

A BIOPHYSICAL STUDY OF BACTERIOPHAGE ØX-174 REPLICATION

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ABSTRACT

Techniques for synchronizing, by cyanide and starvation, the development of ϕ X-174 in all of the infected cells of a culture of E. coli C were developed. The infection of starved cells in buffer, followed by the addition of broth, was found most satisfactory. One-step growth curves of ϕ X and ϕ X ρ^- (a plaque morphology mutant of ϕ X that has a longer mean latent period and will not lyse cells in concentrated cultures) were determined. The rate of phage maturation is constant. A probit plot of the percentage of phage released vs. the logarithm of the time after infection is linear.

The replication of ϕ X DNA was investigated using E. coli CR (a thymine requiring strain), 5-bromodeoxyuridine, the technique of equilibrium CsCl density gradient centrifugation, and the protoplast assay technique for ϕ X DNA. The DNA combining with the infecting single strand to form the first replicative form (RF) is synthesized after infection from low molecular weight precursors from the medium. The rate of increase in the amount of infective RF in the culture during the eclipse period is constant, and the RF molecule containing the parental single strand replicates semiconservatively. The conserved subunit is a single strand of DNA. The

material constituting the progeny single strands is derived directly from the medium, not from RF.

ϕ X carrying 2-4 P^{32} atoms per particle were used to infect cells in cold medium. The phage and early complexes were killed with an efficiency of 1; mature complexes were inactivated with an efficiency of about 0.2, even though unlabeled and infective RF had been synthesized in them. At about the same time as progeny single strands were synthesized the complexes became completely refractory to suicide. Chloramphenicol did not prevent the P^{32} suicide efficiency of the complexes from decreasing to about 0.2; complexes formed in chloramphenicol from cells which had not been starved became fully resistant to inactivation, whereas complexes formed from starved cells did not.

The ultraviolet radiation sensitivity of ϕ X complexes formed with E. coli C, E. coli C_s (a non host cell reactivating strain), and E. coli CR were determined at various wavelengths, at various stages of development, and under various conditions. Free ϕ X is not host cell reactivable; the target that is observed in the Luria-Latarjet curves of the complex is host cell reactivable. The most uv sensitive structure in the complex is the single strand of DNA synthesized after infection and associated with the infecting single strand. Budr present in this newly synthesized strand will sensitize the complex to uv. Experiments entailing a switch between Budr and

thymidine media indicated that the uv sensitive target in the complex could be identified with the single strand of DNA associated with the infecting parental strand. The DNA constituting this strand "turns over" each time the parental RF replicates.

The uv sensitivity of the bacterial capacity to support ϕ X growth was also studied. Although the colony forming ability of C_g is more sensitive to uv than C , the capacity is not. There was evidence that a single nucleic acid like structure and a large number of protein like structures had to be inactivated in order to destroy the capacity. Starvation of CR for thymine, or growth in Budr, increased the uv sensitivity of the capacity; as the cells in a thymine starved culture underwent thymineless death, they also lost their capacity to support ϕ X growth.

Action spectra for the complexes and for capacity are presented. The uv sensitivities of MS-2, Bu- ϕ X, and Bu- λ (in the presence and absence of cysteamine) are described.

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PART I

INTRODUCTION

ϕ X-174 is one of the smallest bacterial viruses known that contains DNA. It is spherical and has a particle weight of 6.2 megadaltons (1). The DNA may be extracted from the virus as a single molecule with a molecular weight of 1.7 megadaltons (2). Spheroplasts made from various strains of E. coli with lysozyme-versene will produce mature ϕ X particles after having been infected with purified ϕ X DNA (3-7). The DNA isolated from the virus is a single polynucleotide chain whose ends are joined to form a ring structure (8-10).

During the intracellular development of ϕ X-174 the infecting single strand DNA is incorporated into a double stranded DNA called the "replicative form" (RF) (11-13). RF has been purified, characterized in more detail (14, 15), and shown to be circular also (16-18). The formation of the RF containing the parental single strand occurs immediately after infection and is quickly followed by the replication of the parental RF, even in the presence of chloramphenicol (11, 12); RF continues to accumulate through the eclipse period at least. The RF does not appear to be a material precursor of progeny single strand DNA (19, 20), and parental DNA is not transferred to progeny (12, 21). Single strand DNA synthesis begins about half way through the latent period and requires the prior synthesis of one or more proteins (22). ϕ X infection neither interrupts protein, DNA, or RNA synthesis by the

host cell (23), nor prevents induction of an inducible enzyme (24).

Inside the infected cell RNA is made that will hybridize with the RF molecules but not with the single stranded DNA isolated from the virus (24). This result suggests that the ϕ X messenger RNA made in the infected cell has a base sequence identical to the single strand DNA contained in the virus. There is evidence that the RF must be circular in order to have only one strand copied (25); if it is not circular both strands are transcribed. Inside uninfected cells there is an RNA complementary to a restricted section of ϕ X-174 DNA (24). Shortly after infection it disappears.

The work reported below describes a set of related studies on the ϕ X infected cell. There were two complementary reasons for performing this work. One was to deduce further information about the nature of the ϕ X replicative process from the response of the phage-bacterium complex to the treatment it received. The other was to gain an increased understanding of the action of various agents on the infected cell from the reaction of the complex to the agent. Since these experiments entail an analysis of the behaviour of complexes when subject to a particular perturbation at defined stages in their development, it is necessary to ensure that all of the infected cells in the population are at a similar stage of

development. Hence in Part II techniques for the synchronization of ϕ X development are described, and certain results of this work are discussed. Some studies on the intracellular behaviour of ϕ X DNA are presented in Part III. Parts IV and V comprise studies on the P^{32} suicide and ultraviolet light sensitivities of the infected cell. Part VI contains a brief synthesis and summary.

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PART II

THE SYNCHRONIZATION OF ϕ X-174 GROWTH:

PHAGE MATURATION AND CELL LYSIS

Introduction

Some characteristics of the ϕ X-174 growth cycle have been reported, but no detailed analysis has been presented. Sinsheimer et al. (1) found that under their conditions the rate at which the phage went into eclipse was such that in a population of infected cells phage-bacterium complexes at all stages of development coexisted. Matsubara et al. (2) reported some experiments in which synchronization was achieved with cyanide; they did not present any details of the growth curve. Eigner et al. (3) found that in their unsynchronized system phage release seemed to precede cellular dissolution. Hutchison and Sinsheimer (4) showed that phage are released in bursts from infected cells.

Two methods have been used to synchronize the development of the infected cells in a culture infected with ϕ X; both act by blocking anabolic processes but not phage adsorption and eclipse. Phage eclipse is defined as the disappearance of mature phage particles in the presence of sensitive bacteria (after lysing the cells with lysozyme-versene) and presumably represents the release of phage DNA from the protein coat. The techniques of synchronization by cyanide (5) and synchronization by starvation (6) were found to effect synchronization of ϕ X development.

Materials and Methods

Starvation Buffer (SB) contains 5 g KCl, 1 g NaCl, 1.2 g tris (Sigma), 0.1 g MgSO₄, and 1 ml of 1 M CaCl₂ in 1 liter of distilled H₂O. It is autoclaved after adjustment of the pH to 8.1 with HCl.

Tryptone Broth (TKB) contains 10 g of tryptone broth (Difco) and 5 g of KCl in 1 liter of distilled H₂O.

Lysozyme solution contains 222 mg/l lysozyme (Worthington, A grade) in 0.033 M tris, pH 8.1. It is made by diluting a stock (kept in the cold) of lysozyme (2 mg/ml lysozyme in 0.25 M tris, pH 8.1) 1 to 9 into 0.033 M tris, pH 8.1.

