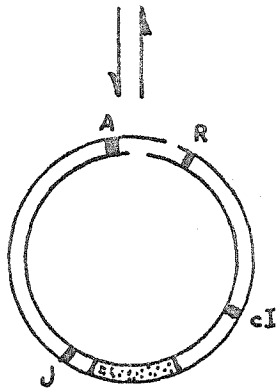
The life cycle of phage lambda has an added dimension compared to that of T4. Upon injection of the lambda DNA into a host, either a process similar to that described above for T4 takes place or the

phage DNA interacts with the host to establish the lysogenic state.

To attain the lysogenic state a number of different steps must be taken (Figure 2). The infecting DNA becomes circular through hydrogen bonding of the cohesive ends and this circular molecule is then closed to become a covalently bonded circle (Young and Sinsheimer, 1964)(Ogawa and Tomizawa, 1967b). A portion of the middle of the vegetative chromosome then pairs with the lambda-specific attachment site of the host chromosome. A lambda directed mechanism then facilitates a reciprocal recombination event leading to insertion of the lambda genome into the host chromosome (Signer and Beckwith, 1966). This model of phage events leading to insertion was first postulated by Campbell (1962) and has since received substantial experimental support (Rothman, 1965)(Franklin, Dove and Yanofsky, 1965).

Some of the phage genes whose functions are necessary for establishing and maintaining the lysogenic state have been identified. Bode and Kaiser (1965) demonstrated that the cII and cIII gene functions are required to establish the lysogenic state but are not required to maintain lysogeny. The cI gene controls the formation of the lambda immunity substance (Kaiser and Jacob, 1957) and is required to maintain the lysogenic state. The immunity substance, or repressor, acts on the prophage DNA in a manner which does not allow the lambda genes to function. This repressor also acts on any superinfecting phage DNA of the same immunity type to keep it from replicating or functioning (Wolf and Meselson, 1963). Thus a cell lysogenic for lambda is also immune to superinfection by lambda phage.

Figure 2. Integration and excision of the lambda genome. At the top
of the figure is a schematic representation of the lambda vegetative
genome after infection of the host. This is interconvertible with the
hydrogen-bonded ring structure shown below the linear molecule. The
hydrogen-bonded ring is interconvertible with a covalently bonded ring
structure shown as the third figure from the top. Pairing of the co-
valent circular molecule is depicted with an arc of the circular host
DNA at the lambda attachment site located between the gal and bio genes.
The dashed lines connecting the two structures represent places where
reciprocal recombinational events could occur. The bottom three arcs
represent the host DNA after insertion of the phage genome. The three
figures represent three ways the prophage ends would be structured if
recombination had taken place at each one of the dashed lines in the
middle figures. The drawing is schematic only and no attempt was made
to draw the structures to scale.

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A   J   cI   R

A   R

cI

J

A   R

cI

J

I : n : III

gal   bio

cI   R   A   J

I   gal   bio

cI   R   A   J

II   gal   bio

cI   R   A   J

III   gal   bio

If the repressor molecules are inactivated, the prophage genes become derepressed and the excision of the prophage genome follows (Lieb, 1966). The excision process is thought to be essentially the reverse of the insertion scheme (Figure 3). The prophage forms a loop with the phage homology region pairing with the host homology region. The lambda integration system then facilitates a reciprocal recombination event to release the phage genome in the form of a circle. After this, the lytic cycle ensues.

There are several unanswered questions relating to the integration-excision mechanism. The integration and excision events are very precise since the majority of the phage issuing from independent lysogens have identical DNA content. Also, lambda only integrates into the host between the gal and bio genes, suggesting that the lambda integration system only recognizes one small portion of the host chromosome - the lambda attachment site. Conversely, the lambda genome is inserted in the same way every time, suggesting that the integration system only recognizes one portion of the lambda genome - the integration site. It is not known precisely where the integration event takes place relative to the phage and bacterial homology regions. If the recombination occurs within the homology region, the phage and host homology regions are deposed to the ends of the prophage genome in some unknown pattern (Figure 2, II at bottom). If the recombinational event occurs at the ends of the homology regions, these homology regions would be distributed one at each end (Figure 2, I or III at bottom). The question of where each region is located in the prophage state

is moot, if the phage homology region and the bacterial attachment site
are indeed homologous and equal in length. If they are, it would not
matter how they are deposed after integration since they would now
be identical.

That the integration-excision mechanism is not always precise is
evidenced by the isolation of defective, galactose-transducing phage,
lambda dg. The defectiveness of these phage is due to the loss of
lambda genes starting with J and continuing in a sequential fashion
up to A, depending upon the dg strain (Campbell, 1963). The dg phage
also contain some of the gal genes derived from the host chromosome and
thus are able to transduce these genes into another host. A proposed
scheme for the formation of the lambda dg genome is shown in Figure 3
(right side). A mispairing occurs following induction. The mispaired
loop is excised with the resulting phage genome carrying some gal genes
in place of phage genes. Alternatively, excision of lambda dg phage
might be due to some mechanism other than the phage excision mechanism.
A random cutting at both ends of the prophage genome with little
specificity would result in the creation of some phage with the host
gal region and a loss of phage genes from the right end of the prophage.
However, the model presented in Figure 3 has been favored, otherwise
an ad hoc assumption would be required to permit the union of the pro-
phage ends after excision by random cuts. The lambda dg chromosome
is thought to be similar in structure to a lambda wild type chromosome
with the prophage ends covalently linked (Hogness and Simmons, 1964).

Much has been said about the phage homology region so far and

Figure 3. Excision of the lambda prophage and the function of lambda dg. At the top of the figure is a representation of the host DNA containing an inserted lambda genome. The left figures represent the looping of the chromosome following phage induction to allow the prophage ends to pair. This results in a recombinational event which leads to the excision of a circular lambda DNA molecule. The figures on the right represent a mispaired loop in which the original prophage ends have not paired. Instead the gal genes of the host are contained in the loop and the J gene of lambda is not in the loop. A recombinational event leads to the excision of a phage genome lacking the J gene but containing the host gal gene. As in Figure 2, the drawings are schematic only and no attempt was made to draw the structures to scale.
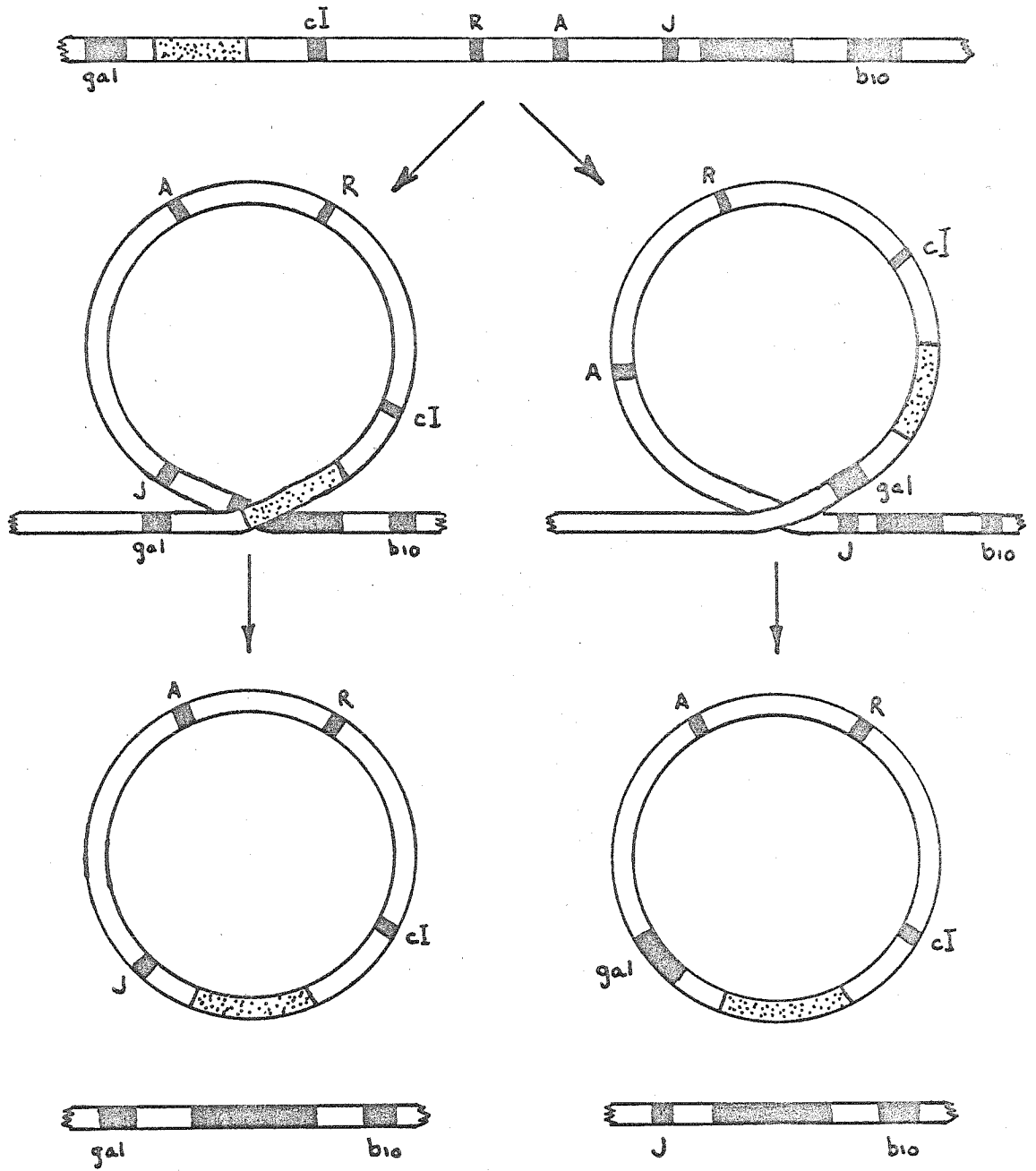
Formation of vegetative λ
from prophage

Formation of λ dg from
prophage

there is some evidence for its existence. The deletion mutant,

lambda b2 (Kellenberger, Zichichi and Weigle, 1961), has 18% of the

DNA between genes J and cIII deleted (Jordan, 1964). This mutant

can perform all of the functions of lambda except the establishment

of a stable lysogenic state. The lysogenic process works up to a

point, but the phage genome is not capable of inserting itself into

the host chromosome. Nevertheless, all other lysogenic functions

occur and the lysogenic state can be established and maintained.

However, since the lambda b2 DNA is repressed but not inserted into

the host DNA, it does not replicate and upon cell division the phage

genome segregates with one daughter cell or the other (Zichichi and

Kellenberger, 1963). Thus one of these daughter cells is no longer

lysogenic and soon acquires sensitivity to lambda (Ogawa and Tomizawa,

1967a).

It has been postulated that the b2 deletion mutant lacks the phage

homology region and thus cannot pair with the host homology region

(Fischer-Fantuzzi, 1967). That the homology region is missing in

lambda b2 is supported by the analysis of the gene order of the pro-

phage which indicates that the homology region must occur somewhere

in the interval deleted in the b2 mutant (Calef and Licciardello,

1960)(Rothman, 1965).

All of the foregoing statements indicate that a major segment of

the lambda chromosome contains non-essential or even non-existent genes.

Phage mutants lacking portions of this region occur. This region may

appear to be dispensable genetically; however, various portions of the

region are involved in the integration and excision functions of the lambda genome. Thus the availability of a variety of b2-type deletion mutants of lambda would be advantageous in the determination of the size of the homology region, the structure of the prophage ends, the process of integration and excision and the identification of any non-essential genes occurring between J and cIII.

These considerations motivated the isolation and characterization of new lambda deletion mutants reported here. A novel approach for selection of deletion mutants involving thermal inactivation is used and experiments on the response of lambda to heat inactivation are described. New deletion mutants have been found and characterized to some degree, with the conclusion that most of these deletion mutants are similar to lambda b2 in several properties but vary considerably in the amount of DNA deleted. A new class of deletion mutants was discovered which displays abnormally high recombination in a localized region of the chromosome. This novel property is correlated with a discontinuity in the phage DNA in this region.

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Part I

ISOLATION AND CHARACTERIZATION OF DELETION

MUTANTS OF BACTERIOPHAGE LAMBDA

INTRODUCTION

Large sections from the central portion of the chromosome of bacteriophage lambda can be deleted without loss of essential viral functions. While these deletion mutants grow normally in their bacterial host, nevertheless, the deleted regions appear to be functionally important for integration into the host chromosome (Kellenberger, Zichichi and Weigle, 1961a) (Franklin, 1967b), and may contain viral genes not directly concerned with vegetative growth and maturation.

The most well characterized deletion mutant of lambda, b2, (Kellenberger, et. al., 1961a) has an 18% deletion of DNA near the middle of the vegetative genome (Jordan, 1964) and is not capable of establishing a stable lysogenic state. It has been suggested that lambda b2 lacks the homology region (Campbell, 1962) necessary for insertion of the phage genome into the host chromosome at the lambda attachment site (Fischer-Fantuzzi, 1967).

Franklin has recently isolated a number of deletion mutants of a Ø80-lambda hybrid phage by induction of a defective prophage. These deletions, as in the b2 mutant, occur in the middle of the chromosome but are of varying lengths. From genetic and physiological experiments, she has shown that all of the mutants like lambda b2 lack the homology region. At least one deletion mutant lacks a gene whose function is necessary for genetic recombination and also the gene which controls the lambda exonuclease. Some of these deletion mutants are also unable to cure lysogenic cells by superinfection. These observations suggest

that between genes J and cI there are at least 3 and perhaps more non-essential genes.

Clearly, the availability of a variety of deletion mutants of lambda would prove useful in attempts to identify in more detail the function and location of genes in the rather large, genetically silent region of lambda between genes J and cIII. Such mutants might also lead to a further understanding of the nature of the integration process.

Previously, several deletion mutants of lambda (Kellenberger, et. al., 1961a) and T5 (Hertel, Marchi and Muller, 1962) have been isolated by selecting for phage of lower density, since phage with less DNA per particle have a lower density. The T5 deletion mutants were found to be more resistant to heat inactivation than wild type. This observation suggested the possibility that selection for heat resistant mutants might co-ordinately select for deletion mutants.

Two instances of lambda heat resistant mutants have been reported. Siminovitch (in Jacob and Wollman, 1954) found that certain clear plaque mutants had increased resistance to heat although the result was not pursued. Groman and Suzuki (1962) isolated a stable heat resistant mutant of lambda vir for use in temperature effects on growth. In neither case was the density of the heat resistant mutants determined.

The work reported here concerns the isolation of deletion mutants by selection of lambda mutants resistant to thermal inactivation. A prerequisite study of various aspects of the heat sensitivity of lambda

is also described. It is shown that lambda deletion mutants are more
heat resistant than the wild type and that it is possible to isolate
pre-existing deletion mutants of lambda using thermal inactivation
as a selective technique.

A number of properties of the new deletion mutants isolated
have been examined with the general conclusion that these new
deletion mutants resemble the lambda b2 deletion mutant in many ways.

MATERIALS AND METHODS

<u>Media</u>. Growth medium for bacteria was either tryptone broth or K medium (Weigle, Meselson and Paigen, 1959). Thiamine was added at 10 μg/ml for growth of strain C600. Bacteria used for adsorbing lambda were grown in media supplemented with 0.2% maltose (Ptashne, 1967). For a solid medium, 1.5% agar was added to tryptone broth. Soft agar for assaying phage particles was tryptone broth with 0.65% agar. All phage dilutions were made in TMG (10^{-2}M Tris, pH 7.4, 10^{-2}M $MgSO_4$ and 0.01% gelatin). Gal^+ transductants were assayed by plating on TTC agar (Weigle, <u>et</u>. <u>al</u>., 1959). Abortive lysogens were identified by stabbing from the center of turbid plaques onto EMB-glucose (0.1%) agar (Zissler, 1967).

<u>Bacterial strains</u>. All bacteria used were <u>E. coli</u> strains and are listed in Table 1. Plating bacteria for phage assays were grown to saturation in tryptone broth with maltose at 37°C, centrifuged, resuspended in TM (10^{-2}M Tris, pH 7.4, 10^{-2}M $MgSO_4$) at 2×10^9 cells/ml and used without further treatment. Bacteria for phage crosses were grown to 10^8 cells/ml in K medium with maltose at 37°C, centrifuged and resuspended in TM at 0°C. Phage at a multiplicity of 5 each were adsorbed to the cells at 2×10^8 cells/ml in the presence of 4×10^{-3}M KCN at 37°C. After 15 minutes adsorption the infected cells were diluted 4×10^4 into K with maltose. The progeny were assayed after 90 minutes at 37°C.

<u>Phage strains</u>. All phage strains used are listed in Table 1. Three different methods were used for preparation of phage stocks.

Plate stocks: Approximately 10^5 phage particles were plated on C600 and after 4½ hours at 37°C, 0.5 ml chloroform was added to the plate. When the chloroform had evaporated, 4 ml of TMG was added to the plate and left at room temperature for 2-3 hours; the liquid was collected and yielded stocks of better than 10^{10} phage/ml.

Ultra-violet induction: UV induction of bacteria lysogenic for lambda was carried out under a dual germicidal lamp for 1 minute at a distance that gives 1 lambda hit/minute. The cultures were grown and induced in K medium.

Temperature induction: Lysogenic cultures carrying lambda cIt1 were induced by placing the culture at 43°C for 25 minutes and transferring back to 37°C until lysis.

Anti-phage serum. Rabbit anti-lambda serum was kindly provided by J. Weigle.

CsCl buoyant density gradients. 2.29 gm of CsCl was added to 2.92 ml of phage suspension in TM or broth; 3 ml of this solution was placed in a nitrocellulose tube and covered with 2 ml of parafin oil. Centrifugation was carried out using an SW39 rotor in a Spinco Model L ultracentrifuge at 22,000 rpm for 12 to 20 hours at 15°C. The gradients were collected and analyzed by the method of Weigle et. al. (1959). For analytical gradients, approximately 0.5 ml saturated CsCl in 10^{-2}M Tris, pH 7.4, was added to 0.5 ml phage suspension in TM and the density adjusted to 1.496 g/cm^3 as determined from refractive index. Of this, 0.7 ml was

added to a 12 mm cell and run in an AN-D or AN-F rotor at 44,770 rpm

at $20^{o}C$ in a Spinco Model E ultracentrifuge. After 24 hours,

pictures were taken using absorption optics and tracings were made

on a Joyce-Loebl microdensitometer. In all runs a density marker

was used and calculations are based on the distance from this

marker (Vinograd and Hearst, 1962).

Band sedimentation velocity. The method of Vinograd, et. al.

(1963) was used to determine sedimentation coefficients by banding

in CsCl. A 12 mm, type III banding cell was used (Vinograd,

Radloff and Bruner, 1965). All banding runs were done at 31,410

or 35,600 rpm in CsCl at a density of 1.36 g/cm^3. Sedimentation

coefficients are expressed as $(s_{20,w})_w$ for the sodium salt of the

macromolecule (Bruner and Vinograd, 1965).

Electron microscopy. Grids were kindly prepared by Mr. J. King.

The grids were parlodion coated followed by a carbon film. Contrast

was increased by staining with 4% phosphotungstic acid at pH 7.0.

The microscope used was a Phillips EM200.

Thermal inactivation. Phage were thermally inactivated by diluting

samples 100-fold into prewarmed buffer of the appropriate ionic

constitution. Time points were taken by diluting aliquots into TMG

maintained at room temperature. The water bath used held the stated

temperature within $\pm$ $0.4^{o}C$. After dilution the time of assay was not

critical as the phage used were all stable in TMG at room temperature.

Multiplicity reactivation. Phage in TM were UV irradiated to a sur-

vival level of less than 10^{-3}. Samples of UV irradiated phage were

then adsorbed at multiplicities of approximately 10 or much less than 0.01 to AB2480 and the infective centers plated on AB2480. The number of infective centers obtained with multiple infection was divided by the number of bacteria in the adsorption tube. This ratio was divided by the ratio of the number of infective centers from the singly infected bacteria to the number of active phage before irradiation. Multiplicity reactivation is evident when the resulting quotient is much greater than the multiplicity of infection.

Table 1. Bacterial and phage strains used in this study.

Bacterial strain	Reference	Obtained from
C600	Appleyard (1954)	J. Weigle
C600(λ)		"
C600(λcItl)		"
W3350	Campbell (1958)	"
594	Weigle (1966)	"
C	Bertani and Weigle (1953)	"
AB1886	Howard-Flanders and Theriot (1966)	P. Howard-Flanders
AB2480	"	"
803	Wood (1966)	W. B. Wood
B707	"	"
B837	"	"

Phage strain

|		
lambda wild type	Kaiser (1957)	J. Weigle
lambda cI26	Meselson (1964)	"
lambda cItl	Lieb (1964)	"
lambda b2	Kellenberger, Zichichi and Weigle (1961a)	"
lambda b2-b5	Kellenberger, Zichichi and Weigle (1961b)	"
lambda b5	"	"
lambda col-co2-b2	Kaiser (1955)	"
lambda susA11	Campbell (1961)	"
lambda susL63	"	"
lambda int4	Zissler (1967)	J. Zissler
lambda susJ33-cItl	Parkinson (1967)	J. Parkinson
$\emptyset$tdel 20R	Franklin (1967b)	N. Franklin

RESULTS

I.   Thermal inactivation of lambda as a function of ionic strength

and temperature of inactivation.

Since thermal inactivation was chosen as a selective enrichment

technique for isolating deletion mutants (as explained later), it

seemed necessary to characterize the heat sensitivity of lambda in

order to use thermal inactivation efficiently as a tool.

The kinetics of thermal inactivation of lambda as shown in

Figure 1, are essentially biphasic, suggesting two components which

are inactivated at pseudo-first order rates. It would appear that in

a lambda wild type population there is a small class of phage which is

more resistant to heat than the majority of the phage population.

Although from the shape of the curve it could be argued that there

may be many classes of heat sensitivity in the population, the curves are

treated as two-component for simplicity. From a plot of log of the

surviving phage fraction versus length of treatment, the rate of inac-

tivation of the major fraction of the population may be determined from

the slope. Extrapolation of the second part of the inactivation curve

to zero time gives the frequency of the relatively heat resistant class

of phage in the total population.

The rates of inactivation of the two classes and the fraction of

phage with higher resistance to heating depends upon the ionic make-up

of the medium and on the temperature of inactivation. Lambda wild type

is relatively resistant to heat inactivation at $60^{o}C$ in 1M $MgSO_4$ but

as the concentration of $MgSO_4$ is decreased the heat sensitivity

Figure 1. Thermal inactivation of lambda wild type. Lambda wild

type diluted 100-fold into 10^{-1}M Tris (pH 7.4) with 10^{-3}M MgSO$_4$ at

60°C. At various times a sample was diluted 10-fold into TMG at

room temperature and assayed for viable phage. Log of the surviving

phage fraction is plotted against time at the inactivating temperature.
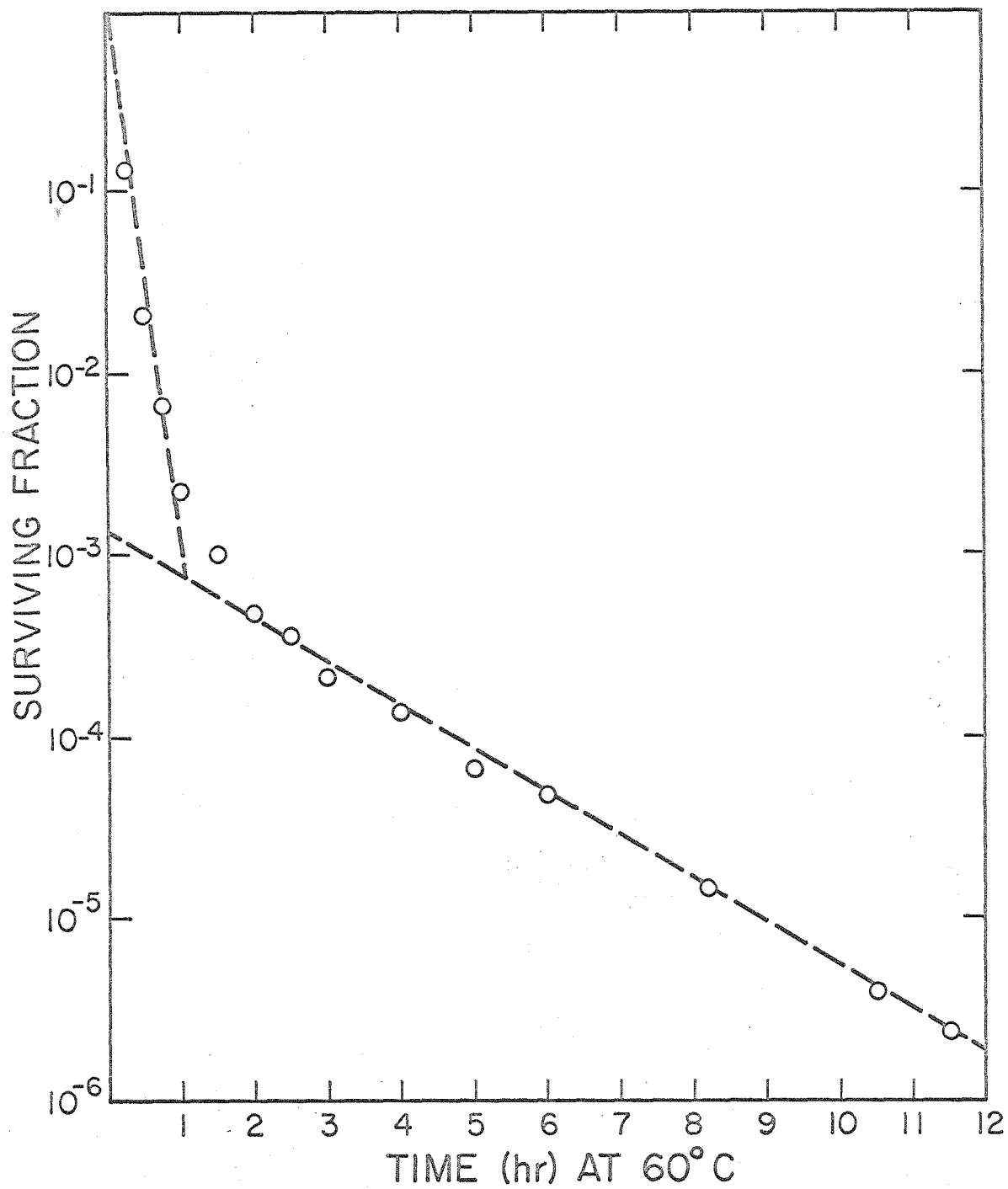
increases and the heat resistant subfraction becomes apparent as seen

in Figure 2. The same effect is seen with NaCl, $CaCl_2$ and $MgCl_2$

although divalent cations are more efficient in protecting lambda from

inactivation than monovalent cations and magnesium is more effective than

calcium as seen in Table 2. This difference in protection afforded by

the various cations may be related to the mechanism of inactivation

and will be discussed later.

The temperature at which inactivation is carried out also strongly

affects the rates of inactivation of the two lambda subclasses and also

the relative frequency of the more heat resistant fraction as seen in

Figure 3. An Arrenhius plot of log rate of inactivation versus the

reciprocal of the absolute temperature gives activation energies of

approximately 20 kcal/mole for both components of a lambda wild type

population. This value of activation energy is similar to that obtained

from most virus heat inactivation curves, although lower than some

(Woese, 1960). Activation energies of this level are higher than

single bond energies but may be due to the breaking of several bonds,

as would be expected in protein denaturation (Eyring and Stearn, 1939).

It is also possible that the activation energies reflect a denaturation

of the phage DNA. However, experiments reported later point to a local

damage in the phage head as the lethal event and not denaturation of

DNA inside the phage head.

II. Origin of phenotypic heat resistance.

Heat resistant mutants do not account for the more heat resistant

class of phage in a lambda wild type population. This minor component

Figure 2. Thermal inactivation of lambda wild type as a function of MgSO$_4$ concentration. Lambda diluted 100-fold into 10^{-1}M Tris (pH 7.4) with various concentrations of MgSO$_4$ at 60°C. Time points taken as in Figure 1. Data is plotted as in Figure 1. (O) 1M MgSO$_4$; (□) 10^{-1}M MgSO$_4$; (△) 10^{-2}M MgSO$_4$; (O) 10^{-3}M MgSO$_4$; (◇) 10^{-4}M MgSO$_4$.
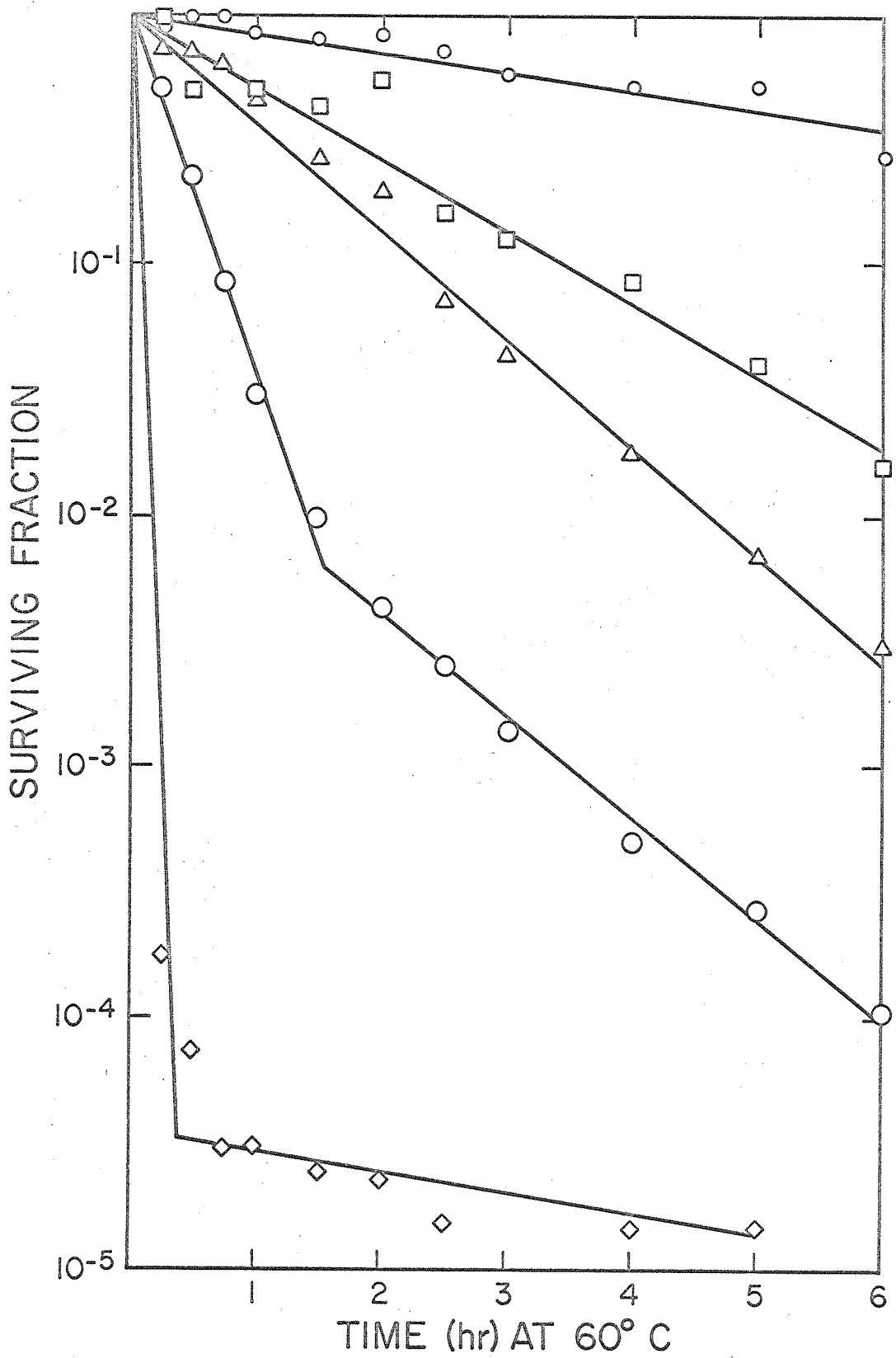
Table 2. Rate of thermal inactivation of lambda wild type at $60^\circ C$ in various salt concentrations.

Rate of inactivation (K, in hr^{-1}) at the stated molarities of:

Salt Molarity	KCl	NaCl	$CaCl_2$	$MgCl_2$	$MgSO_4$
5M	4.4	11	---	---	---
1M	13.6	14	6.3	1.5	0.16
$10^{-1}M$	25	25	6.3	1.2	1.0
$10^{-2}M$	> 24	> 24	14	2.7	2.2
$10^{-3}M$	> 25	> 24	19	18	8.9
$10^{-4}M$	---	---	---	---	> 30

All experiments were done as the one described in Figure 1, using 0.1M Tris with the indicated salts. The rate of inactivation, K, is calculated from the slope of the initial component of the thermal inactivation curve. (Dashes indicate that no test was done.)

Figure 3. Thermal inactivation of lambda wild type at various
temperatures. Lambda wild type diluted 100-fold into 10^{-1}M Tris with
10^{-2}M $MgSO_4$ at $50°C(\bigcirc)$, $60°C(\square)$ and $70°C(\triangle)$. Assays were performed
as described in Materials and Methods. Log of the surviving phage
fraction is plotted against time at the inactivating temperature.
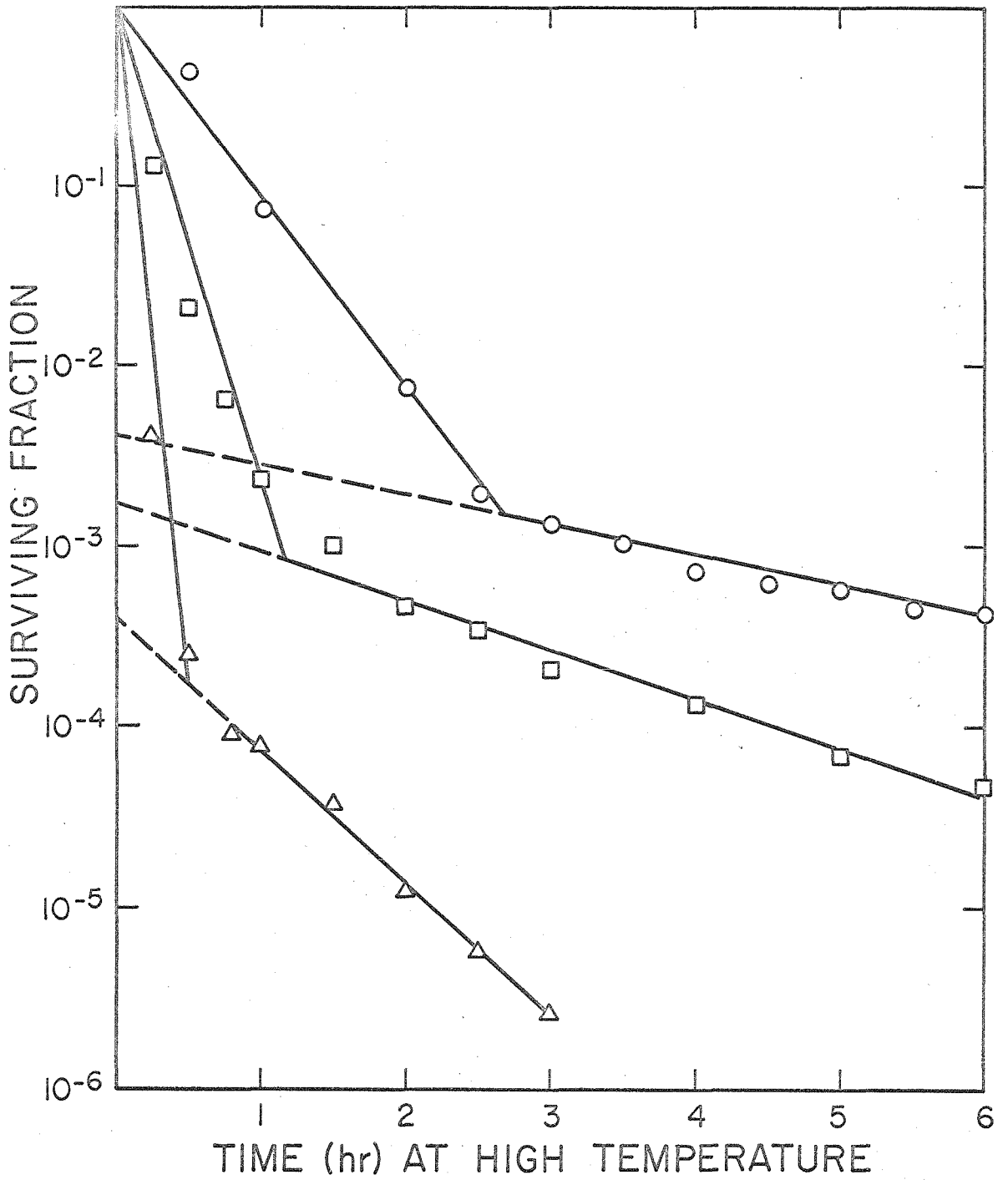
of the population consists of phenotypically heat resistant phage of
lambda wild type genotype as indicated by the following experiments.
Lambda wild type was heated until the more heat sensitive class was
inactivated (Figure 10A). The surviving heat resistant phage were then
passed through several cycles of growth in C600 and the resulting
progeny phage were thermally inactivated. As shown in Figure 10B,
the resulting progeny contain approximately the same proportion of
heat resistant phage as did the original population. This result in-
dicates that the heat resistant component of a lambda wild type pop-
ulation contains mostly phenotypically heat resistant phage and few,
if any, genotypically heat resistant phage.

This class of phenotypically heat resistant phage was found in
T5 populations (Lark and Adams, 1953). Hertel, Marchi and Muller
(1962) showed that these phenotypically heat resistant T5 phage were
also less dense than the more sensitive major class of phage; however,
their progeny were again of normal density.

To see if the same situation held for lambda, lambda wild type
was heated at $60^{o}C$ to a survival level of 10^{-4}. The surviving phage
were centrifuged in a CsCl buoyant density gradient. As shown in
Figure 4, the surviving phage were found to be distributed in two
peaks; one has the density of lambda wild type while the other is
broader and less dense. Plaques made by phage in the lighter peak
were picked and phage from the resulting plaques were tested for heat
resistance by assaying the resuspended plaque before and after heating
at $60^{o}C$. The vast majority of the plaques contained no significant

Figure 4. CsCl buoyant density gradients of thermally inactivated

lambda wild type. Lambda wild type was thermally inactivated for

5 hours at 60°C in 10^{-1}M Tris with 10^{-3}M MgSO$_4$. CsCl was added to

the surviving phage and centrifuged as described in Materials and

Methods. Lambda cI26 was added as a density marker. Phage titer

is plotted versus the percent of the gradient. Density increases

from right to left. A) Density gradient of phage surviving thermal

inactivation. B) Density gradient of the progeny of the phage

surviving thermal inactivation. Lambda wild type (O); lambda

cI26 (□).
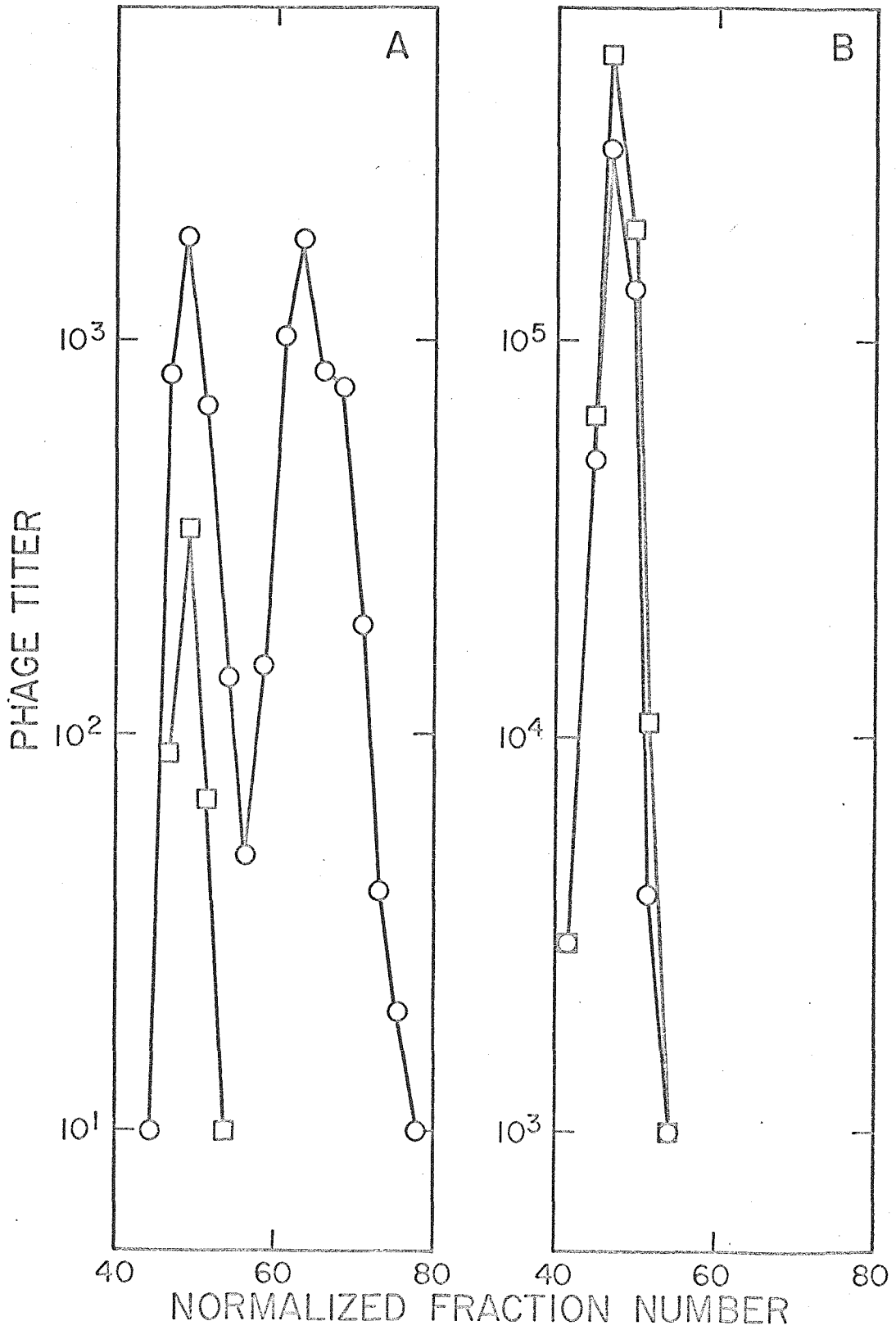
number of heat resistant phage. Furthermore, progeny virus resulting from growth of the less dense phage form a homogeneous peak at the density of wild type (Figure 4B). Thus the heat resistant fraction of a lambda wild type population is less dense but these less dense phage are both phenotypically heat resistant and phenotypically less dense since their progeny have the heat sensitivity and density of lambda wild type.

Meselson and Weigle (1961) found that the distribution of lambda wild type in a CsCl buoyant density gradient is not perfectly Gaussian. There is a "tail" of phage in the lower density region. This tail of phage could be due to phage which are phenotypically less dense. In support of this notion Kellenberger, Zichichi and Weigle (1961a) reported that a fraction of the less dense phage in the tail of a lambda wild type density distribution are less dense when rebanded but the progeny of these phage are again wild type in density. It might be hoped that if only phage from the peak of a lambda wild type density distribution were heat inactivated, the fraction of less dense, heat resistant phage would be considerably decreased. To test this notion a lambda wild type preparation was purified in CsCl and the three fractions containing the peak were pooled, dialyzed to remove the CsCl and heat inactivated. The fraction of heat resistant phage in the purified preparation was not reduced by the CsCl purification as shown in Figure 5. Although the frequency of heat resistant phage as determined from the back extrapolate was greater before purification, the level of heat resistant phage was much higher than was expected if the less dense

Figure 5. Thermal inactivation of CsCl purified lambda wild type.
Lambda wild type was purified by banding in CsCl. Three fractions
containing the peak of phage were pooled and dialyzed to remove the
CsCl. This purified preparation was thermally inactivated at $60^{\circ}C$
in 10^{-1}M Tris with 10^{-3}M $MgSO_4$ as described in Materials and Methods.
Log of the surviving phage fraction is plotted versus length of time
(hours) at $60^{\circ}C$. Lambda wild type before purification ($\square$) and
after purification ($\bigcirc$).
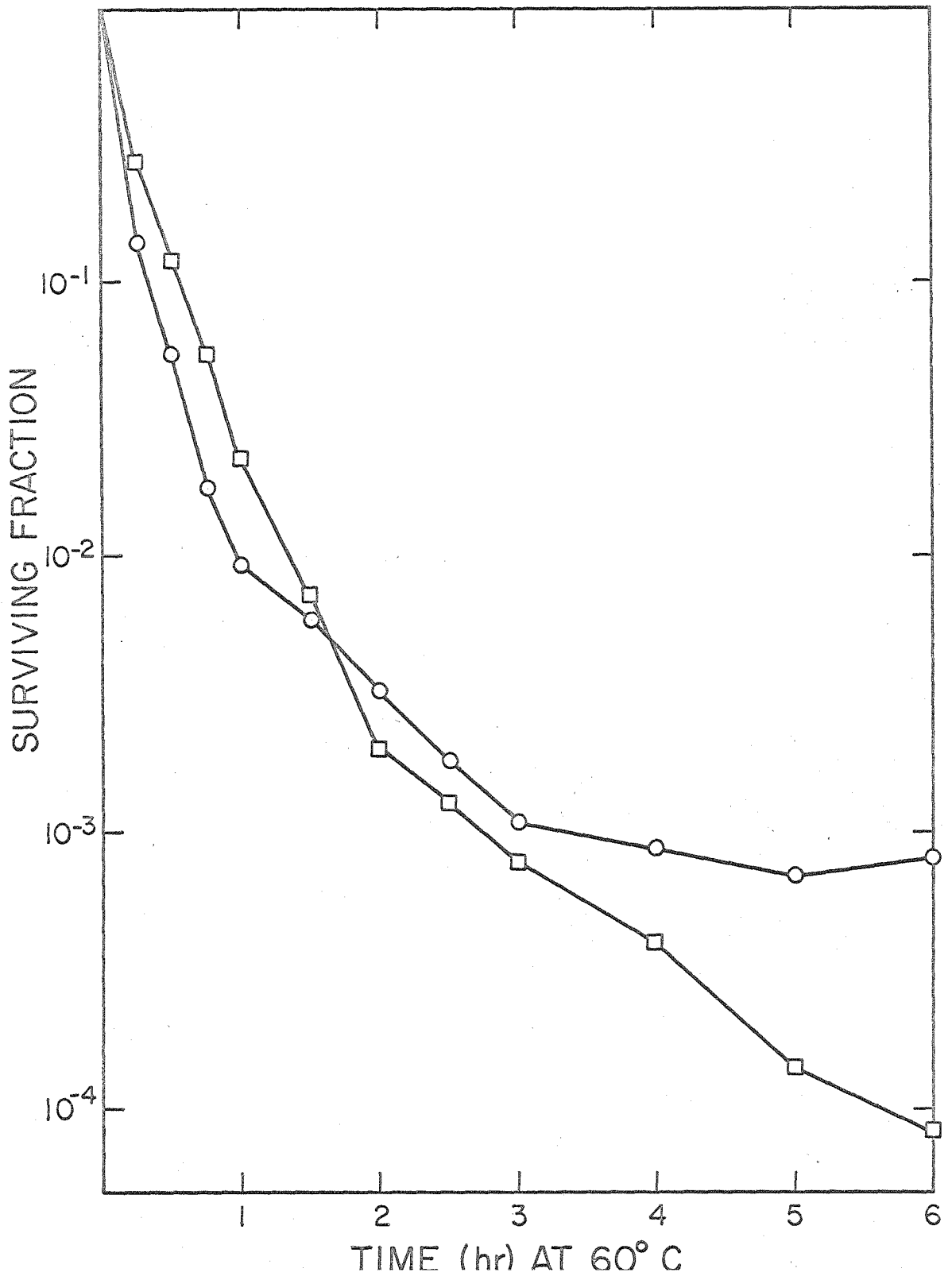
phage in the population accounted for all of the heat resistant phage.

Thus purification by density did not remove a significant fraction of the heat resistant phage. Either the purification by density was not quantitative or if it was, an equilibrium between the heat sensitive and heat resistant forms of lambda may exist. Thus purification of the heat sensitive form would be masked by the formation of heat resistant phage due to this equilibrium.