Versene solution contains 8 g/l versene (disodium salt) in 0.1 M tris, pH 8.1. It is made by diluting a 4% stock solution (in H₂O) 1 to 5 into 0.1 M tris, pH 8.1.

ØX-174.—The bacteriophage characterized by Sinsheimer (7). A single purified preparation has been used in all of the experiments described.

ØX-174 ρ^- .—A mutant of ØX-174 isolated in this laboratory from a preparation of tritium labeled phage made by Dr. Alan B. Stone. The designation "rho minus" signifies "retarded lysis" (see below), in contrast to the rapid (r) lysis mutants of T4. Purified preparations were used in all of the experiments described.

Escherichia coli C.—BTCC #122.

Starvation Synchronization.—Log phase cells grown to a concentration of $1-4 \times 10^8$ cells/ml in tryptone-KCl broth (TKB) containing 2×10^{-3} M CaCl_2 are pelleted, washed once with starvation buffer (SB), and resuspended in SB. In the process the cells are often concentrated 2-4 times. Starvation is accomplished by aerating the cells in SB for 60 to 90 minutes at 37°C . After starvation phage in SB are added, and 5 minutes are allowed for adsorption and eclipse. Then a 1:1 dilution with prewarmed TKB is made to initiate development.

Cyanide Synchronization.—The cells are grown as above to $1-4 \times 10^8$ /ml. Cyanide (0.3 M KCN in H_2O) is added to a concentration of 0.003 M 5 minutes prior to adding the phage. Five minutes after adding the phage the culture is diluted at least 10^{-4} in prewarmed TKB or is filtered, washed, and resuspended in TKB.

Premature Lysis.—E. coli C, infected or uninfected, may be lysed with lysozyme-versene. Samples from the infected culture are diluted 1/10 into 0.9 ml ice cold lysozyme solution; 2 to 20 minutes later 1.0 ml of versene solution is added. After standing in the cold for 0.5 to 2 hours the samples are slowly frozen 1x in the deep freeze or, alternatively, quick frozen 3x with acetone- CO_2 . These procedures do not inactivate the virus and lyse more than 90% of the cells. Treatment with 0.1% sodium dodecyl sulfate does not increase the titer of phage in the frozen

and thawed lysozyme-versene treated samples.

Infective Centers.—Infective centers may be plated immediately, after dilution through room temperature TKB if necessary, or they may be stored without loss in ice cold TKB for several hours before plating. Unadsorbed phage (usually less than 1%) may be removed by filtration or inactivated by antiserum included in the TKB added at 0 minutes with a K of six (K , in min.^{-1}), is the inactivation constant in the equation surviving fraction equals e^{-Kt} (8)).

Lysis and Phage Release.—Lysis of the culture is followed by the decrease in turbidity of the culture as measured on a Bausch & Lomb Spectronic 20 Colorimeter at 440mp. Release of phage is measured by the increase in infective center titer in the culture.

Assay Technique.—In general the methods described by Adams (8) are used. Log phase E. coli C grown to $4-6 \times 10^8/\text{ml}$ in TKB plus 2×10^{-3} CaCl_2 are used as the seed bacteria. Bottom and Top agar are as described by Sinsheimer (7) except that the NaOH is omitted. Plastic petri plates (Falcon Plastics, Los Angeles) containing 10 ml of bottom agar are overlaid with 2.2 ml top agar, 0.3 ml seed bacteria, and an aliquot of phage and incubated at 37°C for $2\frac{1}{2}$ - $3\frac{1}{2}$ hours.

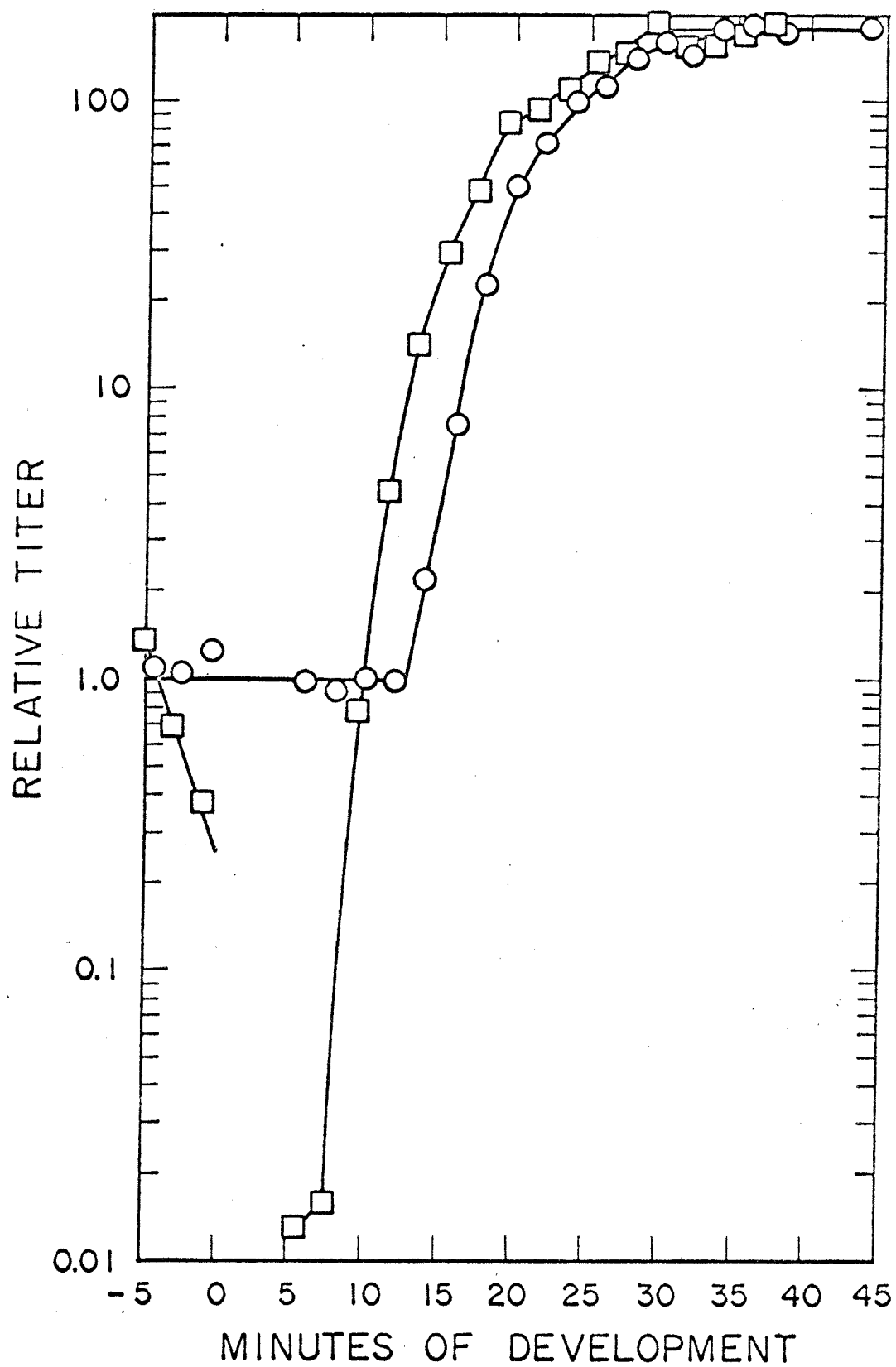
All experiments are done at 37°C , and all cultures are aerated by bubbling air through them.