III. What is the lethal event during heat inactivation?

The following experiments indicate that upon heating lambda phage particles at $60^{o}C$ in $10^{-3}M$ $MgSO_4$, the particles release their DNA. The sedimentation coefficient of lambda phage particles was determined by using the band velocity technique of Vinograd, et. al. (1963) in the analytical ultracentrifuge as outlined in Materials and Methods. The $(s_{20,w})_w$ obtained was 410 which is equal to the published value (Weigle, 1966). After heating at $60^{o}C$ to a survival of 10^{-4} an aliquot of the same preparation of lambda phage, an obvious increase in viscosity was observed. Upon sedimenting in CsCl as before, the $(s_{20,w})_w$ of the UV absorbing material was found to be 35, corresponding to the published values for lambda DNA (Hershey, Burgi and Ingraham, 1963)(Studier, 1965). These observations support the hypothesis that DNA is released from phage particles as a consequence of thermal inactivation.

In support of the foregoing conclusion is electron microscopic evidence that after heat inactivation, the majority of phage particles have empty heads. A preparation of lambda wild type was purified in a

in a CsCl buoyant density gradient to remove pre-existing empty particles. The purified phage were examined in the electron microscope before and after heating. The unheated sample contained 60% full-headed phage particles while the heated sample contained less than 0.1% full-headed particles (Plates 1 and 2). (In order to see the phage particles after heating it was necessary to treat with DNase before applying the sample to the grid.)

IV. Is the loss of DNA related to thermal effects on the head, tail or both?

Weigle (1966) demonstrated that lambda genes A to F are concerned with head formation and genes G to M are concerned with tail formation. A lysate of cells infected with a mutant defective in tail formation contains active heads and a lysate of cells infected with a mutant defective in head formation contains active tails. By mixing two lysates, one containing active heads but no tails and the other containing active tails but no heads, viable phage are formed _in vitro_ from the active heads and tails. Using this _in vitro_ system it is possible to determine the thermal effects on heads and tails independently.

Following the nomenclature of Weigle, defective lysates of lambda sus mutants A11 (tail donor) and L63 (head donor) were prepared by temperature induction of strain 594 lysogenic for lambda susA11-cIt1 and 594 lysogenic for lambda susL63-cIt1. Aliquots of both lysates were placed at 60°C for 2 hours and assayed for surviving heads or tails by subsequent incubation with aliquots of the complementary unheated lysates. The results as shown in Table 3 indicate that free heads are

Plate 1. Electron micrograph of unheated lambda phage particles.
A CsCl purified lambda wild type preparation was applied to a grid
and photographed prior to heat inactivation. The grid was negatively
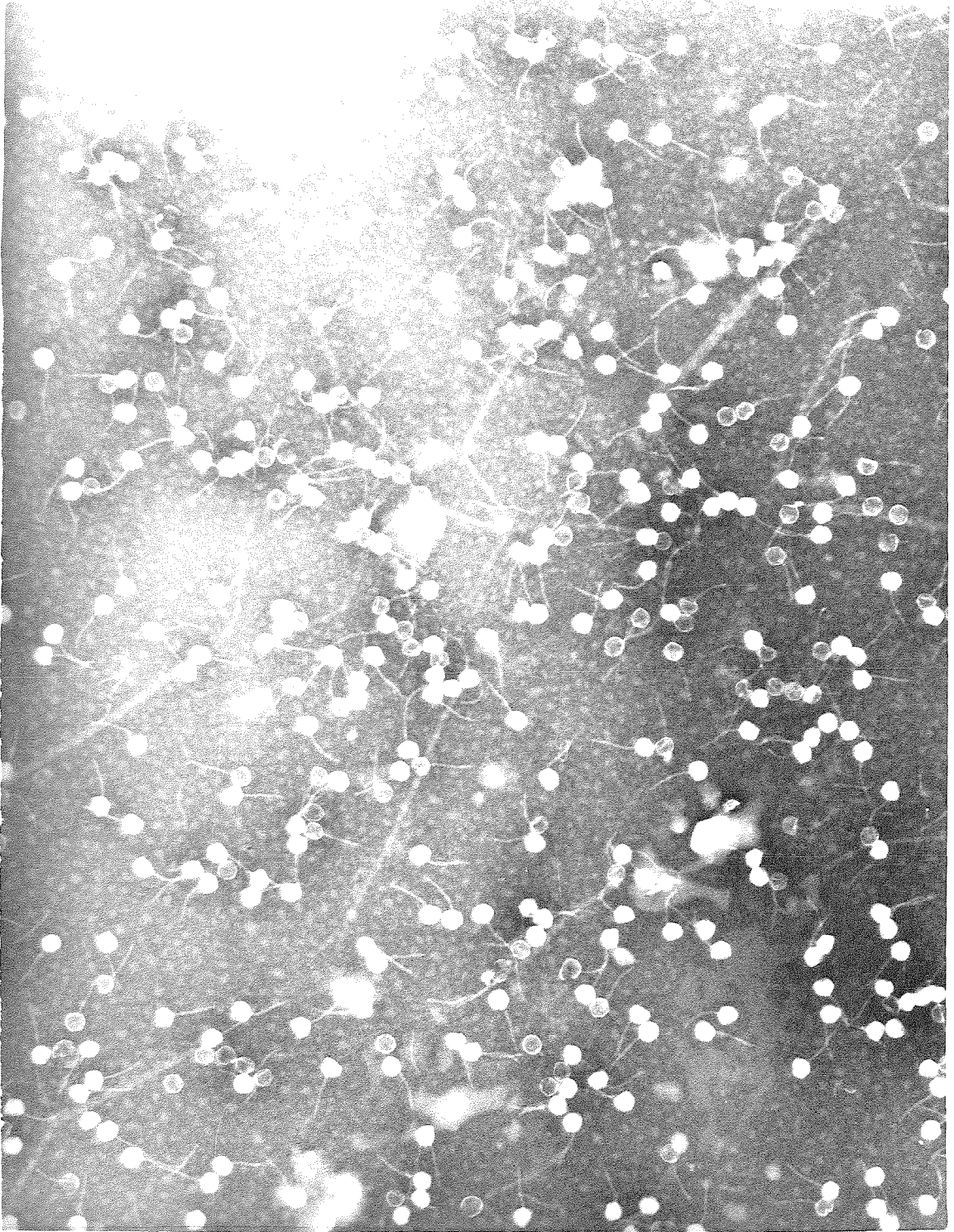stained with phosphotungstic acid.

Plate 2. Electron micrograph of heated lambda phage particles.

A CsCl purified lambda wild type preparation was heated at 60°C to a

survival of 10^{-4}. DNase was added at 1 μg/ml and the preparation

applied to grids. The grid was negatively stained with phosphotungstic

acid.
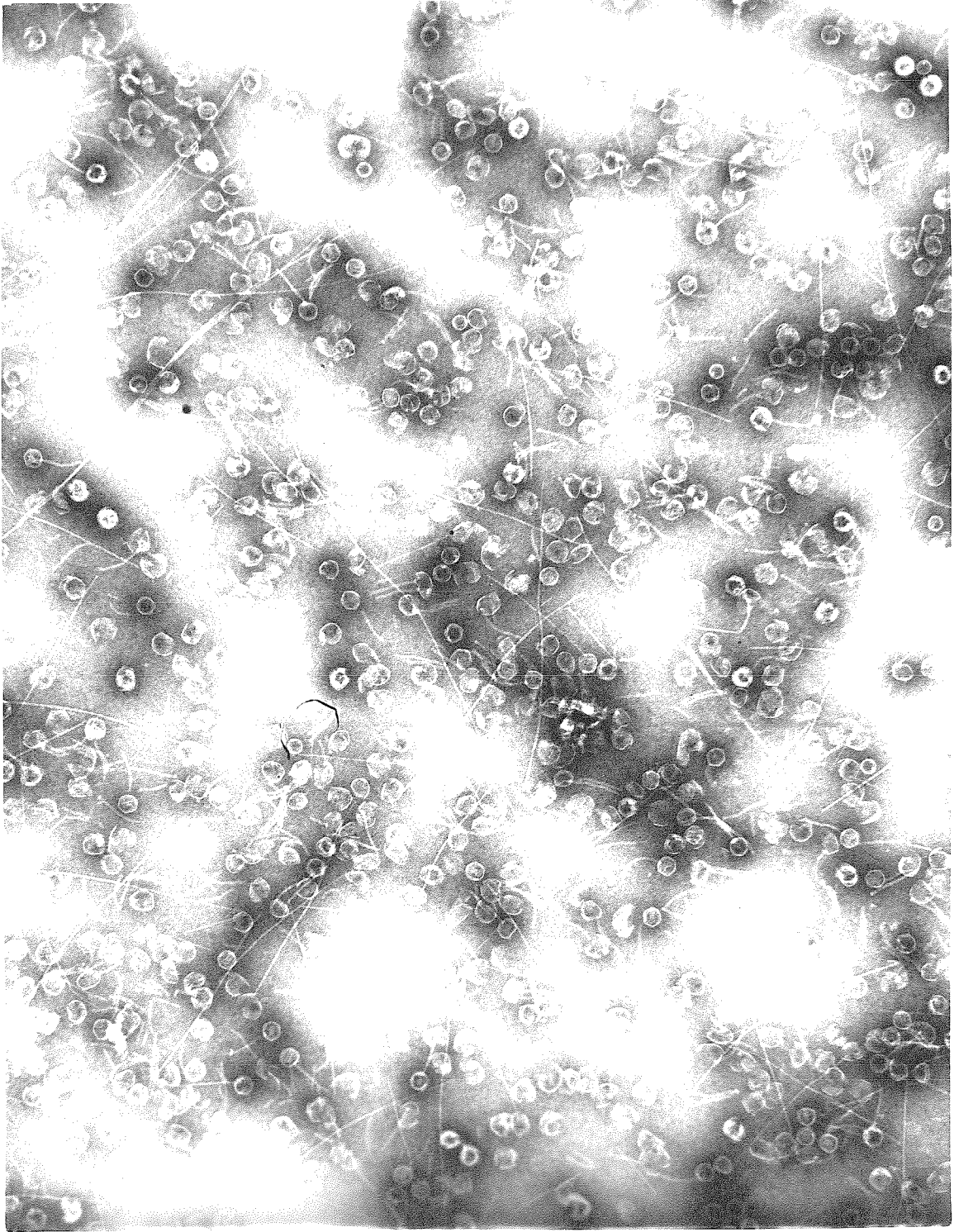
Table 3. Thermal inactivation of lambda heads and tails.

Mixtures	Final titer on C600
1. unheated heads + unheated tails	2.9×10^9
2. heated heads + unheated tails	$< 10^2$
3. unheated heads + heated tails	2.0×10^9
4. wild type phage before heating	8.4×10^7
5. wild type phage after heating	4.2×10^2

Heads and tails of lambda were contained in lysates of lambda sus mutants grown in the restrictive host, 594. Strain 594(lambda susA11-cIt1), a tail donor, and 594(lambda susL63-cIt1), a head donor, were grown to 2×10^8 in K medium and induced by temperature shock. An aliquot of each lysate was placed at 60°C for $1\frac{1}{2}$ hours. Aliquots of each heated lysate were incubated with equal amounts of the complementary lysate for 2 hours at room temperature and assayed for viable phage on C600. A lambda wild type phage control was also thermally inactivated under the same conditions (lines 4 and 5). Viable phage in the defective lysates were less than 10^2/ml.

inactivated to a survival level of less than 10^{-7}. Since the absolute number of heads or tails in the lysates is not known, it is possible that free tails were inactivated to some extent but it was not seen because the tails might have been in great excess as compared to heads. Thus the damage caused by heat inactivation most likely takes place in the phage head rather than the tail.

Phage heads do not contain a significant heat resistant fraction as do whole phage (Figure 6). Thus the phenotypically heat resistant phage may be due to some interaction between the tail and head.

V. Thermal sensitivity of deletion mutants of lambda.

Two non-defective mutants of lambda with DNA deletions have been described. Kellenberger, et. al. (1961a) showed that the lambda b2 mutant is less dense than lambda wild type and the density shift could be accounted for by an 18% loss of DNA. Caro (1965) confirmed this large DNA deletion by measuring the length of the DNA molecule in the electron microscope. Meselson and Weigle (1961) described the other presumed deletion mutant, lambda b5, and calculated it to have a 5% DNA deletion on the basis of its density. The double deletion mutant, lambda b2-b5, occurs as a recombinant in crosses of lambda b2 by lambda b5 and by density analysis has a total of 23% of its DNA deleted. MacHattie and Thomas (1964) confirmed this 23% deletion in lambda b2-b5 by length measurement of the DNA.

As was mentioned earlier, the density mutants of T5 have increased heat resistance and the altered density of these mutants is due to loss of DNA. To see if this was true for the lambda deletion

Figure 6. Thermal inactivation of lambda heads. A defective lysate

containing heads was obtained by temperature induction of 594(susL63-

cIt1). The lysate was placed at 60°C and samples withdrawn, incubated

at room temperature with the defective lysate of a tail donor for

2 hours and assayed for viable phage. A lambda wild type phage control

was thermally inactivated under the same conditions (K medium at 60°C).

Log of the surviving fraction is plotted against length of time at

60°C. Lambda heads (□); lambda wild type phage (O).
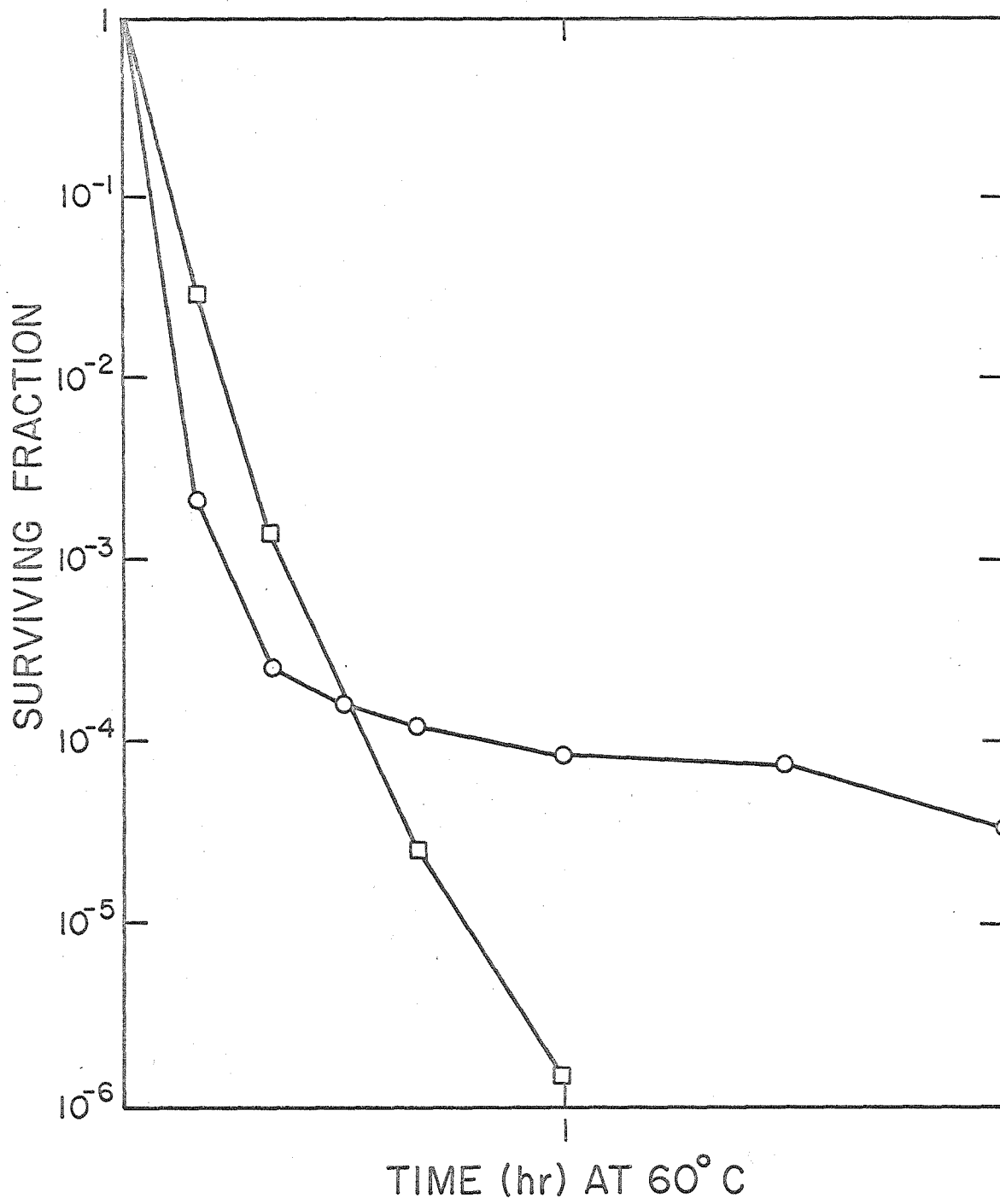
mutants the heat sensitivity of the lambda mutants was determined.
As shown in Table 4, the lambda deletion mutants are more resistant to
heat inactivation than lambda wild type. On the basis of the four
phage strains examined, the larger the deletion the greater the phage
resistance to heat inactivation although it may not be a simple rela-
tionship.

The heat resistance of the DNA deletion mutants may be a con-
sequence of the deletion itself. Alternatively, this heat resistance
may be the result of a genetic difference between the mutants and
lambda wild type affecting some protein subunit of the phage. To test
this notion of a protein difference between the deletion mutant lambda
b2 and wild type, a phenotypic mixing experiment was performed. The
progeny from a mixed infection of lambda wild type and lambda b2 were
separated by density on a CsCl buoyant density gradient and phage from
the different peaks analyzed for heat sensitivity (Figure 7). If the
heat resistance of the deletion mutant is due to a protein difference,
this altered protein should appear on both wild type and b2 phage which
issued from the mixed infection. These phenotypically mixed wild type
phage would then be as heat resistant as the parental b2 phage and the
phenotypically mixed b2 phage would be as heat sensitive as the wild
type parent. That this is not the case is shown by data presented in
Figure 8. The phage from the dense peaks of both the parental mixture
and the progeny behave like lambda wild type upon heat inactivation
and do not contain a significant fraction of heat resistant phage.
Phage from the less dense peaks of both the parental mixture and the

Table 4. Thermal inactivation of density mutants of lambda.

Phage mutant	Density (g/cm^3)	Percent DNA deleted	K (hr^{-1})
wild type	1.508[1]	0	8.9
b5	1.501[2]	5	4.6
b2	1.491[3]	18	1.1
b2-b5	1.484[2]	23	0.75

Phage samples were diluted into 0.1M Tris with 10^{-3}M MgSO$_4$ at 60°C. Samples were removed and assayed for surviving phage at various times. The results are given as a rate of inactivation calculated from the slope of the plot of ln P/P$_o$ versus time. The amount of DNA deleted is explained in the text.

1. Kellenberger, Zichichi and Weigle (1960)
2. Kellenberger, Zichichi and Weigle (1961b)
3. Kellenberger, Zichichi and Weigle (1961a)

Figure 7. CsCl buoyant density gradients of lambda wild type and
lambda b2. A mixture of lambda wild type and lambda b2 was used to
mixedly infect C600 at a multiplicity of 5 each. The parental phage
mixture (A) and the progeny phage (B) were analyzed on CsCl buoyant
density gradients as described in Materials and Methods. Log of
phage titer is plotted versus percent of the gradient. Density
increases from right to left.
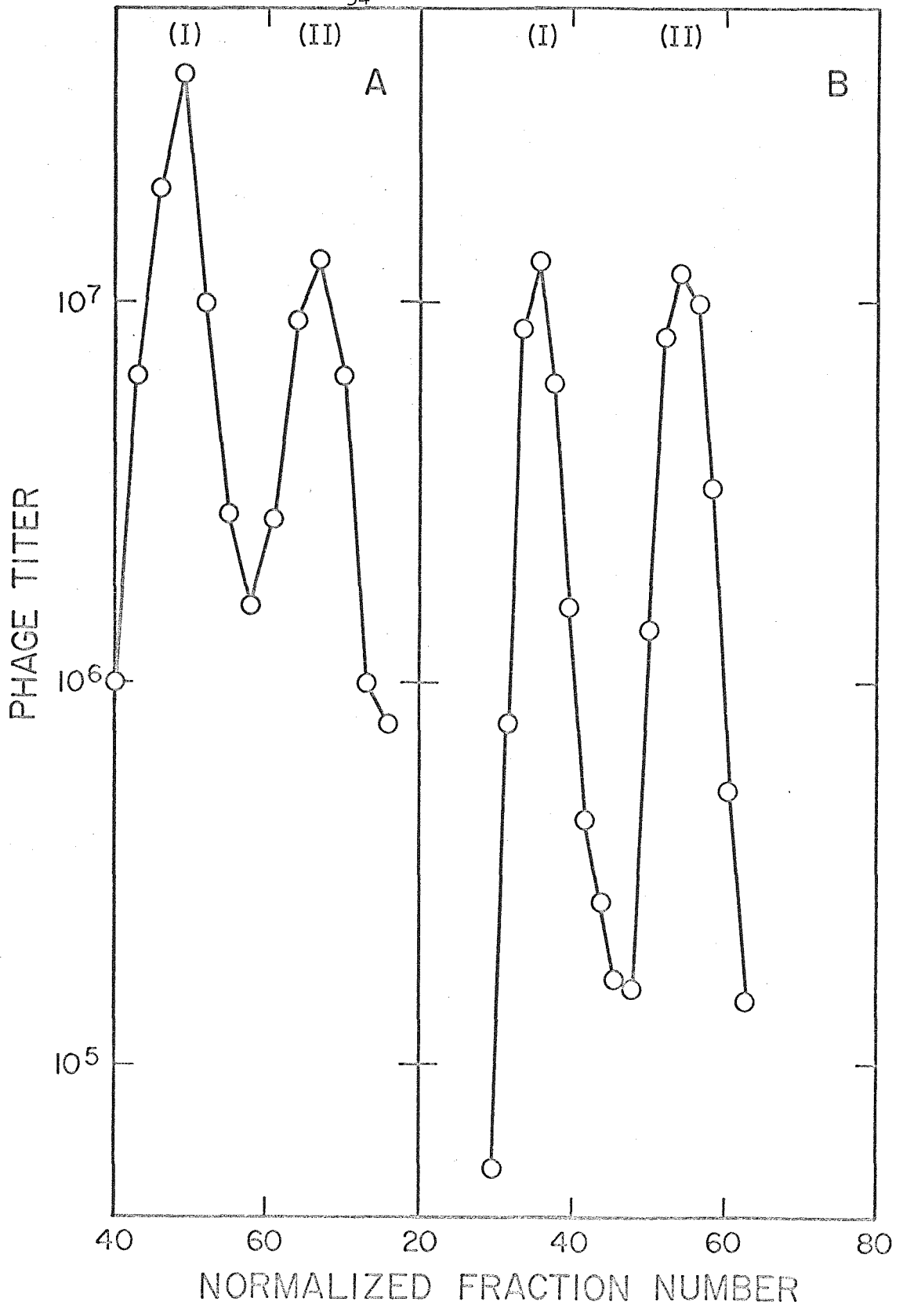
Figure 8. Thermal inactivation of lambda wild type and lambda b2.
Thermal inactivation at 60°C in 10^{-1}M Tris with 10^{-3}M $MgSO_4$ was
performed using the following phage suspensions: a mixture of lambda
wild type and lambda b2 (O); the progeny of a mixed infection of
lambda wild type and lambda b2 (◉); phage from the normal density
peaks (I) of the gradients shown in Figure 7A (□) and 7B (▣);
phage from the b2 density peaks (II) of the gradients shown in
Figure 7A (△) and 7B (▲). Log of surviving phage fraction is
plotted against time at 60°C.
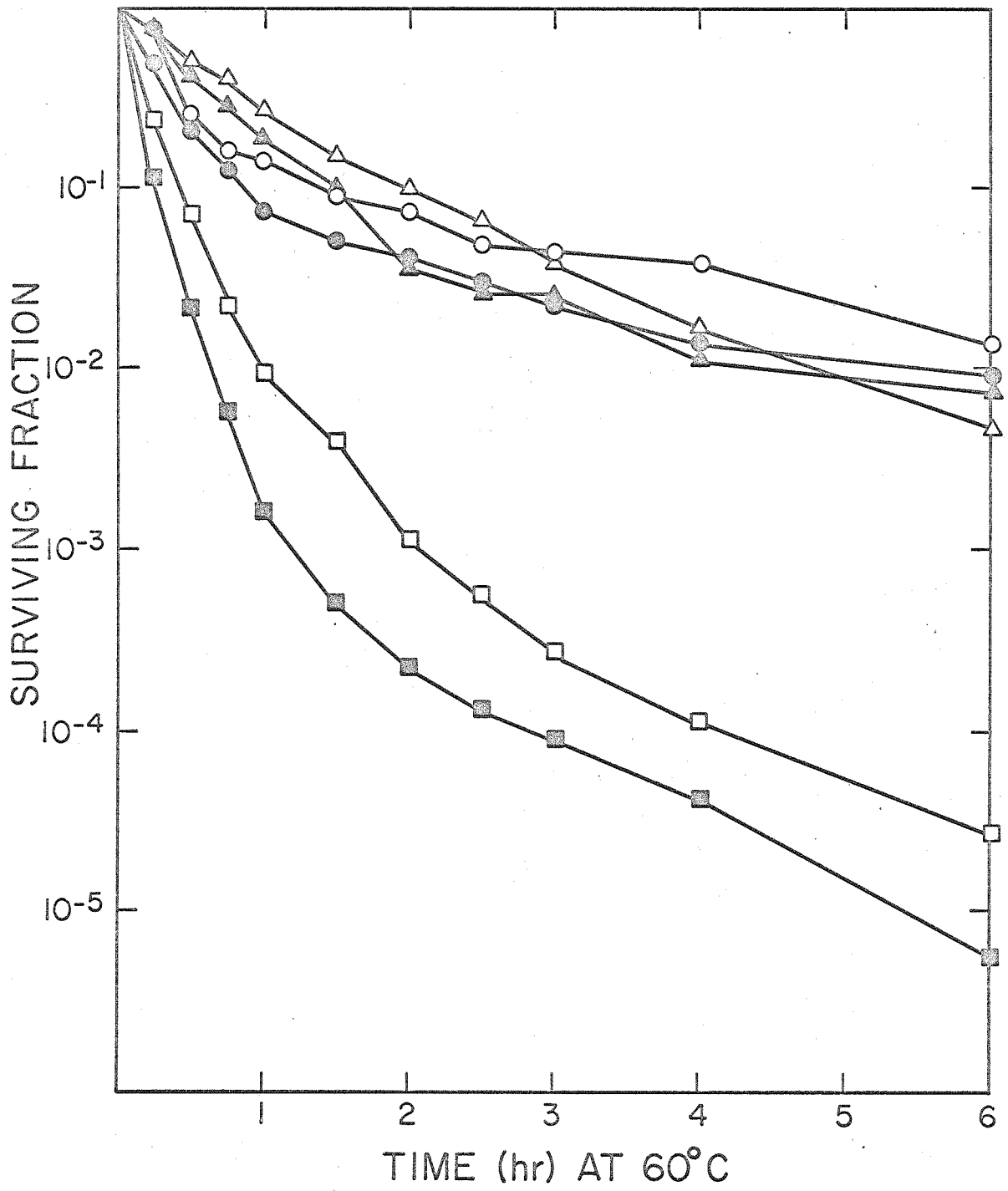
progeny behave like lambda b2 upon heat inactivation. Thus the heat resistance of lambda b2 does not segregate from the density after mixed infection with lambda wild type suggesting that the heat resistance of lambda b2 is not due to a difference in head structure resulting from an altered gene product.

VI. Selection of lambda density mutants.

All of the foregoing observations indicate that lambda deletion mutants are more heat resistant than lambda wild type. It may be possible then to isolate deletion mutants by selecting for heat resistant phage mutants. Due to the presence of the phenotypically heat resistant phage in lambda wild type populations, stable heat resistant mutants are not readily isolated by simply looking at survivors of thermal inactivation. However, the sensitive class in a lambda wild type preparation is inactivated at a much higher rate than the lambda b2 mutant. Thus if deletion mutants pre-existed in a wild type preparation, their frequency would be increased after thermal inactivation. As was indicated earlier, the phenotypically heat resistant phage give rise to progeny which are as sensitive as wild type. Thus cycles of heating followed by multiplication of the survivors would selectively increase the frequency of any stable heat resistant mutants in a wild type population.

In order to test the validity of this selection procedure, the following control was performed. Mixtures of lambda wild type and lambda col-co2-b2 were prepared at various ratios and the mixtures were thermally inactivated as shown in Figure 9. It may be seen that

Figure 9. Thermal inactivation of mixtures of lambda wild type and

lambda b2. Lambda wild type and lambda b2 were mixed at ratios of

1:1 (O); 10:1 (□); 100:1 (△) and 1000:1 (◇), respectively, and

thermally inactivated at $60^{\circ}C$ in $10^{-1}M$ Tris with $10^{-3}M$ $MgSO_4$ as

described in Materials and Methods. Log of surviving phage fraction

is plotted against time at $60^{\circ}C$.
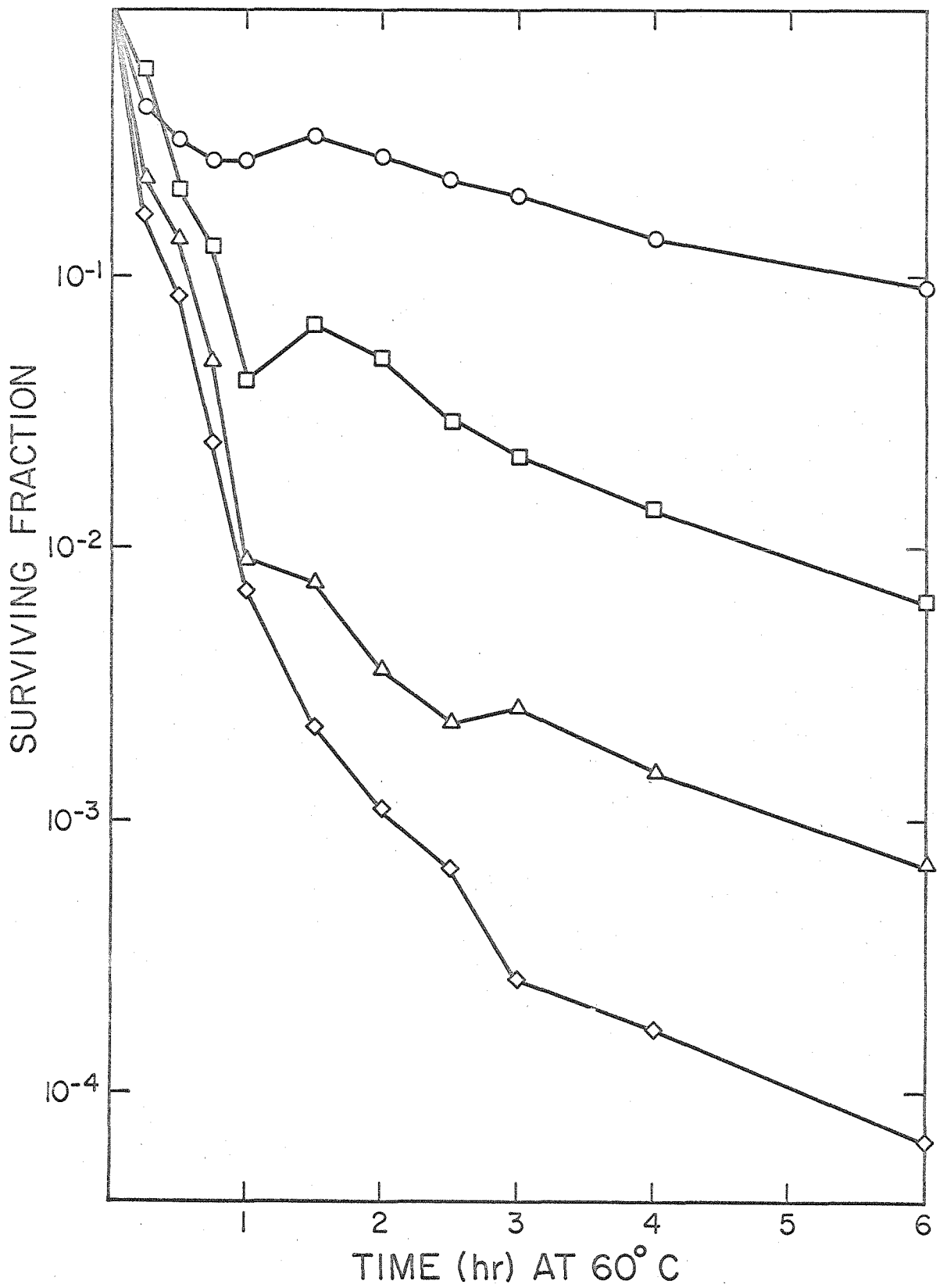
the inactivation curves accurately reflect the original make-up of

the mixtures; that is, the back extrapolate of the heat resistant

component corresponds to the proportion of lambda b2 in the mixtures.

The mixture which originally had a frequency of 10^{-3} lambda b2

contained 33% b2 among the survivors of heat inactivation. This

ratio was the same among the progeny of the survivors. Thus it is

possible to increase the frequency of heat resistant phage in a

lambda wild type population by means of heat inactivation, and growth

of the survivors does not affect the genetic make-up of the resulting

population. One cycle of heat inactivation yields an enrichment of

approximately 300-fold for lambda b2 with respect to lambda wild type.

In an attempt to determine the mode of origin of any deletion

mutants isolated, four independent preparations of normal density

lambda were prepared by different methods. A lambda wild type pre-

paration was made by UV induction of a C600 lysogen; two lambda cIt1

preparations were made from C600 lysogens, one by temperature induc-

tion and one by UV induction; and a lambda cI26 preparation was grown

vegetatively on solid media. These preparations were heated to a sur-

vival of 10^{-4} and the surviving phage were used to make plate stocks

(Figure 10A). The resulting progeny were again heated under the same

conditions and plate stocks were again made of the survivors (Figure

10B). The third heating revealed a significant heat resistant fraction

in each preparation not apparent in the previous cycles (Figure 10C). In

order to determine the density of these heat resistant phage, the pro-

geny derived from the survivors of the second heat inactivation (Figure

Figure 10. Thermal inactivation of lambda wild type prepared by different methods and selection for density mutants. The four lambda preparations were made as follows: lambda wild type from a UV induced lysogen (O); lambda cIt1 from a UV induced lysogen (□); lambda cIt1 from a temperature induced lysogen (◇) and lambda cI26 grown vegetatively on solid media (△). Thermal inactivation was carried out at $60^{\circ}C$ in $10^{-1}M$ Tris with $10^{-3}M$ $MgSO_4$ and assayed as described in Materials and Methods. The data are plotted as in Figure 1. Progeny of survivors were obtained by using the survivors to make plate stocks. A) Thermal inactivation of the lambda wild type preparations. B) Thermal inactivation of the progeny of the surviving phage (6 hours at $60^{\circ}C$) of A. C) Thermal inactivation of the progeny of the surviving phage (6 hours at $60^{\circ}C$) of B.
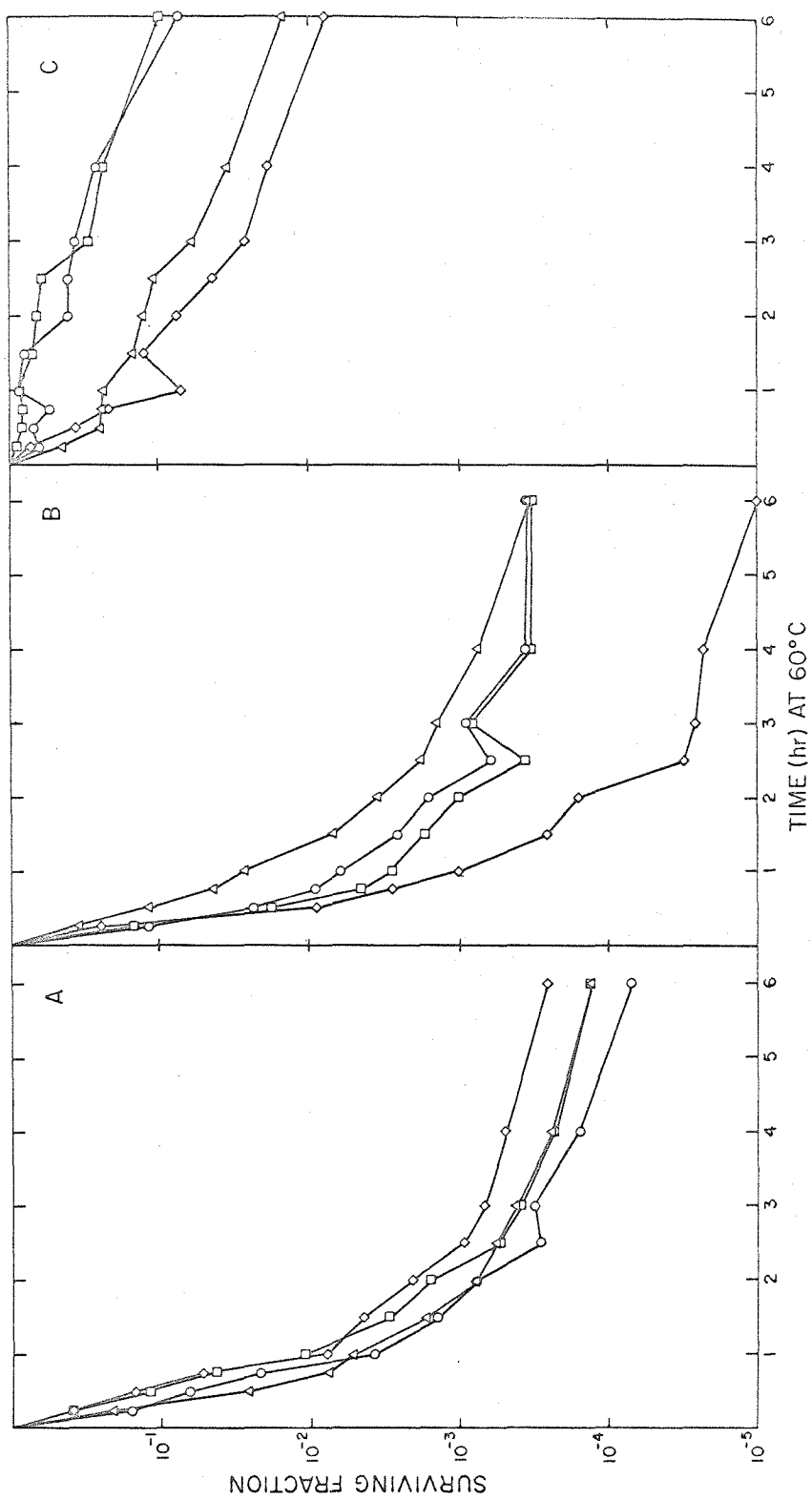
10B) were centrifuged in CsCl buoyant density gradients. As shown in Figure 11, each of the four preparations contained two density peaks; one at the density of lambda wild type and a broader peak at a lighter density. The frequency of heat stable phage as determined from the back extrapolate of the third heat inactivation curve (Figure 10C) was found to be approximately equal to the frequency of less dense phage as determined from relative peak heights in the density gradients (Table 5). This indicates that the majority of heat resistant phage were also less dense. However, in this case the lower density and heat resistance are apparently genetically determined.

Stable heat resistant mutants of lower density were found in the progeny of all four different lambda wild type preparations. Thus it would appear that these mutants can arise during growth following UV or temperature induction or during the lytic cycle of growth. Nevertheless, the frequency of mutants in the different populations varied over a considerable range with the highest frequency of mutants obtained in the UV induced preparations and the lowest in the temperature induced preparation.

The frequency of lambda dg particles in the various initial untreated preparations was also determined. Lambda dg phage are deletion mutants which have lost some of the lambda genes in the left arm of the chromosome and picked up some of the gal genes of the host (Campbell, 1963). As shown in Table 5, the frequency of lambda dg phage parallels the frequency of the less dense phage in that it is highest in the UV induced preparations and lowest in the temperature induced preparation.

Figure 11. CsCl buoyant density gradients of lambda wild type preparations selected for heat resistant mutants. The progeny of the surviving phage (6 hours at 60°C) of thermal inactivation shown in Figure 10B were banded in preparative CsCl gradients as described in Materials and Methods. Phage titer is plotted against percent of the gradient. Density increases from right to left. A) lambda wild type from UV induced lysogen. B) lambda cIt1 from UV induced lysogen. C) lambda cIt1 from temperature induced lysogen. D) lambda cI26 grown vegetatively. E) a mixture of unheated lambda wild type and lambda b2.
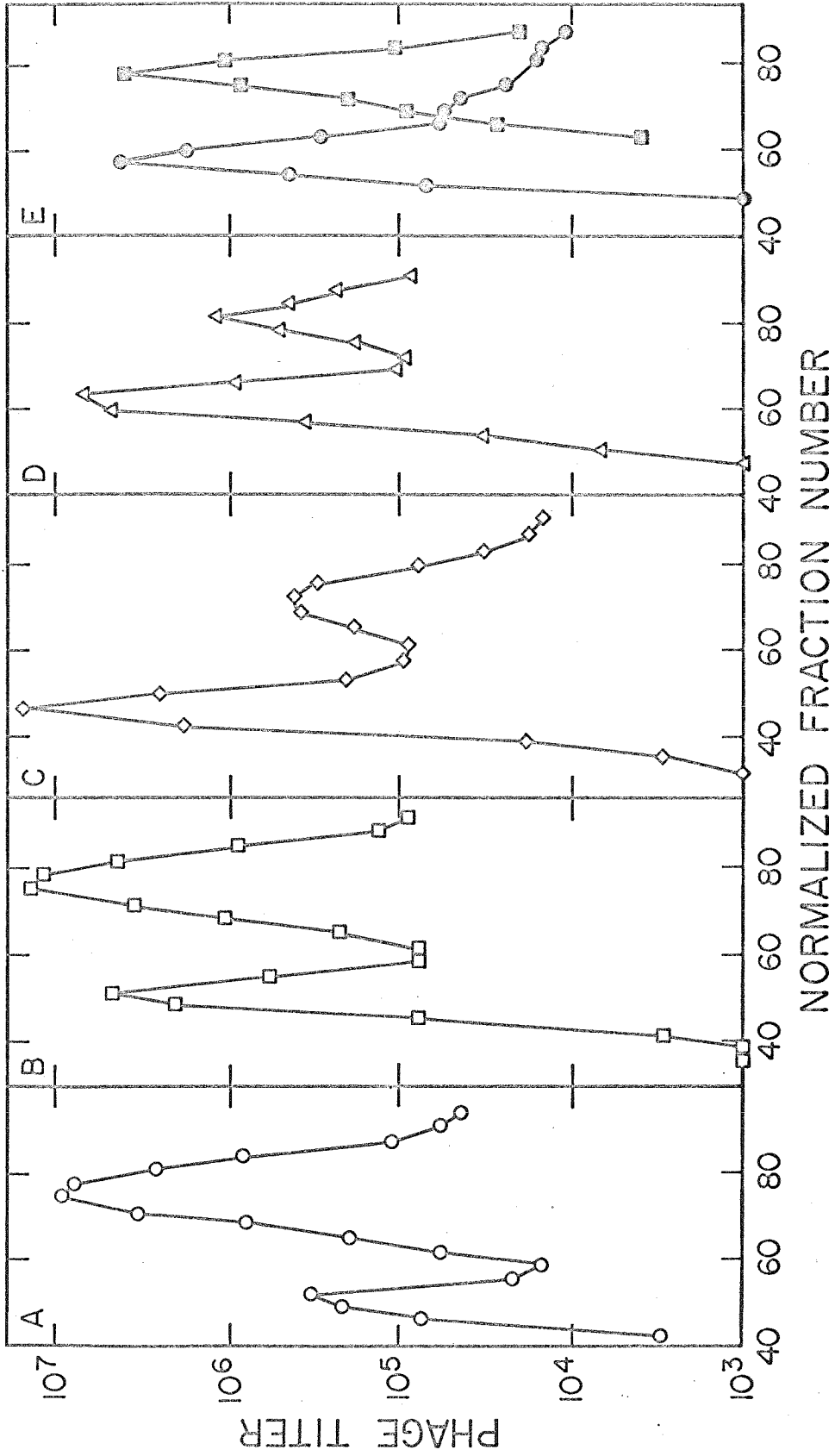
PHAGE TITER

NORMALIZED FRACTION NUMBER

Table 5. Calculated frequencies of mutants in the original lambda wild type preparations.

Phage strain	Method of preparation	A	B	C	B/C
wild type	UV ind.	1.1×10^{-4}	1.0×10^{-4}	1.0×10^{-5}	10
cIt1	UV ind.	1.1×10^{-4}	7.8×10^{-5}	1.7×10^{-6}	46
cIt1	temp. ind.	3.4×10^{-6}	4.1×10^{-7}	2.5×10^{-8}	16
cI26	veg. growth	5.9×10^{-5}	2.6×10^{-6}	---	---

A. The frequency of heat stable mutants in the original lambda wild type preparations was obtained by dividing the enrichment factor for each preparation into the back extrapolate of the resistant fraction of the thermal inactivation curves of Figure 10C.

B. The frequency of less dense phage in the original lambda wild type preparations was obtained by dividing the enrichment factor for each preparation into the frequency of less dense phage as determined from peak heights of the density gradients shown in Figure 11.

C. The frequency of lambda dg particles was determined by plating the original untreated preparations on TTC plates seeded with W3350 and scoring the gal^{+} transductants.

An enrichment factor was calculated for each phage preparation after each cycle of heating by dividing the frequency of survivors in a lambda b2 control by the frequency of survivors in each lambda wild type preparation. The final enrichment factor was the product of the factors for the two cycles of heating.

VII. Characterization of density mutants.

The broadness of the lower density peaks in Figure 11 is indicative of density heterogeneity among the phage in that part of the gradient. It would appear that several density classes exist in these preparations indicating that there may be many mutants of independent origin. Since the isolation of a variety of deletion mutants was the desired result, the occurrence of a large number of independent mutants in one population would lessen the amount of work required to isolate different mutants. The actual number of independent mutants in the selected population depends upon their number in the original preparation and more specifically upon the number of independent mutants among the survivors of the first thermal inactivation. Using the frequencies of mutants as shown in Table 5, there were approximately 10^4 density mutants among the survivors of the heat inactivated lambda wild type shown in Figure 10A. If each mutant had arisen during the excision process followed by normal growth, then approximately 100 classes of independent mutants were present in the phage population following the initial selection. Thus the final selected population would be expected to contain up to 100 different classes of density mutants, suggesting that the isolation of independent mutants from a single population would be highly probable.

The density mutants examined so far were taken from the UV-induced lambda wild type preparation mentioned in the previous section. Plaques (200) were picked from the less dense peak of the CsCl buoyant

density gradient shown in Figure 11A. From the picked plaques, plate
stocks were made and the ensuing tests were performed on these prepar-
ations.

Immunity. The presumed density mutants were tested for lambda immunity
by plating on plates seeded with C600 lysogenic for lambda and none
of the mutants made plaques under these conditions. By this criterion
none of the mutants isolated are like lambda b5 which has a different
immunity from lambda.

Lysogeny. The density mutants were plated on C600 and bacteria from
the center of a turbid plaque were transferred to EMB-glucose agar
to test for abortive lysogenization. Of the 200 phage isolates, 12
gave a stable lysogenic response and the remaining 188 gave an
abortive lysogenic response. Density determinations, as explained later,
showed that the 12 isolates giving a stable lysogenic response had the
density of lambda wild type and were, no doubt, contaminants from the
phage of wild type density in the density gradient. Thus the majority
of the phage isolated behave like the density mutant lambda b2 in giving
an abortive lysogenic response. (100 plaques were picked from the less
dense peak of the gradient of the UV induced cIt1 shown in Figure 11B.
All of these phage also gave an abortive lysogenic response and had the
immunity of lambda.)

Restriction. The DNA of lambda is restricted by the host following
infection unless the DNA has been previously modified by growth on
the same host or a modifying host (Arber and Dussoix, 1962) (Lederberg
and Meselson, 1964). That is, lambda grown on strain C initiates infec-

tion in strain C but on strain K these phage plate with an efficiency
of 10^{-3}. Meselson (personal communication) has evidence that unmodi-
fied DNA is degraded into 4 large pieces under restricting conditions
indicating that perhaps 3 sites on the phage DNA are recognized by the
restriction system.   In order to test the mutants isolated here for
loss of these sites, the phage were cycled on strain C or strain K
and then the efficiency of plating was determined on strains C, K,
$K(P1)$, $K(r^-m^+)$, $B(r^+)$ and $B(r^-)$.   All of the mutants isolated behaved
like the lambda wild type control.   Thus none of these mutants have
deletions involving all three of the restriction sites although the
absence of one such site might not have been detected by this test.