Results

Growth Curves of ϕ X-174 and ϕ X-174 ρ^-

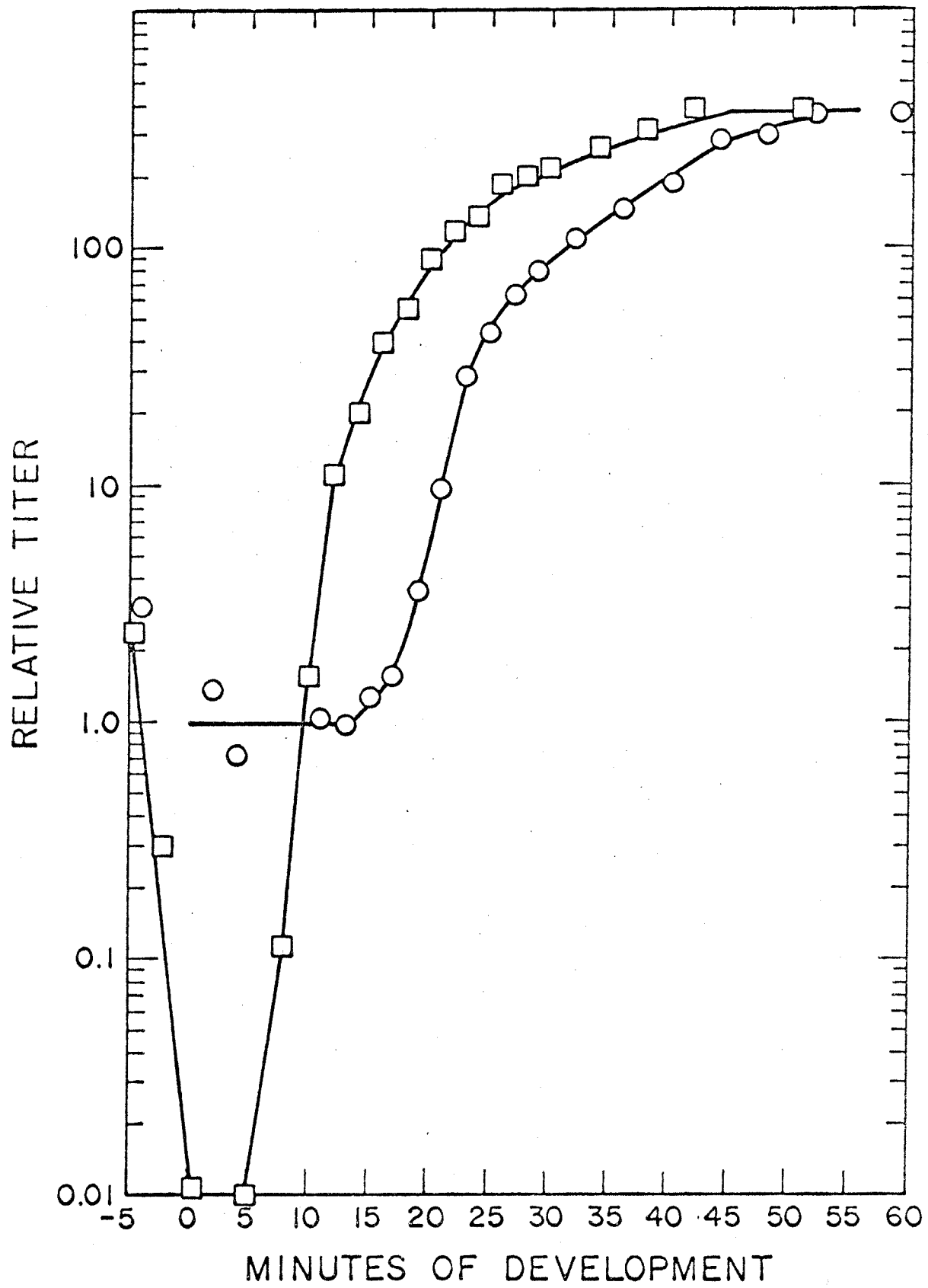
Figures 1 and 2 exemplify growth curves of ϕ X-174 and ϕ X-174 ρ^- respectively. Usually better than 90% of the phage have eclipsed after 5 minutes in the presence of the starved bacteria. If development is not initiated by the addition of broth (0') the infective centers gradually decay, and the phage continue to eclipse at an ever decreasing rate; no progeny phage are made. The residual phage observed after premature lysis in the period between 0 and 7 minutes after growth starts are neither removed by centrifugation or filtration nor inactivated by antiserum; they appear to be reversibly adsorbed to the bacteria and released by lysozyme-versene treatment. ϕ X-174 ρ^- differs from ϕ X-174 in its plaque morphology (smaller and with turbid edges), longer mean and maximum latent periods, larger burst size, and inability to lyse most of the infected bacteria in concentrated cultures (greater than 10^8 cells/ml).

The result of an experiment in which the lysis of a culture infected with ϕ X-174 was compared with the lysis of a culture infected with ϕ X-174 ρ^- under equivalent concentrated conditions is shown in figure 3. Aliquots of the two cultures were diluted, and growth curves in dilute culture obtained as described in the legend. Ten