Density.   Density determinations were carried out using the analytical
ultracentrifuge equipped with absorption optics as outlined in Materials
and Methods.   It was necessary to dialyze the mutant preparation against
TM to remove small UV absorbing material.   The density of 59 of the
isolates was determined and the results are shown in Table 6.   Follow-
ing the nomenclature of Kellenberger, Zichichi and Weigle (1960), the
mutants are designated by a small b and an isolation number ranging
from 101 to 300.   In all cases the UV absorbing bands had the same shape
as the lambda cI26 density marker and no satellite bands were observed.
Since all of the low density mutants are also abortive lysogens, it was
assumed that the low density was due to DNA loss.   Based on this
assumption, the amount of DNA deleted was calculated and is presented
in Table 6.   The isolates range in density from 1.498 g/cm^3 to
1.481 g/cm^3 with several classes at intermediate densities.   On the

Table 6. Lambda deletion mutants: density, calculated DNA loss
and frequency of recombination between J and cI.

Low Recombination Frequency				High Recombination Frequency			
Mutant	Density (g/cm^3)	% DNA del.	% rec.	Mutant	Density (g/cm^3)	% DNA del.	% rec.
++	1.508	0	6.8				
b2	1.491	18	3.5				
b111	1.497	12	3.8	b101	1.498	10	22.0
b104	1.493	16	3.7	b112	1.498	10	19.1
b120	1.493	16	2.1	b130	1.496	13	15.3
b110	1.491	18	2.6	b107	1.495	14	17.2
b147	1.491	18	3.0	b150	1.495	14	21.1
b173	1.491	18	3.4	b193	1.494	15	28.4
b102	1.490	20	2.5	b132	1.493	16	22.1
b103	1.490	20	3.6	b134	1.493	16	17.3
b139	1.490	20	3.8	b144	1.493	16	15.3
b249	1.490	20	3.2	b196	1.493	16	16.0
b153	1.489	21	2.5	b105	1.492	17	15.1
b175	1.489	21	2.4	b149	1.491	18	16.6
b181	1.489	21	3.2	b164	1.491	18	12.0
b215	1.489	21	2.8	b174	1.491	18	15.4
b170	1.488	22	2.7	b131	1.490	20	20.9
b197	1.488	22	2.6	b137	1.490	20	17.8
b178	1.487	23	2.5	b135	1.489	21	12.6
b202	1.485	26	3.2	b136	1.489	21	19.8
b225	1.485	26	2.3	b180	1.489	21	17.7
b243	1.485	26	2.3	b182	1.489	21	18.2
b211	1.484	27	2.7	b192	1.489	21	22.2
b205	1.483	28	2.5	b189	1.488	22	17.1
b229	1.483	28	3.3				
b246	1.483	28	2.1				
b221	1.481	30	2.6				
b142	1.498	10					
b264	1.491	18					
b141	1.490	20					
b143	1.490	20					
b294	1.490	20					
b299	1.490	20					
b253	1.489	21					
b161	1.488	22					
b235	1.488	22					
b239	1.488	22					
b242	1.485	26					
b248	1.483	28					

Recombinations values not determined for
these mutants.

Table 6. (continued)

Densities were calculated as a function of the distance from a
density marker in an analytical CsCl buoyant density gradient. The
percent of DNA deleted was calculated by the method of Weigle, et. al.
(1959). Recombination frequencies were determined by doubling the
frequency of susJ$^+$-c recombinants in the progeny of a mixed infection
of lambda susJ33-cIt1 and the density mutant. Crosses were done in
C600 and the progeny assayed on C600 for total progeny and on 594
at 41°C for recombinants.

assumption that a density difference of 0.001 g/cm^3 is significantly

different, there are 16 different density classes. These densities

correspond to DNA losses of 10 to 30%. The majority of the density

mutants fall in the 1.491 to 1.488 g/cm^3 classes which are close to

the lambda b2 density of 1.491 g/cm^3. Since the mutants were isolated

from the leading and trailing edges of the less dense peak of the

density gradient shown in Figure 11A, the spread in density for the

total population of density mutants may be accentuated. If the change

in density is due to DNA loss, lambda is capable of losing up to 30%

of its DNA without any loss of viability. It would appear that the

homology region of lambda necessary for insertion into the host

chromosome comprises no more than 10% of the lambda DNA since these

mutants having lower density also show abortive lysogeny.

Thermal sensitivity. Since the density mutants were isolated as heat

resistant mutants, the heat sensitivity of several of the density

mutants was determined. The results are shown in Figure 12 with the

log rate of inactivation plotted versus the calculated percent loss

of DNA. Also plotted are the rates of inactivation for other lambda

deletion mutants taken from Table 4. There is a general inverse

correlation between rate of inactivation and amount of DNA deleted.

Although the trend of the two sets of data is the same there is a con-

sistent difference in the actual rates measured. This reflects the

variability of rate determinations for heat inactivation and can be

attributed to small but significant uncontrolled differences in the

two sets of experiments.

Figure 12. Rate of thermal inactivation as a function of amount of DNA deleted for various lambda deletion mutants. Thermal inactivation was performed at $60^\circ C$ in $10^{-1} M$ Tris with $10^{-3} M$ $MgSO_4$ and assayed as described in Materials and Methods. From the plot of log surviving phage fraction versus time at $60^0 C$ the rate of inactivation (K) was calculated in hour^{-1}. The rate of inactivation is plotted versus the percent of DNA deleted (calculated as in Table 6) for each of the new deletion mutants used (◉). Also plotted are data from Table 4 for the deletion mutants b5, b2, and b2-b5 (○).

CALCULATED PERCENT DNA DELETED
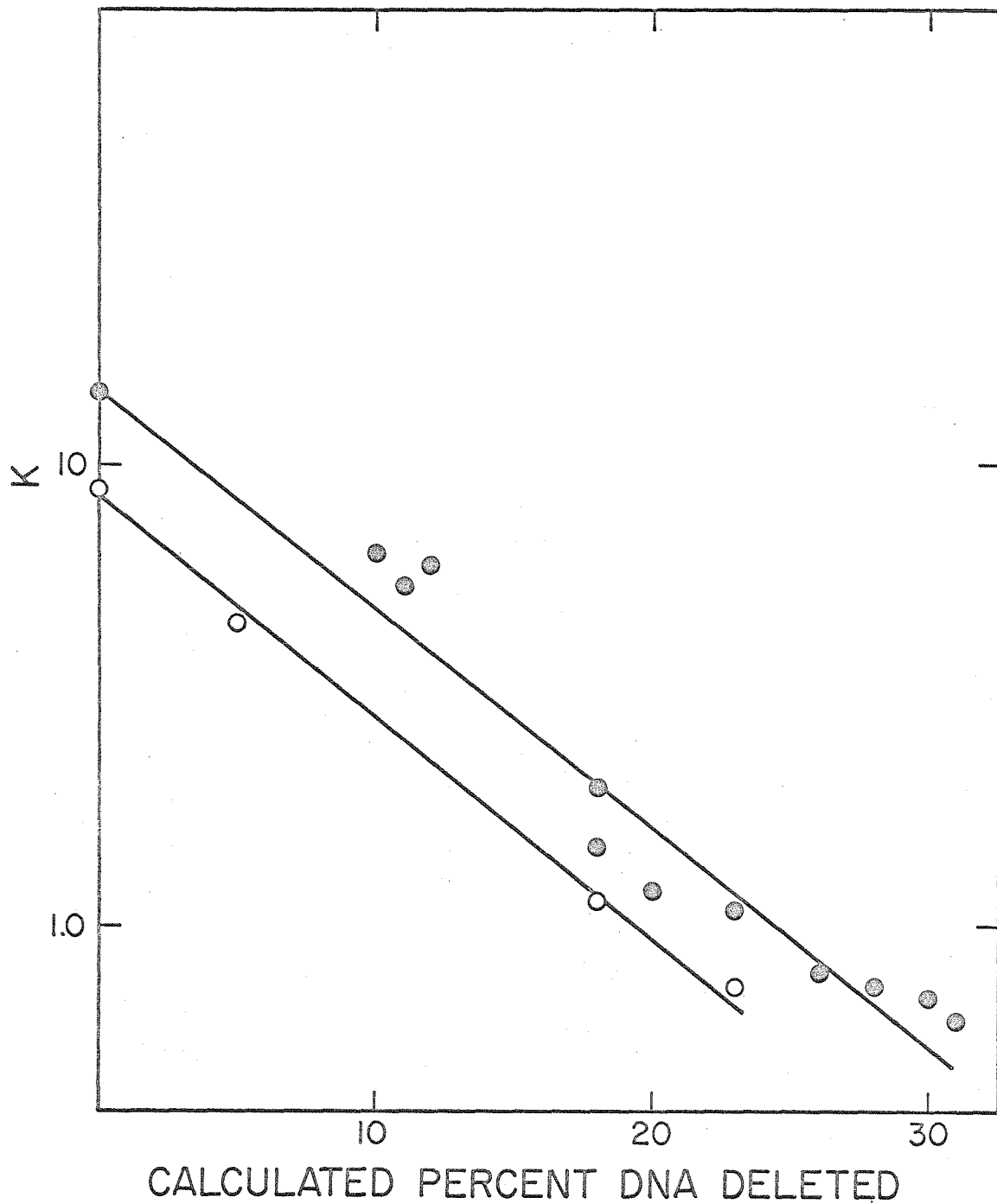
Recombination properties. Jordan (1964) showed that recombination

between the h and c markers of lambda was reduced when at least one

of the parental phage carried the b2 deletion. On the assumption that

a deletion between two genes will lower the recombination frequency

between those genes, some of the density mutants isolated here were

crossed with a double mutant, lambda susJ33-cIt1, and the recombination

frequency was determined by looking for sus$^+$-cIt1 recombinants. As

shown in Table 6, the density mutants fall into two categories with

respect to recombination frequency between J and cI. One class shows

reduced recombination compared to wild type while the other class

exhibits much higher recombination than wild type. The mutants with high

recombination between J and cI have been examined further and these

studies will be reported elsewhere. As determined by a decrease in

recombination frequency, 25 of the density mutants isolated here

appear to have deletions between J and cI similar to the lambda b2
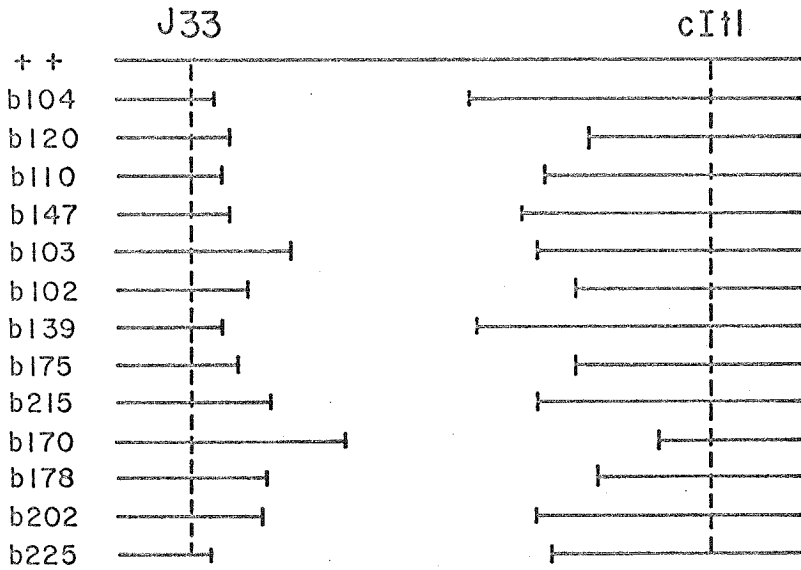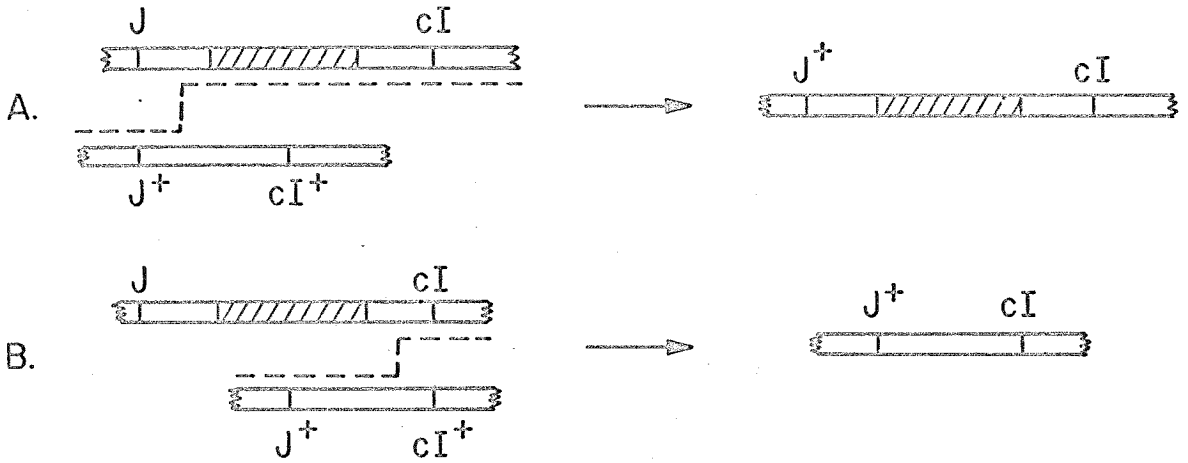
deletion.

On the assumption that the density mutants isolated here are DNA

deletions between J and cI, an attempt was made to map the ends of some

of the deletions by scoring the recombinants of the above crosses for

the segregation of the abortive or stable lysogenic response. That

this phenotype segregates with the deletion is supported by the finding

that the b2 deletion cannot be separated from the abortive response

(Kellenberger, et. al., 1961a). Assuming that recombination frequency

is a measure of physical distance, the ratio of the recombinant types

is a measure of the relative sizes of the genetic segments between J

and the deletion and between cI and the deletion. This ratio should

be insensitive to variations in absolute recombination frequencies due

to environmental variables.

The recombinants tested came from crosses of lambda susJ33-cIt1

with various deletion mutants. The susJ$^+$-cIt1 recombinants were

tested for the abortive or stable lysogenic response by the EMB-glu-

cose method described earlier. Since the recombinants contain the J$^+$

marker from the deletion mutant and the cIt1 marker from the normal

density phage, a recombinant giving a stable response must have had

a recombinational event between J and the left end of the deletion

(Figure 13). Similarly, a recombinant which gives an abortive lysogen-

ic response must have had a recombinational event between cI and the

right end of the deletion. In order to use these data to map the ends

of the deletion, the percent of each type of recombinant was multiplied

times the total recombination frequency for the cross analyzed. This

gives the recombination frequency for the intervals from J33 to the left

end of the deletion and from the right end of the deletion to cI. The

results for 13 of the deletion mutants are depicted in Figure 13 as

deletion maps.

Due to the relatively small numbers of recombinants examined any

interpretation of the results must be limited to an overall summary

rather than individual results. The correlation between the percent

reduction in recombination frequency and the calculated percent

deletion is close but not perfect. With these qualifications in mind,

it appears that the deletions may share a common left terminus but

Figure 13. Deletion maps of new lambda deletion mutants. Crosses were done between lambda susJ33-cIt1 and 13 lambda deletion mutants. Lambda susJ$^+$-cIt1 recombinants were picked and scored for the stable or abortive lysogenic response as described in Materials and Methods. In the top part of the figure is a schematic representation of the formation of the two types of recombinants. If the recombinational event occurred between J and the left end of the deletion (A), the recombinant would not harbor a deletion and would give a stable lysogenic response. If the recombinational event occurred between the right end of the deletion and cI (B), the recombinant would be deleted for the middle segment and would give an abortive lysogenic response. The frequency of each class of recombinants was determined for each mutant crossed and the total recombination frequency (Table 6) was fractionated by the frequencies of the two classes. The resulting frequencies are plotted as lengths from J or from cIt1.

the right ends are clearly different indicating that the mechanism
which produces these deletions may be precise with respect to one
end but not the other.

Physiology.   There are at least two separate physiological functions
of lambda which are apparently non-essential for vegetative growth --
integration and recombination. Zissler (1967) has isolated apparent
point mutants of lambda which give an abortive lysogenic response.
These mutants are reported by him to complement lambda b2 suggesting
that they do not lack the homology region.   One of these mutants
recombines with lambda b2 to give recombinants which give a stable
lysogenic response indicating that lambda b2 does contain the wild
type allele of these mutants.   Zissler called these mutants integration
negative and concluded that they lack an enzyme or enzyme system
necessary for integration of the phage chromosome into the host
chromosome.

Eleven of the deletion mutants isolated here were tested for
complementation with the int4 mutant of Zissler.   A typical result
is shown in Figure 14.   Strain W3110 was mixedly infected with a
deletion mutant and int4 and diluted into K medium with anti-lambda
serum.   At various times aliquots were removed and assayed for total
bacteria and UV-inducible infective centers.   In a positive comple-
mentation test the infective centers would closely parallel the total
cells but in an abortive lysogenic infection the infective centers
would level off while cell count was still increasing.   None of the
mutants tested complemented int4 including the longest and shortest

Figure 14. Complementation of int4. A saturated culture of W3110 was diluted to 4×10^8 cells/ml. Mixed infections of these cells were made with lambda int4-cIt1 and lambda wild type (A), lambda b2 (B) or lambda b104 (C) at multiplicities of 6 each. After 15 minutes adsorption, the infected cells were diluted by 2×10^3 into K medium at $37°C$ containing anti-lambda serum at a K-value of 10 min.$^{-1}$. At various times samples were removed and assayed for viable cells (O). The samples were UV induced and assayed for infective centers ($\triangle$) on plates seeded with C600.
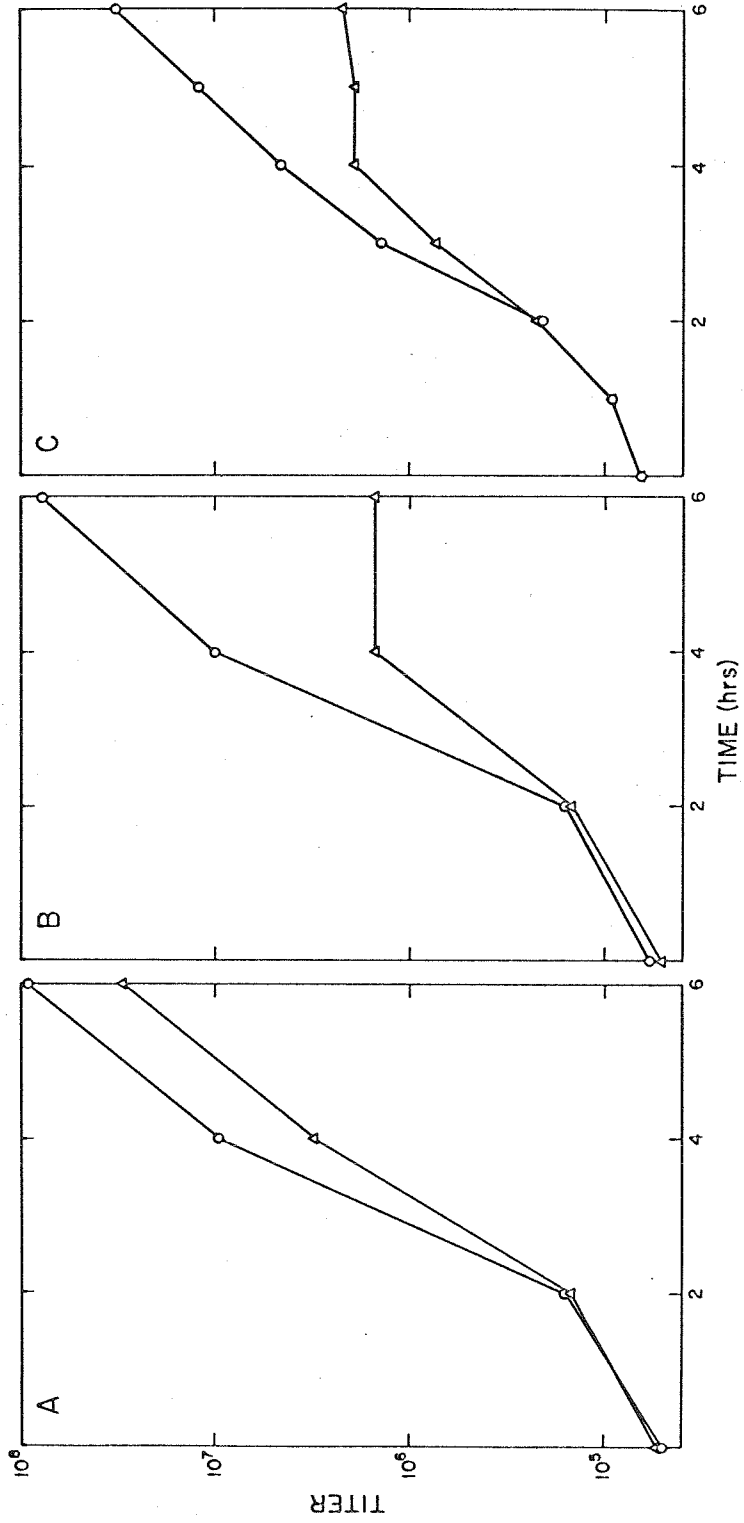
deletions and, contrary to Zissler's results, lambda b2 did not complement int4. Lambda wild type did show complementation with the int⁻ mutant and once integrated cells lysogenic for int4 were stable and inducible by UV. Thus none of the deletion mutants tested contained the integration enzyme system as indicated by these tests. Gottesman and Yarmolinsky (1967) have also isolated int⁻ mutants of lambda and their results show that lambda b2 complements these int⁻ mutants at an efficiency of 5%. Their method of testing for complementation is different than the one used here which may not be sensitive enough to detect 5% or less complementation. However, crosses of int4 by b2 and b130, one of the mutants isolated here, did give recombinants of normal density which exhibited a stable lysogenic response. The progeny of mixed infections of int4 with b2 and int4 with b130 were separated on CsCl buoyant density gradients. Bacteria from plaques made by phage from the normal density peak of each gradient were tested for the abortive or stable lysogenic response. The int4 by b2 cross gave 4.6% normal density recombinants with a stable lysogenic response and the int4 by b130 cross gave 16% of these apparent wild type recombinants. Thus b2 and b130 do not efficiently complement int4 but they do contain the wild type allele for the int4 mutation.

Lambda shows normal recombination in a recombinationless (rec⁻) host (Brooks and Clark, 1967) indicating that lambda contains the information necessary to produce a recombination system. Franklin (1967b) has shown that two of the longer deletion mutants of the ∅80-lambda hybrid exhibit greatly reduced recombination in a rec⁻ host

suggesting that these strains do not contain the phage rec system. In order to test for the ability to undergo recombination a phage strain must contain genetic markers. To avoid the work involved in putting markers into the density mutants isolated here a new test for phage recombination was designed.

It appeared that multiplicity reactivation of UV inactivated phage (Luria and Dulbecco, 1949) might provide an easy test for the presence or absence of the phage recombination system. Earlier work by Epstein (1958) suggested that multiplicity reactivation is, in fact, a reflection of recombination between damaged genomes giving rise to an undamaged genome. The experiments described below give strong support for the recombination basis of multiplicity reactivation.

Various phage suspensions were irradiated with UV and the survivors were plated on rec^--uvr^- cells to determine the rate of survival. The irradiated phage were also adsorbed to rec^--uvr^- bacteria at multiplicities of 5 or more and assayed for infective complexes. The ratio of surviving complexes to initial complexes after irradiation for single infection and multiple infection are compared and if the ratios differ by the same factor as the multiplicity, no multiplicity reactivation has taken place. If the ratios differ by a factor much greater than the multiplicity, the phage have undergone multiplicity reactivation. A control experiment shown in Figure 15 indicates that the ∅80-lambda hybrid deletion mutant ∅tdel 20R which is rec^- and int^- (Franklin, 1967b) does give multiplicity reactivation on a rec^+ host (AB1886) but not on a rec^- host (AB2480).

Figure 15. Multiplicity reactivation of Øtdel 20R. The Ø80-lambda

hybrid deletion mutant Øtdel 20R was irradiated with UV under a

germicidal lamp. At 30 second intervals phage samples were used

to infect cells of strain AB2480 at multiplicities of much less than

0.01 (O) or 3 (□). Also cells of strain AB1886 were infected at

a multiplicity of 3 (△). The infected cells were assayed for

infective centers on plates seeded with AB2480. The resulting titers

were divided by the maximum number of complexes before UV inactivation.
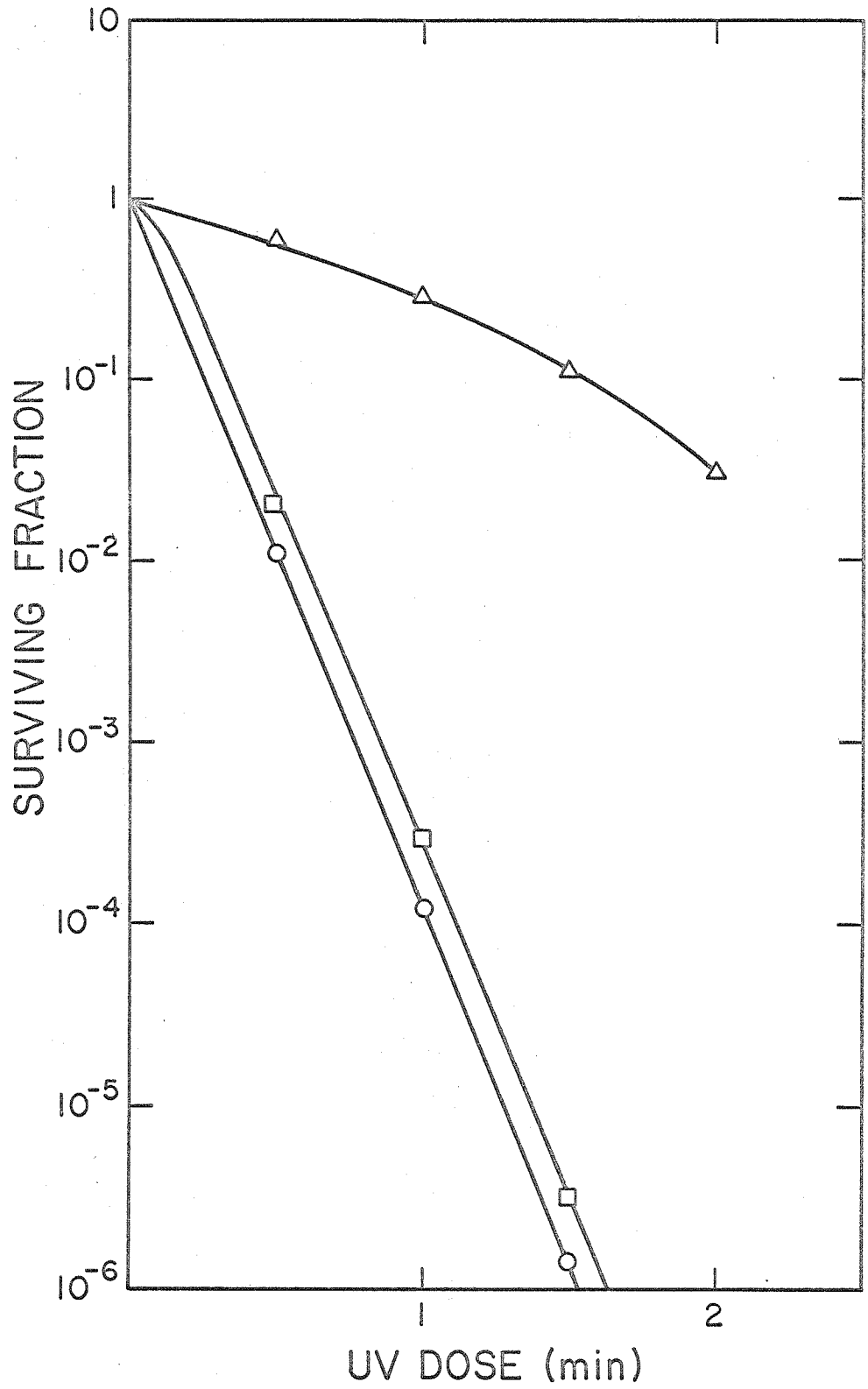
Thus multiplicity reactivation of UV irradiated phage is due to
recombination and does not occur in the absence of both phage and
host recombination systems. All of the lambda deletion mutants tested
(29), including the longest and shortest deletions, exhibited multi-
plicity reactivation in the rec⁻ host indicating that none of these
mutants lack the lambda recombination system.

DISCUSSION

The heat inactivation of bacteriophage T5 was well characterized by Lark and Adams (1953) and their findings are summarized here to show the similarities and differences with bacteriophage lambda. T5 exhibits a biphasic thermal inactivation curve suggesting a low frequency of an apparent heat stable class of phage in a wild type population. However, the progeny of these heat stable phage are wild type with respect to heat sensitivity upon multiplication and Hertel, et. al. (1962) showed that these phenotypically heat stable phage were also phenotypically less dense. The rate of inactivation depends upon the temperature and the ionic make-up of the medium with a higher rate of inactivation at higher temperatures and a higher rate of inactivation at lower ionic strengths. The inactivated phage lose their DNA as well as the ability to adsorb to bacteria, which led to the proposal that the heat inactivation of T5 is a consequence of heat denaturation of the tip of the phage tail. Density mutants of T5 have been isolated which have increased resistance to heat inactivation. Recent evidence shows that these T5 density mutants are due to phage DNA deletions (Abelson and Thomas, 1967).

Thermal inactivation of lambda also results in a two component inactivation curve which reveals an apparent heat resistant phage fraction in a lambda wild type population. The rate of heat inactivation and the back extrapolate of the second component depend upon the temperature of inactivation and the salt concentration of the medium. As with T5, the rate of lambda inactivation by heat increases

with increased temperature or decreased salt concentrations. In fact the results of the salt concentration experiments are very similar to the T5 data with an increasing order of effectiveness in protection from heat inactivation going from potassium to sodium to calcium to magnesium. Lark and Adams (1953) proposed that the ionic effect was due to the requirement of the T5 phage coat for a metal ion and the relative efficiencies could point to the neutralization of two neighboring negative charges leading to a more stable protein configuration. The ionic effect could also be due to a specific binding of the cation to the phage DNA.

The rate of inactivation dependence upon temperature leads to a computation of activation energy for heat inactivation (Daniels and Alberty, 1961). Woese (1960) compiled data from many different studies of viruses with the general conclusion that viruses show activation energies of approximately 20 kcal/mole or 85-100 kcal/mole and in some instances the same virus had a high or low activation energy depending upon the temperature. Woese also surmised that the second component of the heat inactivation curves had a lower activation energy but this is not true for lambda. Both components of a lambda wild type inactivation curve have activation energies of about 20 kcal/mole. T5 has an activation energy of 85 kcal/mole for heat inactivation and this may be an indication of the differences in these viruses with respect to heat sensitivity.

The heat resistant class of phage in a lambda wild type population is largely due to phage with phenotypic heat resistance. These phen-

otypically heat resistant lambda phage are also phenotypically less dense. Phage of phenotypically lower density exist in lambda wild type preparations, but elimination of those phage by CsCl buoyant density gradient purification did not significantly decrease the level of heat resistant phage. Thus during the process of thermal inactivation, the less dense, heat resistant phage appear, apparently as a result of the inactivation process.

As with T5, heat inactivation of lambda is accompanied by loss of DNA. The sedimentation velocity of the heat extracted DNA is the same as that for phenol extracted DNA, which indicates that there is no denaturation or degradation of the DNA. This is not surprising in view of the high melting temperature of DNA (Kaiser and Hogness, 1960). Since deletion mutants are more stable to heating than lambda wild type and this relative stability is correlated with the size of the deletion and is apparently not due to a genetically determined protein difference, the volume of DNA in the phage head must be a factor in the mechanism of heat inactivation. This could explain the cationic effect on heat inactivation. The strong cationic effect, especially with divalent cations, may be due to the cations neutralizing the negative charges of the phosphate backbone of the DNA enabling the DNA to become more tightly packed and thus mimicking a deletion mutant by lowering the DNA volume to head volume ratio.

The cationic effect may account for the low density particles of Fraser (1966) who found that lambda infected cells which are grown to lysis in low salts broth have a lowered burst size and the

phage released are mostly of a phenotypically lower density. I have observed that lambda is unstable at $37^{\circ}C$ but not at $4^{\circ}C$ in the magnesium concentration used in the Fraser experiments and this is most likely an extension of the results on ionic concentration versus rate of heat inactivation. Thus the lowered burst size in low salts medium may be due to inactivation of the normal lambda particles which are present in the burst and the low density particles would appear to be the same class of phage as the phenotypically heat resistant, less dense phage seen here.

Lambda heads are damaged as a result of thermal inactivation at a much higher rate than tails, which may or may not be inactivated under the conditions employed. Free heads also do not exhibit a heat resistant component like whole phage, suggesting a possible role of tails in the formation of phenotypically heat resistant phage. From electron micrographs it appeared that the frequency of phage with ruptured heads had not significantly increased after heat inactivation and the tails were not visibly altered or lost. (Weigle (personal communication) has not seen a significant increase in the number of free tails after heat inactivation of a lambda wild type preparation using the in vitro system.) A possible mechanism to account for these observations is simply a triggering of the head part of the normal lambda ejection mechanism leading to the eventual loss of the DNA. The "trigger" may be activated as a result of the increased kinetic motion of the DNA which would increase the stresses on the head structure.

The two related phenomena of phenotypic heat resistance and phenotypically lower density might be accounted for in light of the challenged evidence of Cummings and Kozloff (1960) which indicates that two head forms of phage T2 can occur, one of which is longer than the other. The shorter, normal form of the T2 head can be converted to the longer form by heating, pH change or by changes in the ionic environment. Contrary to this observation, Kellenberger, et. al. (1965) have failed to observe a longer head form in T4 using similar conditions. Nevertheless, if some such mechanism existed, the heat resistant phage in a lambda wild type population could be the result of a lengthened head form as in T2. Since an increase in head size would increase the phage volume, the density of these proposed long headed phage would be lower than normal. The heat resistance of these proposed long headed phage could be accounted for in light of the heat resistance exhibited by the lambda deletion mutants. The deletion mutants have less DNA in a normal sized head while the proposed long headed phage would have a normal amount of DNA in a larger than normal head. These two situations would resemble each other in heat resistance if heat sensitivity is dependent upon the ratio of DNA volume to head volume.

Bleichrodt and Van Abkoude (1967) have shown that pH changes and ionic conditions can change the heat sensitive form of ØX174 to a heat resistant form or vice versa. Since the same conditions which favor conversion of normal heads to long heads of T2 also favor the conversion of heat sensitive ØX174 to a heat resistant form, it is not

unreasonable to imagine that conversion of lambda heat sensitive phage

to heat resistant phage is simply a lengthening of the lambda head.

Using thermal inactivation as a selective enrichment technique

it was possible to isolate density mutants from a lambda wild type

preparation. Although this procedure selects for heat resistant phage,

the heat resistant mutants isolated were found to have lower density.

This change in density could be due to decreased DNA content per phage

particle, increased protein in the phage coat, increased size of the

phage itself or even different subunit structure of the protein coat

with different salt binding characteristics. However, the evidence

presented here favors the notion that the density mutants isolated

are due to DNA deletions of the b2 type.

All of the low density mutants gave an abortive lysogenic response

indicating that they cannot integrate into the host chromosome, although

since they make turbid plaques, they can lysogenize the host. All of

the density mutants had the immunity of lambda unlike the b5 deletion

mutant indicating that no change had occurred in the immunity region

of the genome. All of the density mutants behaved like lambda wild

type with respect to host modification and restriction. This indicates

that not all of the sites involved in restriction had been changed or

lost due to deletion. This is not unexpected since at least 36% of

the DNA would have to be deleted to remove the three restriction sites

as described by Meselson.

Density determinations confirmed that, in fact, the heat resistant

mutants isolated were stable density mutants. The bands in the ultra-

centrifuge were as narrow as the lambda wild type density marker.

Phage mutants of various densities were observed with the lowest

density at 1.481 g/cm^3 and the highest density at 1.498 g/cm^3.

The majority of the mutants were centered around the density of

lambda b2 which has a density of 1.491 g/cm^3. Since the mutants

were selected from the leading and trailing sides of the lower

density peak of the selected preparation, the distribution of mutants

examined doubtless has a larger spread than the actual distribution

of mutants in the whole preparation. Assuming a density difference of

0.001 g/cm^3 to be significant there were 16 different density classes.

The range of density changes may be limited in the higher end by the

selective technique and the lower end by a physical or genetic require-

ment of the phage but the number of different density types most likely

has not been exhausted and further isolation would, no doubt, uncover

new density classes.

Although no direct evidence such as length measurements is

presented, the tests performed favor the notion that the density

mutants are DNA deletion mutants with the deletion occurring between

genes J and cI. All of the mutants are abortive lysogens, recombination

between J and cI is altered, segregation of the abortive response in

recombinants between J and cI is similar to that shown by lambda b2

and the change in density is centered around the b2 density. All of

these results suggest that these density mutants are due to DNA

deletions of the b2 type.

Based on this assumption the deletion ends of some of the mutants

were tentatively mapped by scoring the segregation of the abortive

character in recombinants between J and cI. Most of the deletions

tested were deleted closer to J than to cI similar to lambda b2.

Since lambda dg phage are deleted in phage genes starting from J and

continuing toward A, the deletion mutants isolated here may have been

produced by the same mechanism that produces lambda dg phage.

The frequencies of lambda dg phage and the low density phage

differed in the various preparations by roughly the same factor.

This would also indicate that these two types of deletion mutants are

produced by the same mechanism. Since the frequencies of lambda dg

and deletion mutants were higher in the UV induced preparations, a

likely candidate for the deletion producing mechanism is the UV re-

pair system of the host (Boyce and Howard-Flanders, 1964) (Pettijohn

and Hanawalt, 1964) (Setlow and Carrier, 1964). The lambda dg phage

as well as the deletion mutants appearing in the non-UV induced

preparations could be due to the background activity of the UV

repair system or to the non-homologous recombination system postu-

lated by Franklin (1967a). These considerations suggest that the mutants

analyzed were formed during the excision of the prophage from the

host chromosome. If so, it is possible that the density mutants ob-

tained from the population which had undergone only vegetative growth

arose via a different mechanism. However, this notion has not yet been

explored.

An attempt was made to determine if any non-essential genic

functions of lambda were missing in the deletion mutants. Under the

conditions used none of the mutants tested complemented the integration negative mutant of Zissler. The test, as explained earlier, may not be sensitive enough to detect a low level of complementation. Whether these mutants do contain the integration function or not is not definitely decided and a better method of testing for this function must be devised. Two density mutants, b2 and b130, did recombine with int4 to give phage of normal density which give a stable lysogenic response. Thus these mutants contain the wild type allele of the int4 mutation which is presumed to affect the formation of the integration enzyme. These apparently contradictory observations remain to be explained.

The fact that all of the mutants tested showed multiplicity reactivation suggests that they are not deleted in the recombination gene or genes of lambda. The test used offers further support for the notion that multiplicity reactivation is the result of recombination between the UV inactivated phage genomes, since a rec$^-$ phage does not show multiplicity reactivation in a rec$^-$ host but does show multiplicity reactivation in a rec$^+$ host. A useful by-product of this work is the possible use of multiplicity reactivation as a tool for selecting point mutants of lambda which are defective in the recombination gene.

Since deletion mutants with as small a DNA loss as 10% were found, the size of the lambda homology region necessary for successful insertion into the host chromosome would appear to be less than 10% of the phage genome. The isolation of even shorter deletions is necessary to put a lower limit on the size of this region. However, at least 30%

of the genome of lambda can be deleted without loss of any essential

or perhaps even non-essential functions although part of this is

required for homology necessary for integration.

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Part II

GENETIC AND PHYSICAL STUDIES ON LAMBDA b130

INTRODUCTION

In the first part of this thesis, the isolation and character-
ization of density mutants of phage lambda were described. Some
of these mutants showed abnormally high recombination frequencies
for the central chromosome segment between genes J and cI. Exper-
iments designed to explore further the nature and reasons for this
high recombination are reported here.

MATERIALS AND METHODS

Media. Growth media have been described previously.

Bacterial strains. E. coli strains C600 and 594 were used and have

been described previously.

Phage strains. All phage strains used in this study are listed in

Table 1. The use of the new lambda sus mutants isolated by J. S.

Parkinson and the int4 mutant isolated by J. Zissler is gratefully

acknowledged. Methods of growth of phage and conditions for genetic

crosses have been described previously.

DNA extraction. Phage at 10^{12} particles/ml were mixed with equal

volumes of water-saturated, recently distilled phenol and the tube

gently rotated by hand. After 5 minutes, the suspension was centri-

fuged, the phenol removed and the extraction repeated. After a

second centrifugation the aqueous layer was removed and dialyzed

against 0.6M NaCl with 0.01M EDTA at pH 7.0. The concentration was

determined by absorbance at 260mμ using the relationship of

1 OD_{260} = 50 μg/ml (Strack and Kaiser, 1965).

Analytical sedimentation velocity. Procedures for band sedimentation

velocity experiments in CsCl have been described previously.

Analytical CsCl buoyant density gradients. Procedures for analytical

CsCl buoyant density gradient experiments have been described previously.

Electron microscopy. Grids were coated with parlodion. DNA at 1 μg/ml

with cytochrome C at 0.1 mg/ml in 1.5M ammonium acetate was layered onto

0.15M ammonium acetate (Kleinschmidt, et. al., 1962). The grids were

fixed and dried in 95% ethanol followed by isopentane. The prepared

grids were shadowed with platinum to increase contrast. All measurements were calibrated through the use of a diffraction grating of known size. The electron microscope used was a Phillips EM200. The technical assistance of Mr. D. Mohr is gratefully acknowledged.

Preparative sucrose density gradients. Linear, 5ml sucrose gradients of 20-5% sucrose in TCM(10^{-2}M Tris, pH 7.4, 10^{-2}M CaCl$_2$, 10^{-2}M MgSO$_4$) were prepared by the method of Britten and Roberts (1960). Centrifugation was carried out in an SW39 rotor in a Spinco Model L ultracentrifuge. After centrifugation the bottom of the tube was pierced and the drops collected in separate tubes. Sedimentation values were calculated by the method of Martin and Ames (1961).

DNA infectivity assay. The assay for lambda DNA infectivity was essentially that of Strack and Kaiser (1965). Helper-infected bacteria were 594 infected with a lambda sus mutant at a multiplicity of 5. Infected bacteria were plated on 594.

Table 1. A list of phage strains used.

Phage strains	Reference	Obtained from
lambda wild type	Kaiser (1957)	J. Weigle
lambda b2	Kellenberger, Zichichi and Weigle (1961)	"
lambda cI26	Meselson (1964)	"
lambda cIt1	Lieb (1964)	"
lambda susB20-cIt1	Parkinson (1967)	J. S. Parkinson
lambda susJ33-cIt1	"	"
lambda susP22-cIt1	"	"
lambda susR54	Campbell (1961)	J. Weigle
lambda susB20-susJ33	Parkinson (1967)	J. S. Parkinson
lambda susB20-susP22	"	"
lambda susP22-susR54	"	"
lambda susJ6-cIt1	Campbell (1961)	J. Weigle
lambda b130	Huskey (1967)	
lambda b144	"	

RESULTS

The observation which prompted the work to be described was
the unusually high frequency of recombination between genes J and cI
in crosses involving certain lambda density mutants. Although 22
mutants described previously showed high recombination in the J to cI
interval, the experiments presented below deal largely with one of
the mutants, lambda b130.

I.   Genetic studies.

To determine if the high frequency of recombination observed
was due to an over-active but non-specific recombination system,
crosses were done to measure the recombination rate in another
region of the genome. As shown in Table 2, the recombination fre-
quencies for lambda b130 and b144 in the region of the right
chromosome arm between cI and P were not significantly different
from those of lambda wild type and lambda b2. Parallel controls
confirmed the high frequency of recombination between J and cI for
crosses involving these mutants as compared to lambda wild type and
lambda b2.

To investigate this high recombination effect throughout the genome
and also to determine if the deletion mutants contained some gross
rearrangement in gene order, markers were crossed into one of the
high recombining phage, b130, by crosses with appropriately marked
lambda strains of normal density. The appropriate recombinants were
selected by the phenotype of the marker used and the abortive lysogenic
response of lambda b130. That the recombinants had the density of

Table 2. Recombination frequencies in crosses of lambda and lambda
density mutants.

Parental strain 1	% recombination with parental strain 2 of the indicated constitution	
	lambda susJ6-cIt1	lambda susP22-cIt1
lambda wild type	6.9	4.4
lambda b2	3.5	4.6
lambda b130	22.1	4.8
lambda b144	16.1	5.0

Mixed infections of C600 were made with equal multiplicities (5)
of each parental 1 strain and one of the two genetically marked
parental 2 strains. Recombination values are expressed as twice
the number of sus$^+$cIt1 recombinants divided by the total number of
progeny and the resulting fraction expressed as a percentage.

lambda b130 was verified. Using the various strains thus constructed, recombination frequencies in the intervals B to J, J to cI, and cI to R were determined in three different situations: (A) both parents of normal density, (B) both parents of b130 density and (C) one parent of each density type. The results are shown in Figure 1 in the form of genetic maps with distances in percent recombination frequency.
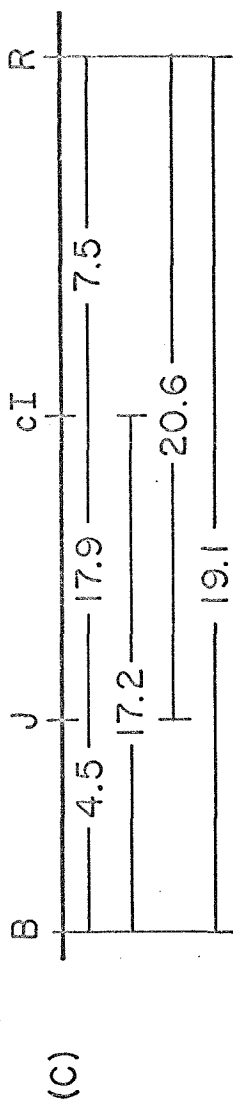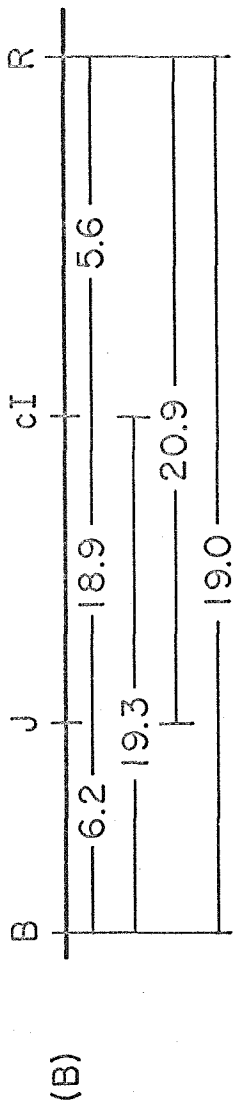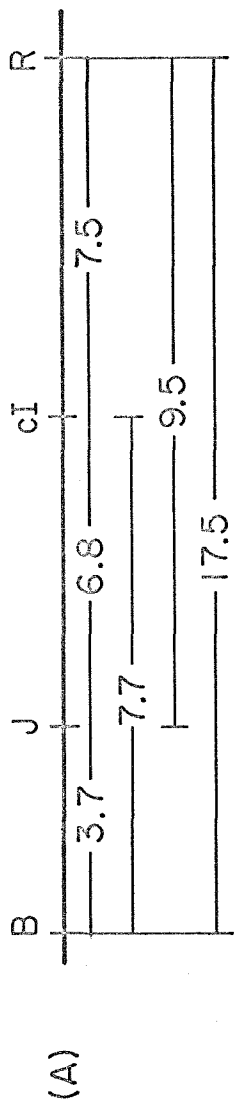
Recombination in the intervals B to J and cI to R is uninfluenced by the composition of the parental phage mixtures with respect to density. However, in the crosses where one or both parents are of lambda b130 density, the frequency of recombination is exceptionally high in the interval between genes J and cI. In fact the B and J markers cannot be ordered with respect to the cI and R markers in crosses involving phage with b130 density. These results indicate that the left and right arms of the lambda genome behave as though they were genetically unlinked in crosses in which one or both parents contained the b130 deletion.

II. Physical studies.

The genetic evidence for two linkage groups in lambda b130 might be accounted for by a physical singularity in the DNA of the phage. To investigate this notion, the DNA of lambda b130 was phenol extracted and its physical properties examined.

Band sedimentation velocity experiments of b130 DNA in neutral CsCl under conditions which separate the cohesive ends of lambda wild type DNA (Wang and Davidson, 1966) revealed three bands of

Figure 1. Genetic maps of lambda as determined by two-factor crosses. Pairwise crosses of lambda strains containing sus mutants or cIt1 were performed. Distances are expressed as percent recombination frequency. All values are an average of three independent determinations. The three maps represent crosses in which both parents were of normal density (A), both parents were of b130 density (B), or one parent was of normal density and one parent was of b130 density (C).