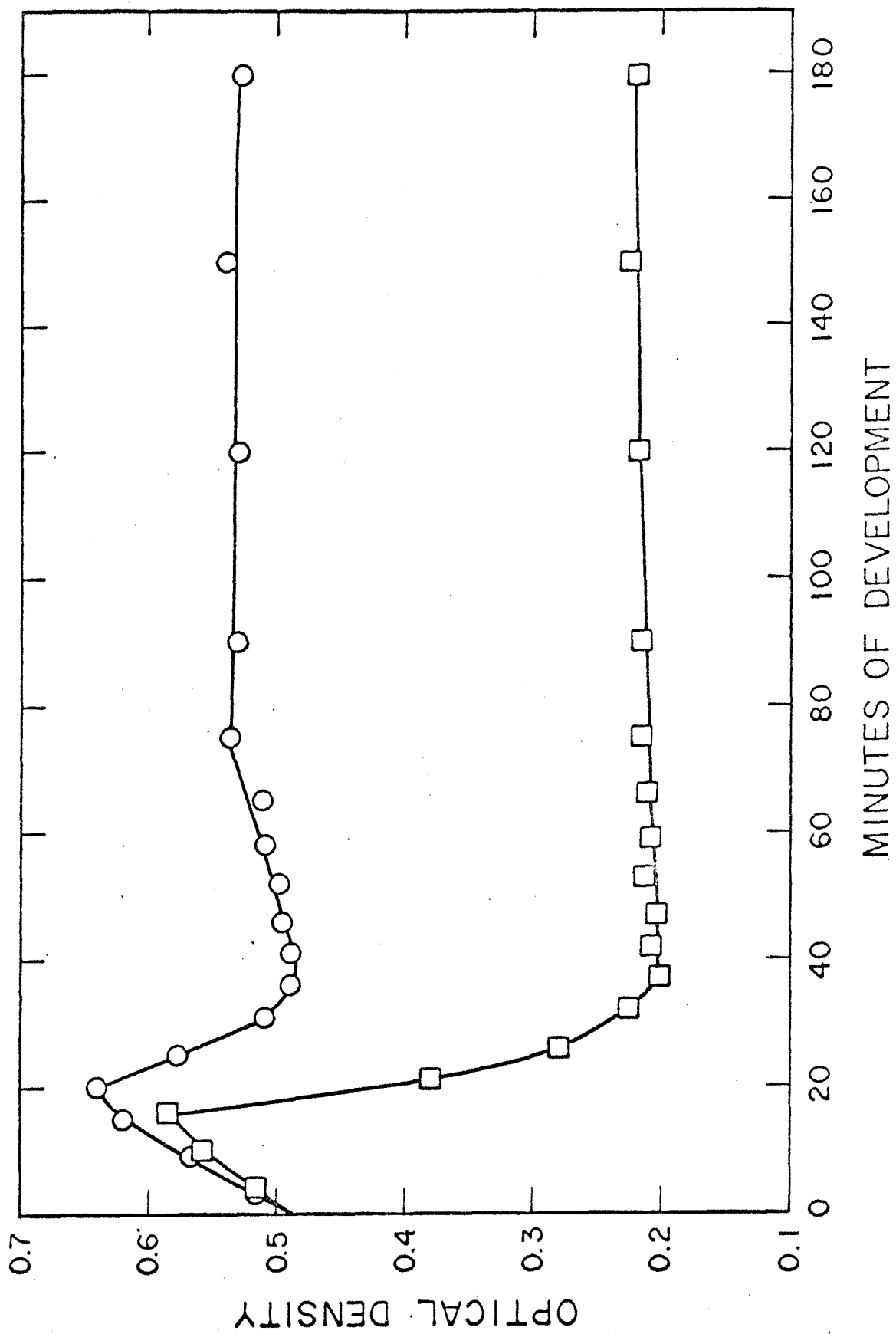
Legend to figure 1

A growth curve of ϕ X-174 in dilute culture at low multiplicity. A culture of E. coli C was starved for 75' at a cell concentration of 3.7×10^8 /ml, and ϕ X was added at -5' to a multiplicity of 0.087. TKB plus anti-serum was added at 0'. The culture was diluted 10^{-3} , 10^{-4} , and 2×10^{-6} at 3 minutes. Infective centers (circles) were plated directly, in most cases, from the appropriate dilution. The total number of mature phage (squares) in the culture was determined after premature lysis. The eclipse period was 10', the minimum latent period, 13', the mean latent period, 21', and the average burst size, 180.



Legend to figure 2

A growth curve of $\phi X-174\phi^-$ in dilute culture at low multiplicity. A culture of E. coli C, starved in SB for 60' at 1.9×10^8 cells/ml, was infected with $\phi X-174\phi^-$ at a multiplicity of 0.074. At 0' prewarmed TKB was added; at 6' the culture was diluted 5×10^{-2} , 2.5×10^{-4} , and 2.5×10^{-6} . Infective centers (circles) were assayed in most cases by direct plating from the appropriate dilution. The total number of mature phage in the culture (squares) was determined after premature lysis. The eclipse period was 9.5', the minimum latent period, 14', the mean latent period, 39', and the average burst size, 380.



Legend to figure 3

The evolution of the optical densities of cultures infected with ϕ X-174 and ϕ X-174 ρ^- . A log phase culture of E. coli C at 1.8×10^8 cells/ml in TKB plus 2×10^{-3} M CaCl_2 was divided into two equal portions. One portion was infected with ϕ X-174 (squares) at a multiplicity of 3.4, the other with an equal volume (in SB) of ϕ X-174 ρ^- (circles) at a multiplicity of 3.2. Both cultures were aerated together in a 37°C water bath. Between 6' and 8' both cultures were diluted 10^{-2} and 10^{-4} . Growth curves (unsynchronized) in both the dilute and concentrated cultures were followed. For the dilute ϕ X-174 culture the eclipse period was 11'; the minimum latent period, 14'; the mean latent period, 23'; and the average burst size, 140. For the dilute ϕ X-174 ρ^- culture the eclipse period was 10.5'; the minimum latent period, 14'; the mean latent period, 31'; and the average burst size, 440.

to thirty percent of the ϕ X-174 ρ^- infected cells in the concentrated culture did lyse; the surviving cells resembled in the phase contrast microscope cells in the early stages of protoplast formation by the lysozyme-versene technique. They have deformed and irregular shapes. Superinfection at 10 minutes of ϕ X-174 and ϕ X-174 ρ^- infected cells with ϕ X-174 or ϕ X-174 ρ^- (in the four possible pairwise combinations) did not affect the course of lysis or non-lysis as instituted by the original infection.

The characteristics of growth curves obtained in several experiments in dilute cultures under a variety of conditions are listed in tables 1 and 2. Growth curves determined in concentrated cultures are complicated by the readsorption of released phage to bacterial debris; in concentrated cultures the eclipse period and minimum latent period are occasionally a few minutes longer than the values shown in table 1. When all conditions but the multiplicity of infection are identical the characteristics of the growth curves are found to be independent of the multiplicity of infection.

The Kinetics of Phage Maturation and Cell Lysis

Cells infected with ϕ X lyse very soon after the onset of phage maturation; in some growth experiments, particularly in concentrated cultures, the curve

Table 1: Characteristics of ØX-174 Growth Curves

Date	(a)	(b)	(c)	(d)	(e)	(f)	(g)
1/12/63	0.03	9.5	13	28	65	0.73	0.46
1/25/63	0.07	10.5	12.5	35	240	0.84	0.36
1/25/63	7	11	12.5	35	96	0.88	0.36
3/30/63	0.04	10.5	14	32	150	0.75	0.44
4/3/63	0.09	13	15	35	180	0.87	0.43
4/10/63*	0.29	9.5	13	30	390	0.73	0.43
4/10/63**	0.4	11.5	15	35	430	0.77	0.43
5/19/63	0.09	10	13	30	180	0.77	0.44
5/19/63	8.7	9.5	14.5	28	120	0.65	0.52

* Synchronized by diluting away from cyanide

** Synchronized by filtering away from cyanide

a = the multiplicity of infection

b = the eclipse period in minutes, defined (8) as the time at which there is an average of one phage per infected cell in the culture

c = the minimum latent period in minutes

d = the maximum latent period in minutes

e = the average burst size

f = the ratio of the eclipse period to the minimum latent period

g = the ratio of the minimum to the maximum latent period

Legend to Table 1

The characteristics of ØX-174 growth curves determined in dilute culture. All growth curves were synchronized by starvation unless otherwise noted. The minimum latent period and the maximum latent period are defined respectively by the time at which the infective center curve starts to rise and the time at which it reaches its final value. The significance of the ratios is discussed in the text.