(A)

B   J   cI   R

3.7   6.8   7.5

7.7   9.5

17.5

(B)

B   J   cI   R

6.2   18.9   5.6

19.3   20.9

19.0

(C)

B   J   cI   R

4.5   17.9   7.5

17.2   20.6

19.1

UV absorbing material (Figure 2). These bands have sedimentation values of 32.7s, 28.9s and 23.1s corresponding to molecular weights of 26.5, 18.7 and 9.7×10^6, respectively, as calculated by Studier's formula (Table 3). Lambda wild type DNA under these conditions appeared as a single band with a sedimentation coefficient of 35.2s. The fastest sedimenting material of lambda b130 DNA had a calculated molecular weight equal to 80% of lambda wild type DNA which is in fairly good agreement with the lower density of b130 phage which gives a calculated loss of DNA of 13-15% as compared to wild type. The two slower sedimenting bands represent approximately 70% and 30% of the molecular weight of the larger b130 DNA molecule. Approximately 23% of the b130 DNA is present as the fast sedimenting species and the other two species are equally divided at 38% apiece.

These results could be interpreted in two ways. Conceivably 23% of the phage contain complete DNA molecules and 76% of the phage contain genomes composed of two hydrogen-bonded partial molecules. Alternatively all of the phage particles contain genomes comprised of two hydrogen-bonded partial molecules and the classes observed in the above experiment arise as a consequence of incomplete denaturation of the hydrogen-bonded fragments during heating. Since DNA is completely denatured into single strand molecules above pH 12.0 (Baldwin and Shooter, 1963) an alkaline sedimentation experiment should resolve the two possibilities mentioned. If single stranded molecules of whole length are obtained, it would indicate that at least one DNA strand of the whole molecules seen in a neutral gradient does not

Figure 2. Analytical band sedimentation velocity of lambda b130 DNA in neutral CsCl. Shown are densitometer tracings of sequential exposures (A to E) taken at 4 minute intervals during a band sedimentation velocity experiment in a neutral (pH 7.4) CsCl solution. Exposure time was adjusted to give a linear response to optical density. Centrifugation procedure is described in Materials and Methods. The DNA was heated at $60^\circ C$ for 5 minutes and rapidly cooled at $0^\circ C$ prior to use.
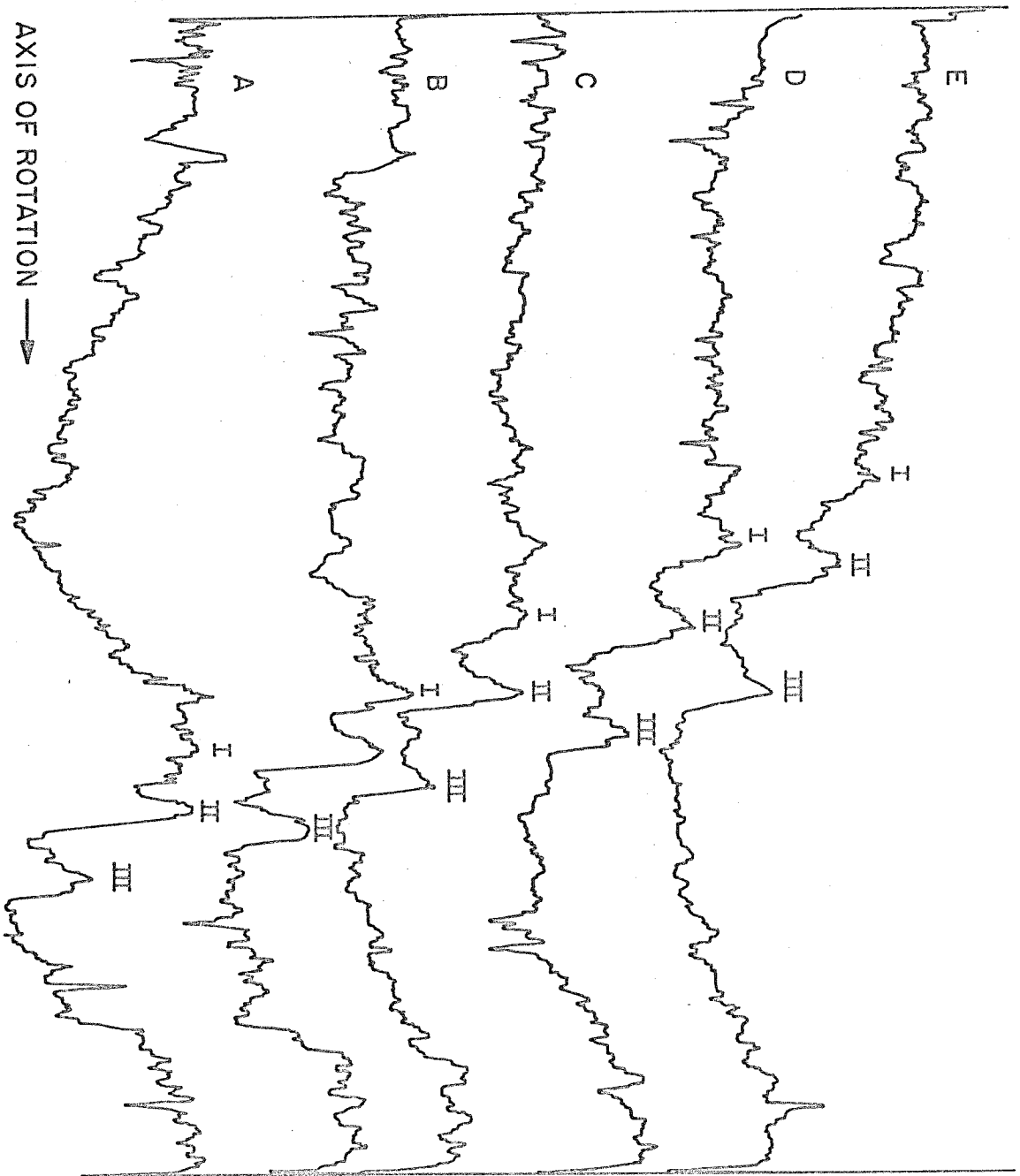
Table 3. Neutral $(s_{20,w})_w$ values for lambda wild type and lambda b130 DNA.

DNA extracted from	$(s_{20,w})_w$	Molecular weight	Fraction of DNA molecule relative to		Mole frac. of total DNA
			$+$ $+$	b130	
lambda wild type	35.2	33.1×10^6	100	---	---
lambda b130 I	32.1	26.5×10^6	80	100	.23
II	28.6	18.7×10^6	56	70	.38
III	22.9	9.0×10^6	29	36	.39

The $(s_{20,w})_w$ values were calculated as described in Materials and Methods. The DNA used was treated to break cohesive ends before sedimentation. Molecular weights were calculated using Studier's (1965) formula for the relation between sedimentation value and molecular weight for large DNA. The molar fraction of each sedimenting species was obtained from the area under each peak and dividing by the calculated length fraction for that species.

contain a single strand break.

The results of an alkaline CsCl gradient are shown in Figure 3. Two peaks were observed with sedimentation values of 39.7s, and 32.0s which give calculated molecular weights of 15.4 and 9.0×10^6 (Table 4). The faster sedimenting species has the sedimentation properties of a complete single strand while the slower species behaves as a single strand of the 70% molecule. Single stranded material of 30% was not seen although there may be some trailing material in the slower peak. Lambda wild type DNA gives one peak in alkaline CsCl with a sedimentation value of 40.6s.

Since the phage had been purified on a CsCl gradient before DNA extraction and no satellite bands were observed, it is reasonable to assume that each phage contains a complete DNA complement. From the above consideration it would appear that 23% of the DNA molecules from b130 particles have at least one strand intact whereas 76% of the phage contain DNA which can be degraded by conditions which break cohesive ends to partial molecules of approximately 1/3 and 2/3 in size.

In order to confirm the existence of the various length classes, b130 DNA was examined in the electron microscope. Since the observed lengths of DNA depend upon the salt concentrations used when applying the molecules to grids (Inman, 1967), the lengths given here for lambda DNA may not agree with published values but in every experiment a lambda wild type control was used as a length standard.

In recently extracted but unheated b130 DNA the length distribution is very closely centered around 12.6μ, which is equal to 85% of

Figure 3. Analytical band sedimentation velocity of lambda b130 DNA

in alkaline CsCl. Shown are densitometer tracings of sequential

exposures (A to F) taken at 4 minute intervals during a band sedimen-

tation velocity experiment in an alkaline (pH 12.8) CsCl solution.

Exposure time was adjusted to give a linear response to optical

density. Centrifugation procedure is described in Materials and
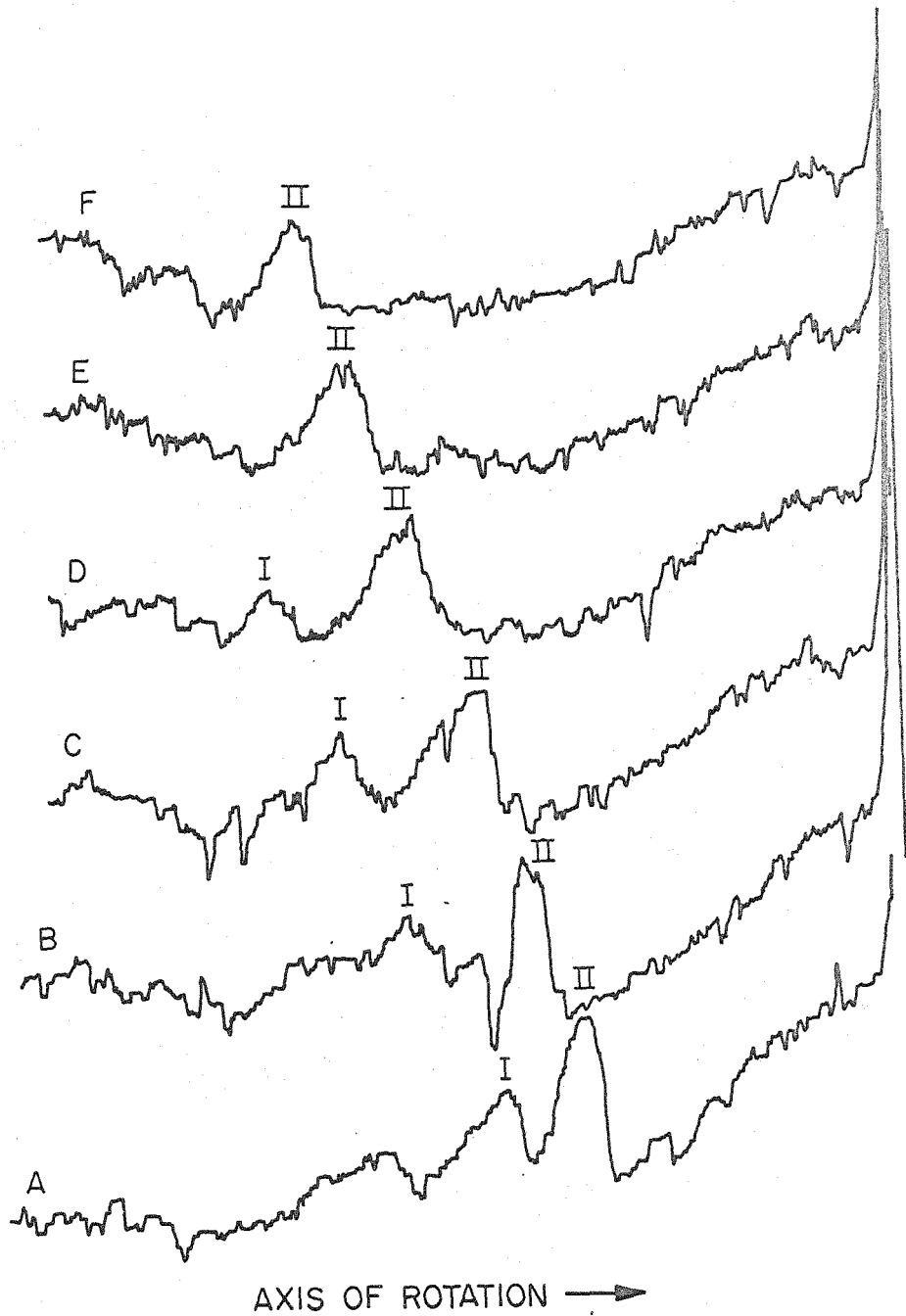
Methods.

AXIS OF ROTATION ⟶

Table 4. Alkaline $(s_{20,w})_w$ values for lambda wild type and lambda b130 DNA.

DNA extracted from	$(s_{20,w})_w$	Molecular weight	Fraction of undenatured DNA from Table 3
lambda wild type	40.6	16.4×10^6	50% whole + + DNA
lambda b130 I	39.7	15.4×10^6	48% whole b130 DNA
II	32.0	9.0×10^6	48% 2/3 b130 DNA

The $(s_{20,w})_w$ values were calculated as described in Materials and Methods. The CsCl solution used to sediment through was adjusted to pH 12.8 before the experiment. Molecular weights were calculated using Studier's (1965) formula.

lambda wild type DNA (14.4μ) measured under the same conditions (Figure 4A). This would indicate that lambda b130 phage contain single linear molecules of DNA with a 15% deletion relative to lambda wild type. However, when the DNA is heated to 60°C and rapidly cooled to break cohesive ends, the length distribution is divided into three classes of 4.0μ, 7.8μ and 12.7μ (Figure 4B). The longest molecules are 88% of wild type in length which is commensurate with density and sedimentation calculations. The two shorter classes represent approximately 1/3 and 2/3 whole molecules of b130 DNA corresponding to the sedimentation results. Thus some of the DNA molecules from lambda b130 phage particles can be separated into two molecules of 1/3 and 2/3 lengths when the DNA is treated to break cohesive ends.

When b130 DNA is heated to 60°C and cooled slowly to allow cohesive ends to anneal, circular molecules are observed as well as linear molecules equal in length to monomers, dimers, trimers, and one molecule equal to 1 2/3 length (Plate 1). The DNA concentration was somewhat higher than that used by Wang and Davidson which perhaps accounts for the large number of dimers present.

The partial molecules occur as discrete classes suggesting that the discontinuity which gives rise to them has a specific location. Kaiser and Inman (1965) showed that even partial molecules of lambda were infective in the helper assay system as long as the molecule had at least one cohesive end. Since the partial molecules are joined by some type of cohesive ends it seemed likely that the partial molecules

Figure 4. Length distribution of lambda b130 DNA. Lambda b130 DNA was examined in the electron microscope as described in Materials and Methods. Electron micrographs were taken of measureable molecules and tracings made from the enlarged negative. Molecules were measured by tracing the contour with a Dietzgen map measurer. Number of molecules is plotted versus length as determined from the magnification factor. A) recently extracted b130 DNA. B) b130 DNA heated at 60°C for 5 minutes and rapidly cooled at 0°C prior to application to the grid.
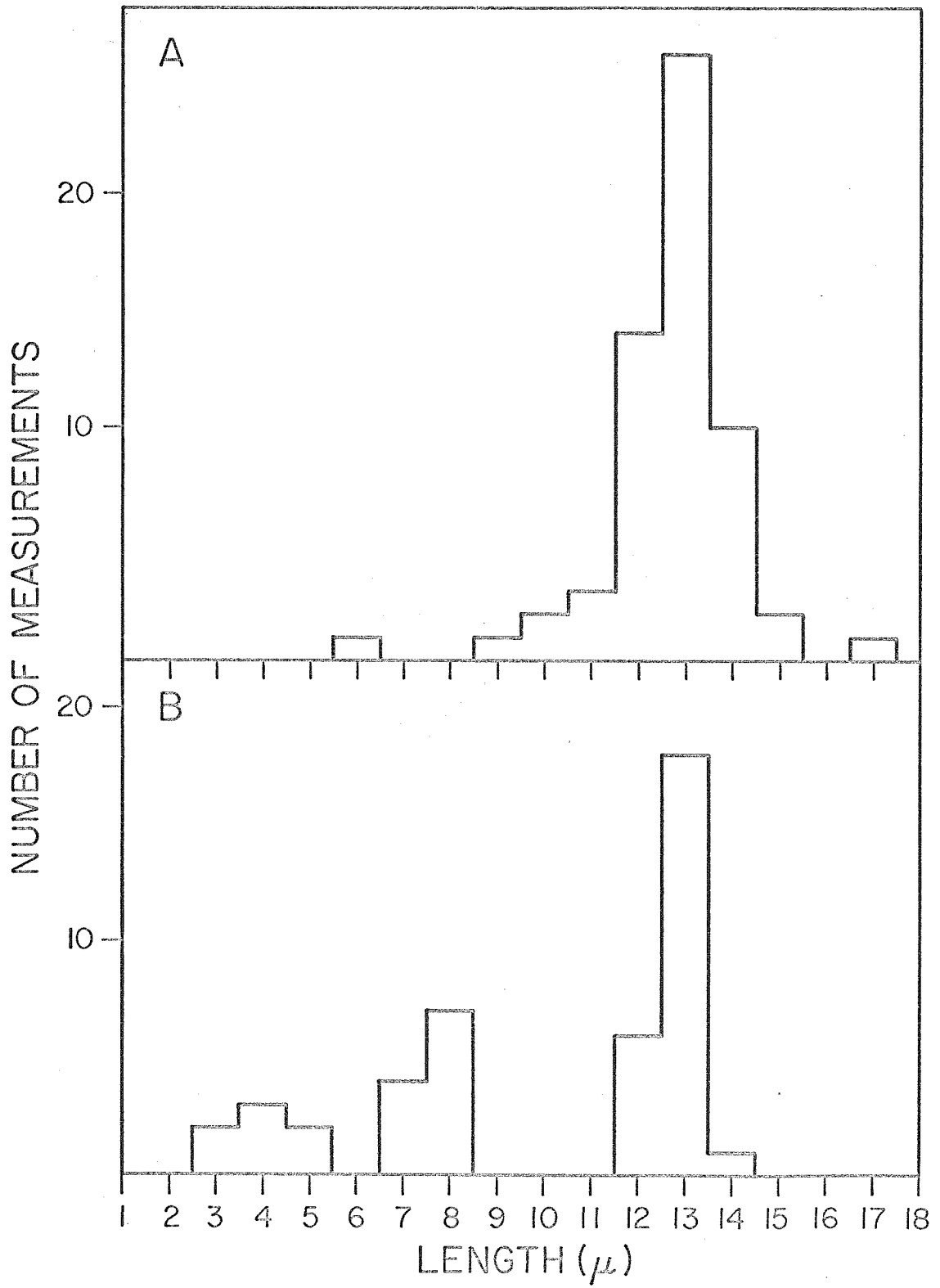
Plate 1. Electron micrograph of lambda b130 DNA. Lambda b130 DNA at approximately 20 μg/ml was heated at 60°C for 5 minutes and cooled slowly at 30°C for 2 hours. Electron microscope procedures are described in Materials and Methods. Above the middle of the picture, on the left is a circular molecule and below the middle on the right is a linear monomer. Also shown are parts of some unmeasureable molecules.
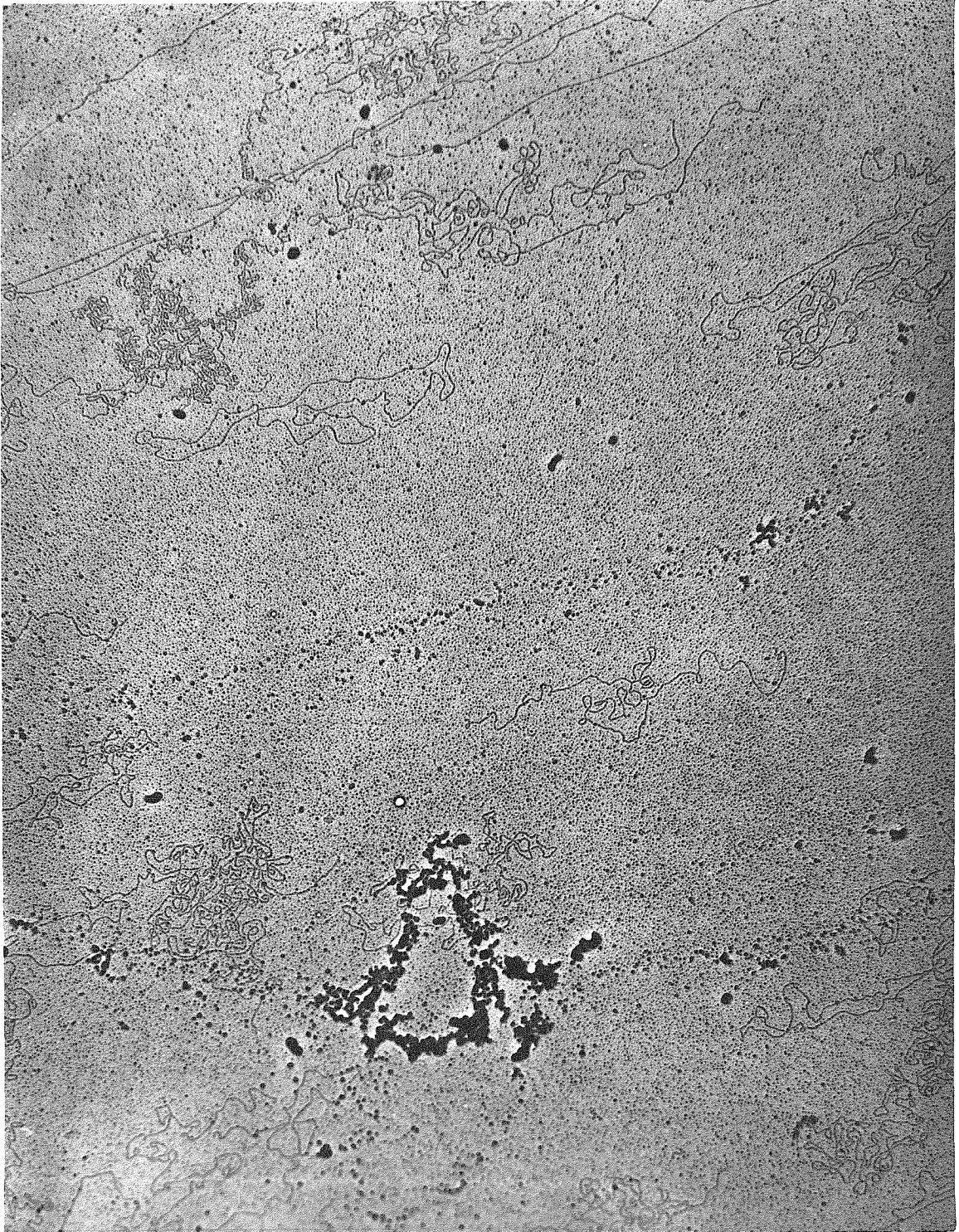
of b130 DNA would be infective. Thus the genetic composition of these
partial molecules could be determined using the DNA infectivity assay.

The partial molecules were separated by centrifuging heated,
fast-cooled b130 DNA through a preparative sucrose gradient. The
gradient fractions were analyzed for the presence of molecules of
different genotypes by the helper system (Kaiser and Hogness, 1960)
for assaying lambda DNA. Since partial molecules were to be assayed,
the assay used was based on marker rescue of conditional lethal
mutants under restrictive conditions. The assay method has reduced
efficiency but the background is low resulting in better resolution
of the peak fractions.

E. coli 594, which is restrictive for lambda sus mutants, was
infected with either lambda susB20-susJ33, lambda susP22-susR54 or
lambda susB20-susP22 and used as helper-infected bacteria. These
strains were used to assay for molecules containing markers from the
left arm of the lambda genome (B and J), the right arm (P and R) or
both arms (B and P). After adsorption with the gradient fractions,
the helper-infected bacteria were assayed for infective centers on
plates seeded with strain 594. Thus only those bacteria which picked
up DNA containing wild type markers homologous to the two sus markers
of the helper phage could initiate an infective center. If the 1/3
molecule contains the B and J genes, the bacteria infected with
B20-J33 helper phage should show two peaks of activity - one at the
sedimentation value of whole molecules, since whole molecules contain
all the lambda sus markers, and one peak at the sedimentation value

of 1/3 molecules. Similarly, if the 2/3 molecules contain the P and

R genes, the bacteria infected with P22-R54 helper phage should also

show two peaks - one at the s-value of whole molecules and one at

the s-value of 2/3 molecules. The bacteria infected with B20-P22

helper phage should exhibit only one peak of activity at the sedimen-

tation value of whole DNA if the discontinuity occurs between genes

J and P.

The results shown in Figure 5 reveal a slight peak of activity

on all three kinds of helper-infected bacteria at an $s_{20,w}$ of 32s.