Table 2: Characteristics of $\phi X-174e^-$ Growth Curves

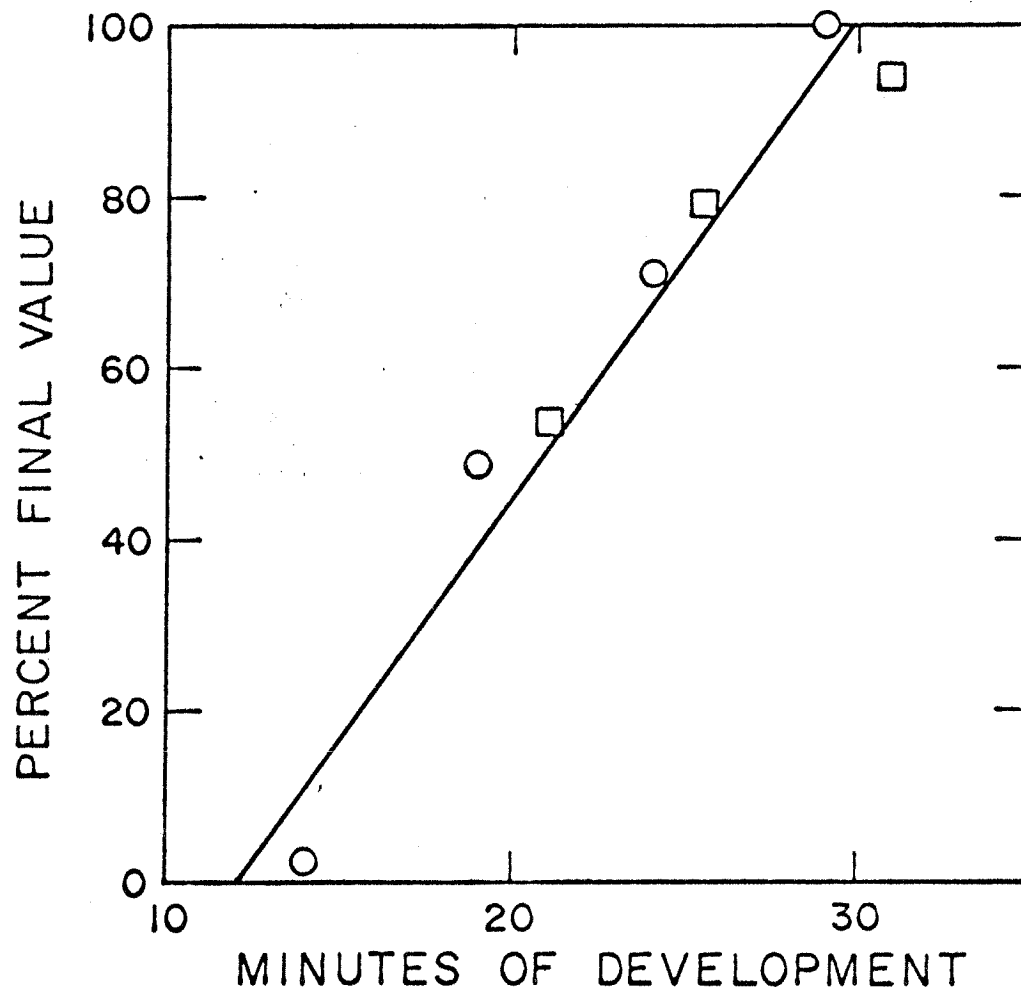
Date	(a)	(b)	(c)	(d)	(e)	(f)	(g)
12/8/63	0.15	11	15	50	275	0.73	0.3
2/8/64	3.1	10.5	13	45	440	0.81	0.29
2/11/64	0.07	9.5	14	53	380	0.68	0.26
4/20/64	0.05	10	14	65	840	0.72	0.22

Legend to Table 2

The characteristics of starvation synchronized $\phi X-174e^-$ growth curves obtained in dilute cultures. The significance of the numbers in each column is described in the legend to table 1.

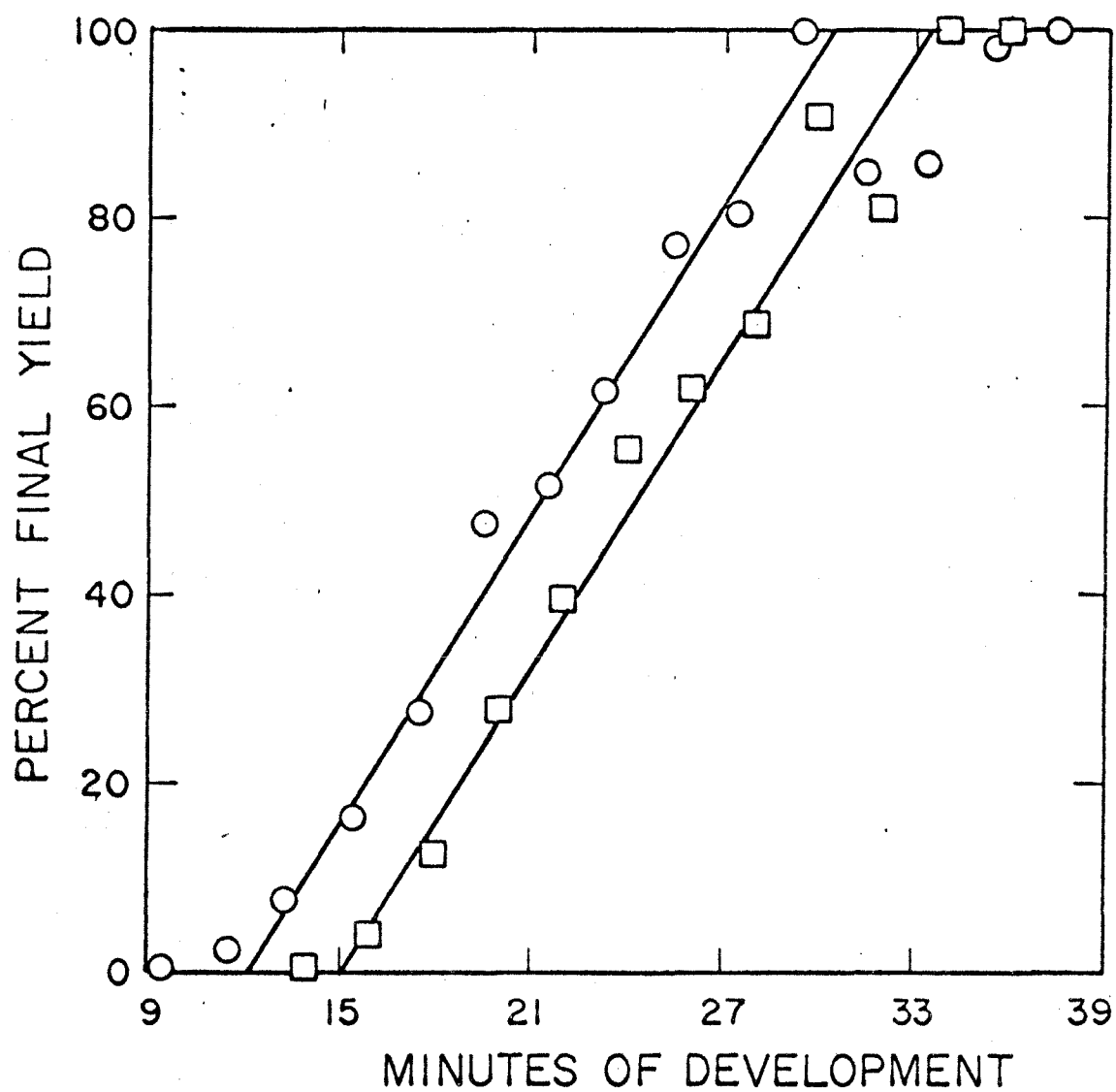
representing the increase in infective center titer is practically superimposable upon the curve representing the increase in intracellular phage. Figure 4 shows that lysis as measured by cell dissolution and lysis as measured by phage release correspond. The ordinate of figure 4 represents two different percentages for the two types of symbols as described in the legend.

The kinetics of phage maturation and phage release are shown in figure 5. The data are from figure 1. Although the rate of appearance of the first few phage in the culture is approximately exponential, most of the phage mature at a constant rate, one every six seconds in this experiment, at least when averaged over the culture. The rate of phage maturation varies considerably depending upon the conditions and the nutritional state of the cells. Phage are matured more rapidly in cells that have not been starved - hence the higher average burst size obtained in cyanide synchronized infections. When cells from the same culture are infected under similar conditions at high and low multiplicities the rate of phage maturation is observed to be independent of the multiplicity. The kinetics of phage maturation and phage release are similar and the former precedes the latter by 2-4 minutes. Similar results are obtained for $\phi X\phi^-$ except that the two curves are displaced from each other by 6-9 minutes.



Legend to figure 4

The correspondence between phage release (circles) and cell lysis (squares) for ØX-174. The squares denote the percentage of the total final optical density decrease that has occurred at the time indicated in the concentrated culture. The circles show the percentage of the total final phage yield that has been released at the time indicated in the dilute culture. The data resulted from the experiment described in the legend to figure 3; the optical density is plotted in figure 3 (squares), and the ØX growth curve in dilute culture was described in the legend.



Legend to figure 5

The kinetics of phage maturation (circles) in and phage release (squares) from ØX-174 infected cells. The circles represent a plot against time of the percentage of the final phage yield that has matured. The squares represent a similar plot of the percentage of the final phage yield that has been released. The data are from the experiment described in the legend to figure 1.

Discussion

The one step growth curve of ϕ X-174 resembles the growth curves of other bacteriophages (8, 9). There is an eclipse period during which no intact phage particles can be found in the infected cell. The synthesis of mature phage particles commences at the end of the eclipse period and continues at a constant rate until the culture lyses. Lysis defines the end of the latent period and results in both dissolution of the cell and release of a number of phage—the burst.

The relative lengths of the various phases of the growth curves for different phage-bacterial systems serve as a means of comparison. Adams (8) states that the eclipse period is usually 0.5 to 0.75 of the minimum latent period; the data in the penultimate column of table 1 indicate that for ϕ X the eclipse period is a significantly larger fraction (average value, 0.78) of the minimum latent period. The ratio of the minimum latent period to the maximum latent period (ultimate column) for both ϕ X (average value, 0.43) and ϕ X ρ^- (average value, 0.26) is smaller than the ratio of 0.68 observed by Doermann (10) for T4.

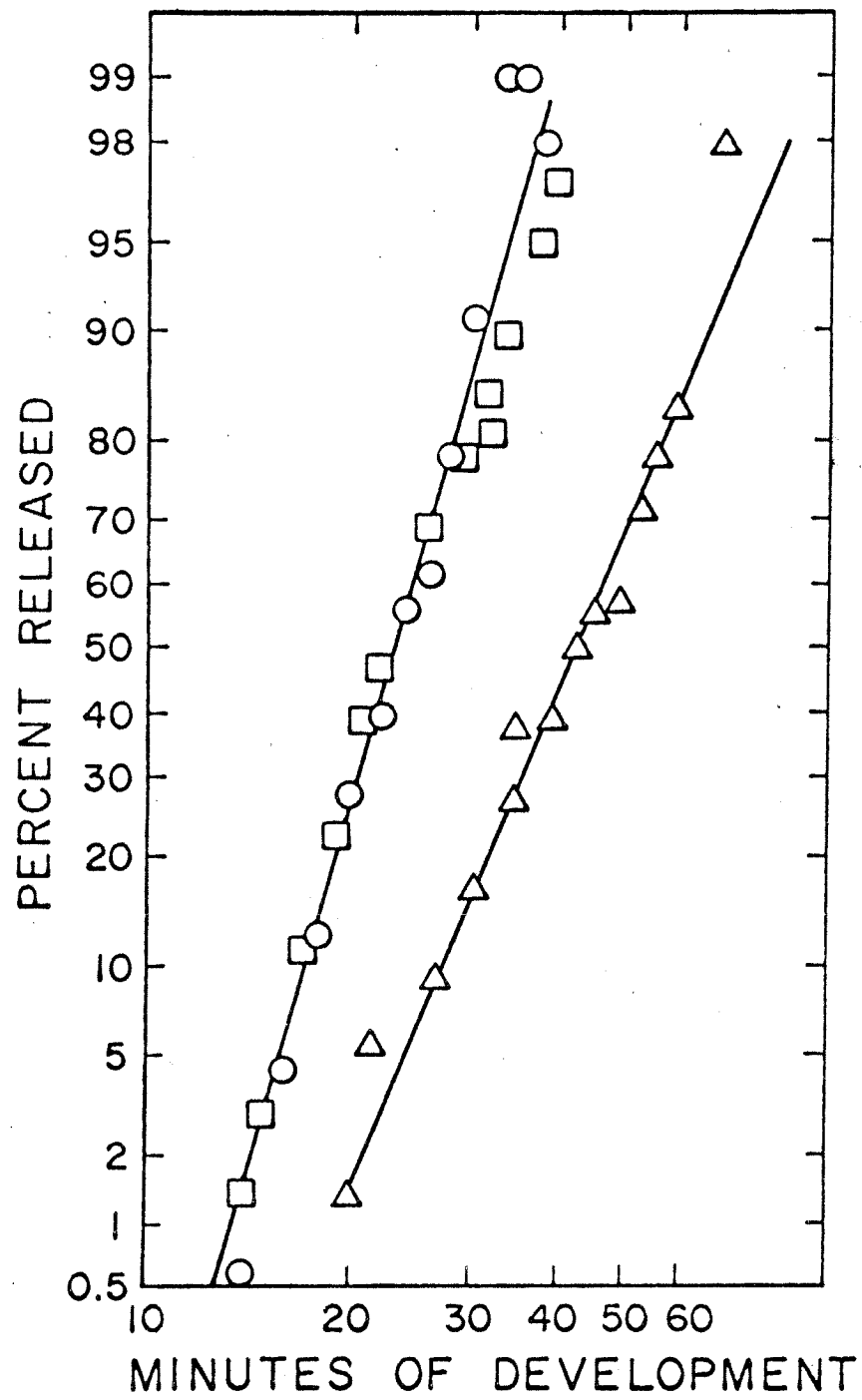
Adams and Wassermann (11) showed that for the T phages the lytic process could be analyzed on a probit plot (12); they found a normal distribution in the times

at which the infected bacteria released phage. The release of ϕ X from infected cells is skewed toward later times (4) with the result that a probit plot of phage release vs. time does not yield a straight line. A linear plot does result if a logarithmic time scale is used as shown in figure 6.

In the rather complicated system studied by Eigner et al. (3) it appeared that phage release preceded cellular dissolution. Hutchison and Sinsheimer (4) showed that phage were released in bursts, presumably accompanied by dissolution of the cell. The data shown in figure 4 clearly suggest that cell lysis and phage release occur simultaneously.

Delbrück (13) and Doermann (10) found with T phages that high multiplicity infections and low multiplicity infections produced identical growth curves and burst sizes. That ϕ X behaved similarly was unexpected since the host bacteria exist predominantly as doublets. However, Delbrück (14) observed that cells which appeared to be doublets behaved as a unit during the lytic event.

The constant rate of phage maturation, at least when averaged over the culture, found for ϕ X also obtains for most virus systems studied (10, 15, 16). The rate of maturation depends on the metabolic state of the cell but not on the multiplicity of infection. The significance of these facts is uncertain. In poorly synchronized



Legend to figure 6

A probit plot (12) of the percentage of the final phage yield released vs. the logarithm of the time after infection. The circles are from the experiment described in figure 1. The squares are from the data presented in figure 1 of the paper by Hutchison and Sinsheimer (4). The triangles represent the data for $\phi X-174\phi^-$ shown in figure 2. The mean latent period (when 50% of the phage have been released) for $\phi X-174$ is about 23', for $\phi X\phi^-$ it ranges between 30' and 45'. In all cases phage release was followed in dilute culture.

systems the existence of an approximately exponential process for the onset of virus maturation may mask an inherent linearity of the process itself, particularly if there is a low average burst size.