There is an additional peak of activity on P22-R54 infected bacteria

at an $s_{20,w}$ of 28s. The higher sedimentation value is similar to the

analytically determined value for whole molecules of b130 DNA and

since all four genetic markers are associated with this sedimentation

value it can be assumed that this peak represents the whole molecules

in the population which are not broken by conditions which break

cohesive ends. The additional peak of activity on P22-R54 infected

bacteria has a sedimentation value similar to that of the 2/3 molecules

as determined in the analytical gradients. The tentative conclusion

is that the 2/3 partial molecule includes the right chromosome arm

containing genes P and R. Although no activity was detected for the

1/3 partial molecule, by inference it probably included the left

chromosomal arm containing the B and J genes. The fact that no acti-

vity for the 1/3 molecule was detected is unexplained. However, the

frequency of marker rescue for B20-J33 infected bacteria was less than

for P22-R54 infected bacteria using b130 DNA prior to sedimentation,

Figure 5. Preparative sedimentation velocity of lambda DNA. Lambda

DNA at 20 μg/ml was heated at 60°C for 5 minutes and rapidly cooled

at 0°C prior to sedimentation. 0.1 ml of the DNA suspension was

layered onto a 20-5% sucrose gradient and centrifuged at 37,000 rpm

at 5°C for 5½ hours in an SW39 rotor. Samples were collected into

0.2 ml of helper-infected bacteria. The first fraction and every

third fraction thereafter was collected into 594 infected with lambda

susB20-susJ33. The second fraction and every third fraction thereafter

was collected into 594 infected with lambda susP22-susR54. The third

fraction and every third fraction thereafter was collected into 594

infected with lambda susB20-susP22. After 25 minutes adsorption, DNase

was added at 1 μg/ml for 5 minutes and the fractions assayed on plates

seeded with 594. The results are plotted as titer versus percent of

the gradient. Axis of rotation is to the right. BJ infected 594 (O).

PR infected 594 (□). BP infected 594 (△). A) lambda b130 DNA.

B) lambda wild type DNA.
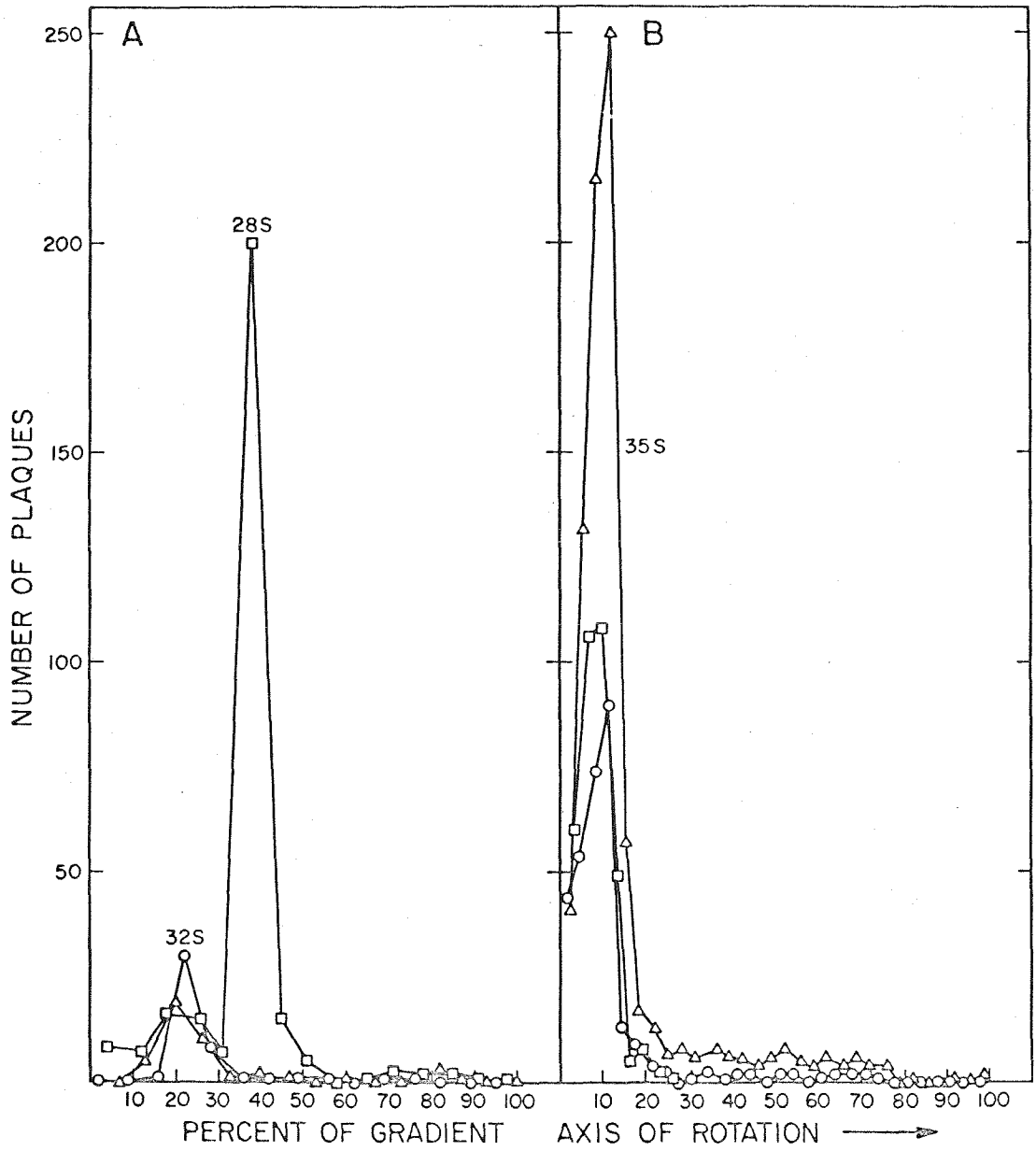
suggesting a lower marker rescue frequency for B and J than for P and R.
A control of lambda wild type DNA was also analyzed by the sucrose
gradient method outlined above and only one peak of activity was ob-
served on all three types of helper-infected bacteria with an $s_{20,w}$
of 35s (Figure 5).

The experiments described indicate that the lambda b130 phage
particles exist as two different kinds of particles with one class
containing whole DNA molecules and the other class containing two
partial DNA molecules joined by cohesive ends. The following exper-
iment was designed as an attempt to determine if these two classes of
phage are infective. A mixture of lambda cI26 and lambda b130 was
centrifuged in the analytical ultracentrifuge in a CsCl buoyant
density gradient. The relative amount of each type of phage was
determined by plaque count and from the optical density as measured
by the absorption optics of the ultracentrifuge. These results are
given in Table 5. If there had been an excess of inactive b130 phage
by a factor of 2 or more, the ratio of the relative amount of each
density class should have been greater than the ratio of the plaque
assay for the two classes. Since the ratio of lambda b130 to lambda
cI26 by plaque assay was slightly larger than the ratio obtained from
optical density, the indication is that the majority of lambda b130
phage particles are active. However, this experiment has a major
drawback. If the lambda cI26 preparation contained a large fraction of
inactive phage which banded at normal phage density, the result would
be the same as that obtained if a smaller fraction of the b130 phage

Table 5. Comparison of plaque forming units to optical density.

Method of analysis	Ratio of amount of lambda b130 to lambda cI26
Plaque assay	3.2
Weight of peaks from tracing of CsCl buoyant density gradient	2.3
Area of peaks from tracing of CsCl buoyant density gradient	2.9

A mixture of lambda cI26 and lambda b130 was placed in a CsCl solution with a final density of 1.495 g/cm^3. The plaque forming units of each type were assayed by plating on C600 and counting clear and turbid plaques. The solution was placed in a 12 mm cell and run in an analytical ultracentrifuge for 24 hours at 44,700 rpm. Pictures were taken with UV light to obtain absorbance. Tracings were made with a microdensitometer and the relative amounts of each density type were determined by weighing the peaks from the tracing or by counting the squares included in each peak. The majority of the absorbance is due to absorbance by DNA. Since lambda b130 contains 13-15% less DNA per phage particle, the ratios obtained from the densitometer tracings were corrected by dividing by 0.85.

were also inactive. Thus this experiment does not, in itself, estab-
lish the notion that the b130 phage particles containing partial
molecules joined by cohesive ends are infective.

DISCUSSION

In the density mutant, lambda b130, recombination frequencies

within the right or left chromosomal arms are not significantly

different from those observed in lambda of normal density. However,

crosses designed to measure recombination between the chromosomal arms

exhibit very high recombination values when at least one of the paren-

tal phage is lambda b130. Indeed, the recombination frequency for the

middle segment of the genome in such crosses is near maximum for lambda,

and as a consequence it is difficult to order markers in the two arms

with respect to each other.

Since the left and right arms of the genome are genetically un-

linked in b130, it appeared possible that a discontinuity existed in

the DNA molecule of this phage. Examination of the phage DNA did in

fact reveal a physical discontinuity in a sizable proportion of the DNA

molecules. As determined by sedimentation velocity and observation in

the electron microscope, approximately 76% of the b130 DNA molecules

could be broken into two discrete classes of 2/3 and 1/3 the length of

the whole molecules by heating at $60^{\circ}C$ and rapidly cooling. Under

these same conditions 23% of the DNA molecules were not degraded. This

procedure of heating and fast cooling breaks the cohesive ends of lambda

wild type DNA (Hershey and Burgi, 1965) but does not denature the whole

DNA molecule (Kaiser and Inman, 1965). Alkaline sedimentation demon-

strated that whole molecules of b130 DNA have at least one and perhaps

both strands intact, ruling out the notion that all of the phage DNA

molecules are identical with respect to occurrence of single strand

interruptions. Circular molecules were observed under conditions which promote annealing of cohesive ends. These might have arisen by circularization of the whole molecules. The linear molecules seen at the same time could be due to reassociation of the 2/3 and 1/3 partial molecules.

From the results of the infectivity assay of b130 DNA fractionated by sucrose gradient centrifugation, it appears likely that the DNA discontinuity occurs between genes J and P. The 2/3 partial molecule contains genes P and R marking the right arm and, by inference, the 1/3 molecule probably contains genes B and J which were used to mark the left chromosomal arm.

These experiments indicate that two types of b310 phage particles exist. One type contains a single DNA molecule which is similar to lambda wild type DNA but with a 13-15% deletion. The other, more frequent class consists of particles containing two partial DNA molecules of 2/3 and 1/3 length which are connected by hydrogen bonds similar to the cohesive ends of lambda wild type DNA. It seems likely that both types of particles are infective and either type can, upon growth, give rise to the two types, although these last assertions have not been demonstrated conclusively. A more detailed analysis of the structure of these chromosomes must await further examination. However, possible structures are presented in Figure 6.

Since it is not known if one or neither of the single strands of the whole molecules contain interruptions, classes 1 and 2 of Figure 6 represent possible structures for the whole molecules. Markers in one
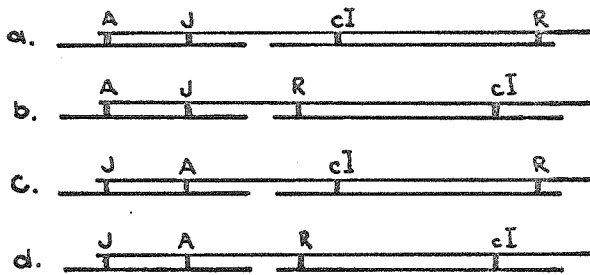
Figure 6. Possible configurations of the lambda b130 chromosome.
These are schematic representations of models for the b130 chromosome.
No attempt was made to draw these structures to scale. The horizontal
lines represent single DNA strands. Lambda genes A, J, cI, and R
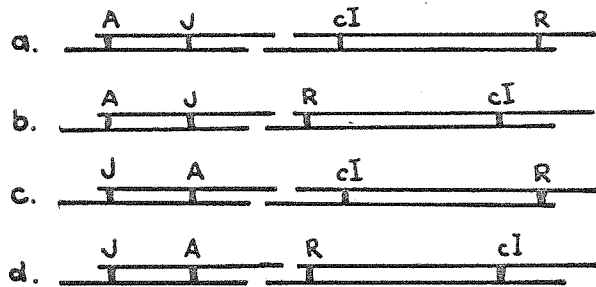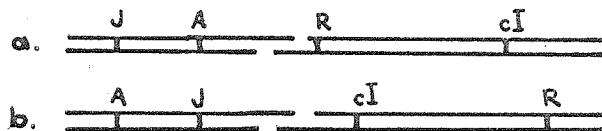are shown to aid in understanding the permutations presented.
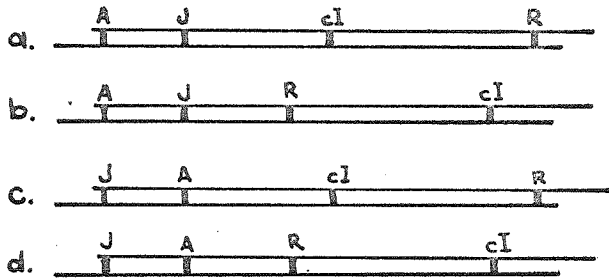
arm cannot be ordered with respect to markers in the other arm and the four categories in each class of Figure 6 represent four possible inversion structures. In 1-a the chromosome is similar to lambda wild type, in 1-b the right arm is inverted and in 1-c the left arm is inverted. In 1-d both arms are shown to be inverted. (The a, b, c and d categories of class 2 are similar to those of class 1.)

In classes 3 and 4 of Figure 6 are presented models of the hydrogen-bonded b130 DNA molecules. In class 4-a it has been presumed that a double strand break occurred between J and cI and the partial molecules have annealed by the cohesive ends. In 4-b a translocation of the cohesive ends to the middle of the chromosome is shown. In both class 4 models the ends of the molecule are not cohesive; however, current evidence points to the involvement of circular DNA in the lambda replication cycle (Young, 1967) and further, no mechanism for repairing a double strand break has yet been described. These difficulties render the class 4 structure unappealing.

The class 3 models for b130 partial molecules involve the creation of a new set of cohesive ends between J and cI. Thus 3-a and 3-d show the partial molecules joined by the new ends or the original cohesive ends. If the postulated new ends are identical to the original ends, two more possibilities for partial molecule association result, and are shown in 3-b and 3-c. The molecules depicted in class 1 or 2 could arise from those of class 3 but not of class 4. For example, the whole molecules of class 1-a or 2-a could result from the filling in of the single strand break(s) of the class 3-a structures by the

action of an enzyme similar to the E. coli ligase (Gellert, 1967)

(Olivera and Lehman, 1967). On the basis of the above considerations,

the model for b130 chromosome structure represented in class 3 is

favored.

The existence of a new set of cohesive ends in the middle of the

b130 chromosome could conceivably account for the increase in recom-

bination frequency in this region. It has been demonstrated that

phage recombination can result from breakage and reunion of whole DNA

molecules (Meselson and Weigle, 1961)(Meselson, 1964). Furthermore,

phage DNA structures similar to those depicted in class 3 of Figure

6 have been demonstrated as intermediates in T4 recombination (Anraku

and Tomizawa, 1965). In addition, single strand breaks occur at

specific locations in T5 DNA molecules which are healed after infec-

tion but recur before maturation (Abelson and Thomas, 1967). Prelim-

inary results of Lanni (in Abelson and Thomas, 1967) indicate that all

of the mutants in one group of closely linked T5 markers show uniformly

high recombination with a mutant in an adjoining region. This last

observation parallels to some extent the findings reported here with

lambda b130.

Two explanations of the bizarre structure of the b130 chromosome

have been considered. The cohesive ends of lambda wild type DNA consist

of complementary single strands of about 15 bases at either end of the

lambda DNA molecule (Wu and Kaiser, 1967). These single strands anneal

after infection and the molecule becomes covalently bonded into a

closed circle (Ogawa and Tomizawa, 1967). Before maturation some

specific mechanism must put single strand breaks into the circular molecule to reform the cohesive ends. This mechanism must be highly specific for this 15 base sequence since the cohesive ends always appear at the same place. If this same 15 base sequence appeared in another region of the DNA molecule, it would be recognized as a cohesive end site and the resultant molecule would have two identical sets of cohesive ends. Since lambda b130 DNA has a 13-15% deletion relative to wild type, it is possible that the deletion resulted in the juxtaposition of two short sequences of bases which together resemble the cohesive ends. This would lead to the occurrence of two sets of cohesive ends in one DNA molecule. If b130 is a simple deletion mutant of phage lambda, this model encounters a major difficulty. There are several density classes among the mutants isolated (see Table 6 in the first part of this thesis) which display high recombination between genes J and cI. The differences in calculated percent deletion of DNA for these mutants are sizable. This explanation would thus require that a number of different sequences exist in the phage, which if juxtaposed would then resemble the site from which cohesive ends are derived. This complication, of course, presupposes that all of the high recombining density mutants have similar chromosome structure to b130.

Another explanation considered, while somewhat involved, has definite appeal in that it makes a number of testable predictions. To recapitulate, any explanation for the formation and structure of b130 must explain three basic observations - (1) high localized

recombination, (2) two sets of cohesive ends and (3) inability to establish stable lysogeny. Since the high recombination of b130 is localized in the same region as the new cohesive ends, the two are probably interrelated. Furthermore, the integration process, which b130 cannot carry out, also takes place in this center segment of the chromosome suggesting that all three observations about b130 are dependent upon one circumstance.

It is reasonable to assume that the structure of b130 was determined as a result of excision from the host. Excision and integration are reverse processes and on a conservative basis the two processes are most likely brought about by the same enzyme system. This system shows great specificity for recombination at a specific chromosomal locus. As has been mentioned before, the integration-excision (I-E) system is regulated in some fashion so as to only promote recombination between the phage and the host chromosomes and not between two phage chromosomes. If this second observation were not the case, the phage would show increased vegetative recombination for the middle segment in proportion to the physical length but vegetative recombination in this segment is proportional to physical length (Jordan and Meselson, 1965).

If the new properties of b130 arise as an excision mistake, then the properties may not be due so much to a deletion as to an inclusion of a piece of host DNA. This postulation is not without precedent since the lambda dg phage contain some host genes in place of some phage genes. But available evidence indicates that the lambda dg phage do not behave in the same manner as the b130 phage (Hogness

and Simmons, 1964)(Tomizawa and Anraku, 1965)(Hogness, et. al., 1966)
so the inclusion of host DNA must come not from the left prophage end
as in a lambda dg but from the right prophage end. This raises the
question of how the two prophage ends must differ.

In Figure 7 a model for the prophage ends is presented. Since
the I-E (integration-excision) system functions to recombine the phage
and host DNAs, the proposal is made that the I-E system recognizes a
difference between the two structures as well as a similarity. That
is, the homology regions (H) of both phage and host are identical and
provide the homology required both for recombination and to bring the
phage (b) and bacterial (B) specific recognition sites into close
association. These recognition sites are different in base sequence
and the I-E system recognizes this difference and carries out a reci-
procal recombination event at or near the adjacent homology site. On
this model the phage and bacterial recognition sites are distributed
at either prophage end with the phage site (b) on the left end and the
bacterial site (B) on the right.

Thus if a b130 chromosome inclusion comes from the right prophage
end, it would contain an homology region and the bacterial recognition
site. A concomitant deletion of the phage recognition site together
with a segment of non-essential genes from the left end of the prophage
would account for the 13-15% deletion in b130. The inability of b130
to insert its chromosome into that of the host is accommodated on this
model since both lambda b130 and the host bacterium contain the bac-
terial recognition site (B) and the I-E system, as has been proposed,

Figure 7. Schematic representation of the proposed model for lambda

integration. The top two figures represent the pairing of the phage

and bacterial chromosomes through interaction of their identical

homology regions (H). Also shown are the specific phage (b) and

bacterial (B) recognition sites of the integration-excision system.

The third figure represents an inserted phage chromosome in a lyso-

genic cell. The bottom five figures represent the chromosomes of the

five types of detectable phage particles which result from excision
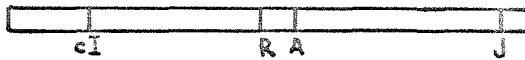
mistakes as described in Table 6.
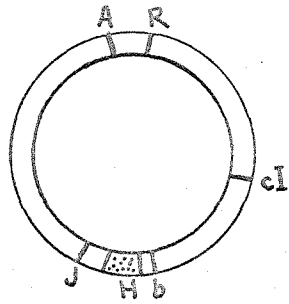
would not be activated.

High recombination observed in lambda b130 by lambda wild type crosses might possibly be due to the I-E system which recognizes the difference in recognition sites between the two phage. However, this system would not account for the high recombination observed in crosses of b130 by b130 where recombination would not be promoted by the I-E system.

The resemblance of the physical discontinuity of b130 DNA to the cohesive ends of lambda wild type makes it reasonable to assume that the physical discontinuity is a new set of cohesive ends.

I propose that high recombination between b130 phage chromosomes and the physical discontinuity can be explained as the consequence of another highly specific lambda enzyme system, the cohesive-end-cutting (C-E-C) system.

The C-E-C system shows similarity to the I-E (integration-excision) system in its high specificity for a chromosomal locus. There is also endonuclease-type activity associated with both systems which leads to the placement of closely occurring single strand breaks in the DNA. Indeed the C-E-C system may have a direct bearing on normal lambda recombination. The DNA replicates as a circle but the vegetative genetic map is linear. The genes nearest the cohesive ends also occur as the endmost markers in the genetic map. Thus the C-E-C system manifests itself in high recombination between genes A and R, the specific site of its action. The similarities between the integration-excision (I-E) system and the cohesive-end-cutting (C-E-C) system make it not

unreasonable that the base sequences at the site of action of each system may resemble each other but be sufficiently different to impart specificity to each system.

Based on the foregoing speculations the proposal is made that lambda b130, by virtue of an aberrant mode of excision, has acquired a new site for the action of the cohesive-end-cutting system. This new site is the association of the bacterial recognition site with the homology region. The localized high recombination in crosses of b130 by b130 is a consequence of the new set of cohesive ends just as the normally high recombination between genes A and R is due to the original cohesive ends.

This model is somewhat complex and does contain several ad hoc assumptions. Nevertheless, it has several redeeming qualities. The model is very specific as to the description of the integration process. (1) The actual point of insertion is near gene J, resulting in the middle third of the vegetative chromosome ending up on the left prophage end. (2) The integration-excision system requires a region of identity (the homology region) and a small region of specific difference (the recognition site). This model is similar to the one proposed by Fischer-Fantuzzi (1967), although the present model is more specific.

The model also suggests that the site of action of the I-E system (the homology region) resembles the site of action of the C-E-C system (the cohesive ends located between genes A and R).

The production of various different aberrant phage types by cuts misplaced from the normal prophage ends is detailed in Table 6. The

Table 6. Consequences of miscutting at prophage ends based on the proposed model for lambda integration.

Misplaced cut occurring at the prophage end indicated.		Phenotype of resultant phage particle.	Homology region?	Recognition site.
Left end	Right end			
Normal	Normal	lambda ++	+	b
Normal	Left	lambda defective	+	b
Normal	Right	(too large)		
Left	Normal	(too large)		
Left	Left	lambda dg	+	b
Left	Right	(too large)		
Right	Normal	lambda b2-type (low recombining)	-	-
Right	Left	lambda defective	-	-
Right	Right	lambda b2-type (high recombining)	+	B
		lambda dbio	+	B

(Left margin, vertical text: Direction of misplaced cuts)

Refer to Figure 7 (third figure from top) for orientation of miscutting. It is assumed that any genome longer than that of lambda wild type is too large to be wrapped up and will not appear in a phage particle. Note that only lambda wild type, a lambda defective type and lambda dg are of the genotype H-b and can therefore integrate.

schematic representation of a few of these phage is shown in Figure 7 (bottom). The model proposed above, in addition to its preciseness, makes several testable predictions involving these aberrant phage types. (1) Lambda dbio DNA molecules should share the physical discontinuity of the b130 molecules. (2) The high recombination properties of b130 are due to structural rather than functional properties. Thus the high recombination should not be manifest between normal lambda chromosome structures even when present in the same cell with b130 chromosomes. (3) The integration-excision system should contribute to high recombination only in crosses of normal lambda by lambda of the b130 type if at all. (This notion is testable since mutants defective in this system have been isolated.) (4) The new set of cohesive ends of lambda b130 should resemble the original cohesive ends enough to allow annealing to occur between appropriate complementary strands of the two sets of ends. Thus even the 1/3 and 2/3 partial molecules should form hydrogen-bonded circular molecules and circles of 1 1/3, 1 2/3, 2 1/3, etc., length should be obtainable.

In the introduction to this thesis it was stated that the isolation of a variety of deletion mutants of lambda might lead to further insight into the lambda integration system. In actuality, not much direct information on this system has been uncovered as yet using the mutants isolated. However, in attempting to explain the DNA structure of one of the mutants, b130, a speculative model for the lambda integration system is proposed. Although the model involves several ad hoc assumptions, it does make some predictions which are testable using some of the mutants isolated.

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