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PART III

THE REPLICATION OF ϕ X-174 DNA

Introduction

The genetic material, or chromosomes, of a wide variety of organisms has been shown to replicate in a semi-conservative fashion (1); Thomas (2) has recently summarized the available evidence. Although semi-conservative replication is a well established fact, the nature of the conserved subunit has not been clearly ascertained in all cases (see, for example, Cavalieri et al. (3-6)). Schildkraut et al. (7) studied the nature of the conserved subunit observed by Meselson and Stahl (8) during the replication of E. coli DNA and concluded that it was a single polynucleotide strand. This conclusion has been supported both by an autoradiographic study by Cairns (9) and by a study of the alkaline transition of 5-bromouracil labeled DNA by Baldwin and Shooter (10). The semi-conserved unit of lambda (11, 12) and T7 (13, 14) also seems to be a single strand of DNA. Wake and Baldwin (15) found that the first products of the action of DNA polymerase were duplex DNA hybrids containing one strand of primer and one strand of newly synthesized material.

The replication of ØX-174 DNA offers an uniquely different approach to the problem of DNA replication because it is possible in this case to label a single well-defined polynucleotide strand. Hence a study of the formation and replication of hybrid RF molecules labeled

specifically in the parental single strand should reveal the nature of the conserved subunit.

The other questions posed in this part are: 1) What is the origin of the material combining with the infecting single strand to produce the parental RF DNA duplex? 2) What are the kinetics of DNA synthesis? 3) What is the origin of the material composing the progeny single strands? Hofschneider (16) has already posed, but not answered, the first question. Matsubara et al. (17) have studied the second question by examining the kinetics of the incorporation of C¹⁴-thymidine into single and double stranded DNA. They observed that the synthesis of double stranded DNA, which included both coli DNA and RF in unknown proportions, continued throughout the latent period; whereas the synthesis of single stranded DNA began only about 9 minutes after infection and continued at a constant rate up to the end of the latent period when about a thousand ϕ X DNA equivalents had been synthesized. The third question has also been investigated by Matsubara et al. (18), and they concluded that the immediate precursor of single stranded DNA was not double stranded DNA but rather low molecular weight acid soluble material. In addition to the objection (that the data presented by Matsubara et al. (18) did not exclude the possibility that double stranded DNA was a precursor for much of the single strand DNA formed) raised by Singh (19) to this conclusion,

there is also the objection that the label in the double stranded DNA very likely did not represent just RF. As pointed out by Sinsheimer et al. (20), the absence of parental to progeny transfer suggests that RF (at least RF's like the parental RF) is not a precursor to progeny single strand DNA.

Materials and Methods

ϕ X-174, E. coli C, and synchronization techniques were described in Part II. SB = starvation buffer and TKB = tryptone, KCl broth (page 9).

yh.—An extended host range temperature sensitive mutant of ϕ X isolated by Mr. Clyde Hutchison, III. It, but not wild type ϕ X, will plate on E. coli C₁ at 30°. ϕ X, but not yh, will plate on E. coli C at 40°. Thus, the two phage strains may be independently assayed in the presence of each other.

Escherichia coli CR.—A strain isolated by Mrs. Regine Weil from a cross of E. coli CR34 Hfr thy⁻leu⁻lac⁻ Sm^S ϕ X^r (from Dr. R. Edgar) and E. coli C416 F⁻Tl^rtry⁻Sm^r met⁻ ϕ X^SPur⁻ (from Dr. E. Bertani). Its only requirement is thymine.

Defined Medium.— (DM) contains, per 200 ml of distilled H₂O, 200 mg each of arginine, tryptophan, aspartic acid, alanine, adenosine, methionine, glycine, glutamic acid, histidine, valine, and lysine (all Cal. Biochem.); 800 mg each of Tris, NH₄Cl, Na₂SO₄, KH₂PO₄, and pyruvate; 8 g each of glucose and glycerin. The pH was adjusted to about 7.0 with HCl. The solution was heated to 100°C for 10' and stored frozen; a slight precipitate which often materialized was ignored. It was diluted 1/10 with SB immediately before use (to give

diluted defined medium, DDM).

5-Bromodeoxyuridine.—(Budr), A grade, Cal. Biochem. A solution, usually 1 mg/ml, in SB at pH 7.5 was prepared, stored in the dark at 4°C, and used for several weeks. The optimum concentration (in terms of phage yield) for infected cultures was found to be 5 mg/l in DDM.

Thymidine.—A grade, Cal. Biochem. Prepared as a solution in SB at 2 mg/ml, and used at a concentration of 20 mg/l to grow E. coli CR in DDM.

Medium Transfers.—Budr to thymidine transfers were made by adding 50 mg/l thymidine to the Budr (5 mg/l) culture at the desired instant. Thymidine cultures were filtered (Millipore, HA), washed well with cold starvation buffer, and resuspended in Budr medium to achieve thymidine to Budr transfers.

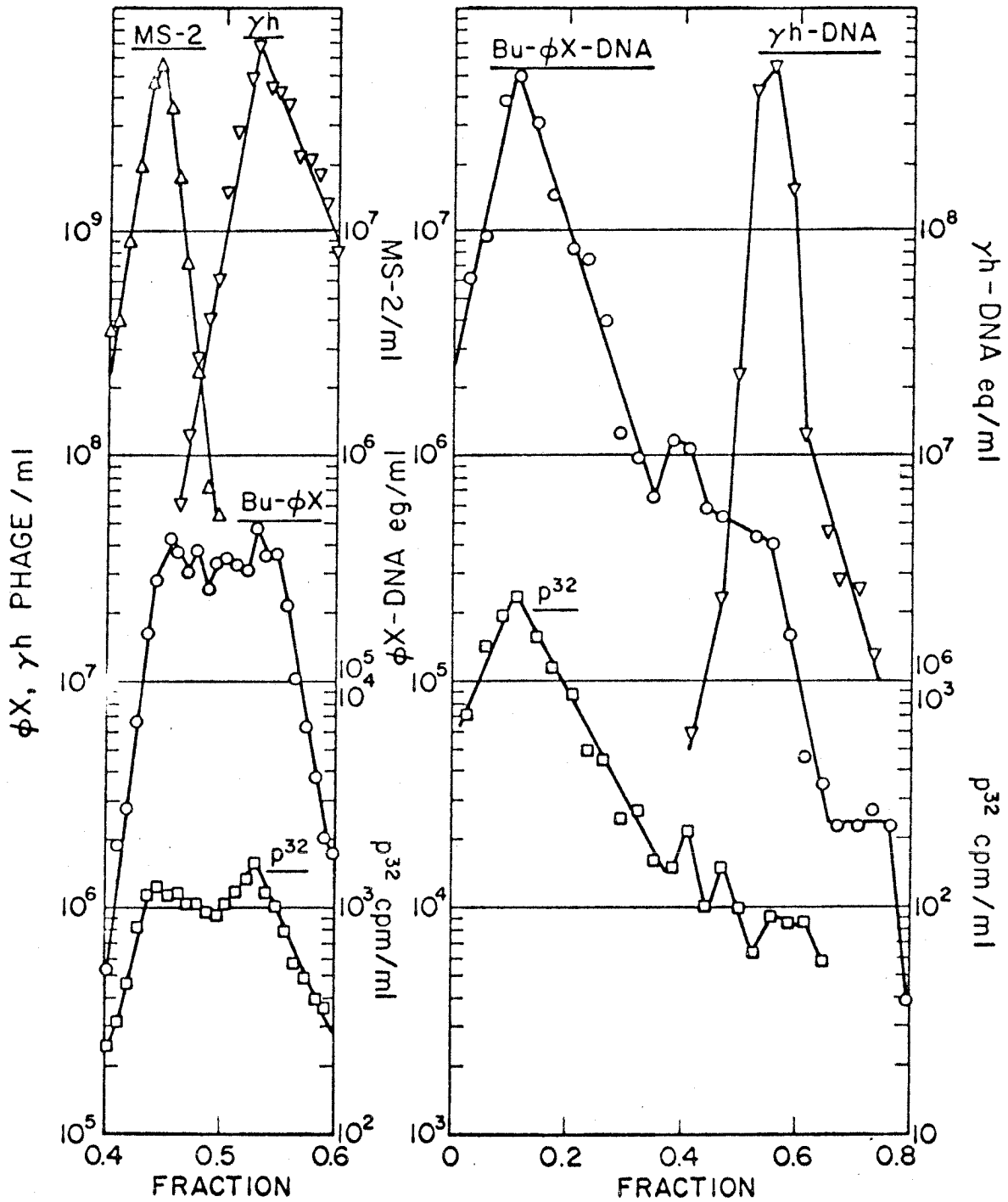
Drop Collecting Medium - (DCM) contains 10 mg/l EDTA, 0.1 mg/l bovine albumin (Armour), and 0.1 mg/l RNA (yeast) in 0.05 M KH_2PO_4 , pH 7.5.

Preparation of P^{32} Labeled Phage.— P^{32} - ϕX was prepared, except as noted below, according to the scheme described by Sinsheimer et al. (20). The TPG medium was modified as follows: the MgCl_2 and Na_2SO_4 were replaced with 0.2 g/l MgSO_4 ; the FeCl_3 was omitted; the phosphate concentration was reduced to give 4-5 mg/l of phosphorus (sufficient for log phase growth through at least $4-5 \times 10^8/\text{ml}$). The radiophosphorus was added only 5-30 minutes before the

phage. When lysis of the culture did not occur (with $\phi X\phi^-$ and occasionally with ϕX when the specific activity of the medium was above 100 mc/mg) the cells were pelleted and lysed with lysozyme-versene (see below). The lysed pellet was dialysed 1x against $\frac{1}{2}$ saturated borate and then 2x against tris buffer (0.1 M NaCl + 0.001 M tris), pH 7.5. When the culture did lyse (50-90 minutes after a high multiplicity infection) it was spun at 10,000g for 10 minutes; the pellet was resuspended in 3-4 ml of saturated borate and dialysed against 3 changes of the tris buffer just described. Borate detaches reversibly adsorbed phage from debris. Dialysis against tris establishes suitable conditions for treatment with DNAase, RNAase, and trypsin as described by Sinsheimer et al. (20). Sephadex chromatography and density gradient centrifugation were performed as described (20). The fractions from the last CsCl centrifugation were collected in SB, and the phage containing fractions were pooled and dialysed against starvation buffer. Kept at 0°C they were quite stable.

Preparation of 5-Bromouracil Substituted ϕX .—The best yields (5-25 phage per infected cell) have been obtained by infecting E. coli CR with a high multiplicity (3) of ϕX in the presence of thymidine (20 mg/l) and chloramphenicol (Parke-Davis, 30 mg/l added 5' before the phage), allowing the infection to proceed for 5' in the presence of thymidine, filtering, washing (cold SB),

and resuspending the infected cells in fresh DDM + Budr at 5 mg/l. The infected cells are incubated for 60' in the presence of Budr. Most of the cells do not lyse when development proceeds in Budr, and it is necessary to pellet and lyse them as described below. When P^{32} labelling is also desired the cells are grown in TPG supplemented with thymidine and uridine (both at 20 mg/l) and infected as just described; the P^{32} is added to the Budr (5 mg/l) plus uridine (20 mg/l) supplemented TPG to which the infected cells are transferred. Uridine was added to hinder any possible effect Budr might have on RNA synthesis. The phage were purified as described above except for modifications instituted in order to avoid exposing the phage to high pH or bright lights. The lysed pellet was dialysed 1x against 0.1 M phosphate, pH 7.5, and then 2x against tris-NaCl. Also 0.1 M phosphate, pH 7.5, instead of saturated borate, was used to elute the phage from Sephadex. The drops from the density gradients were collected in DCM; the pooled phage peak was dialysed 1x against 0.05 M tris, pH 8.1, for 2 hours in the cold and then for 2 hours against SB adjusted to a pH of 7.5. The recovery of phage was 10-30%, and they remained fairly stable for several weeks when kept at 0°C in darkness. The sensitivity to ultraviolet light of phage prepared in this way is usually uniform (cf. figure 9), even though the density is heterogeneous. Figure 1 shows equilibrium



Legend to figure 1

The banding patterns of Bu-P³²-ØX and Bu-P³²-ØX-DNA in CsCl. The phage were banded at 37000 RPM at 7°C in a model L ultracentrifuge for 40 hours in 3.0 ml at a mean density of 1.43 g/ml. One drop fractions were collected (117 total) in 1 ml of DCM and assayed for phage and P³². MS-2 ($\rho = 1.46$ g/ml, (21)) and γ h ($\rho = 1.43$ g/ml, (22)) were added as density markers. The heavy peak of Bu-P³²-ØX is at a density of about 1.458 g/ml. The DNA was banded at 37000 RPM at 16°C for 52 hours in 2.6 ml at a mean density of 1.71 g/ml. Three drop fractions were collected (102 drops) in 0.25 ml of DCM and assayed for both P³² and DNA infectivity. The peak of Bu-P³²-ØX-DNA is at a density of 1.83 g/ml relative to the 1.725 g/ml density of γ h-DNA (20). The phage were labeled with about 0.3 P³² atoms per particle.

CsCl density gradient banding patterns of a preparation of Bu-P³²-ØX and of the DNA isolated from these same phage by phenol treatment. The reason for the heterogeneity in density of the phage particles, but not in the density of the DNA or the uv sensitivity of the phage, is not known. The degree of substitution has not been directly determined; however, the reproducibility of the density and uv characteristics of Bu-ØX prepared in several different ways would suggest that the substitution is complete, as would the observed density of 1.83 g/ml for the DNA, since the expected density for fully substituted Cs-DNA is only 1.81 g/ml (vide infra).

Lysing Procedure.—The cells to be lysed are pelleted at 3000-4000g and resuspended in 2-5 ml of cold 0.25 M tris, pH 8.1. They are washed if the experiment calls for it. To the resuspended cells 0.2 ml of a lysozyme solution (2 mg/ml Worthington A grade in 0.25 M tris, pH 8.1) and 0.3 ml of a versene solution (4% disodium salt in H₂O) are added. The sample is allowed to sit at 0°C for 2 - 4 hours and then slowly frozen by placing it in a -40°C deep freeze. Upon thawing the sample is usually quite clear and very viscous; occasionally when a large (10¹⁰) number of cells were being lysed it was necessary to add 0.1 ml of a 2% SDS solution (sodium dodecyl sulfate in H₂O) to obtain complete clearing.

Phenolation procedure.—The sample to be phenolated

is combined with an equal volume of distilled phenol equilibrated with saturated borate (or with 0.1 M phosphate, pH 7.5, when Bu-substituted DNA is present), alternately placed in a beaker of water at 70°C and mixed vigorously on a vortex mixer for a total of 3 - 5 minutes, chilled, and centrifuged to separate the phases. The aqueous layer is removed and extracted 3x with an equal volume of ether at room temperature to remove the phenol. The ether is removed by bubbling air through the sample on ice for about 30'.

Banding Procedure.—The sample to be banded (usually 2.0 ml) is combined with 1.26 g/ml CsCl to give a density of 1.705 ± 0.01 g/ml, placed in a 5 ml lusteroid tube with sufficient white paraffin oil to fill the tube to within 2 mm of the top, and spun at 37,000RPM at 15-16°C for 46-50 hours in a model L ultracentrifuge. Drops are collected in half-dram vials containing a small volume (usually 0.25 ml) of DCM using an hydraulic apparatus to supply positive and negative pressure. One drop (about 0.037 ml) is usually collected in each vial from unphenolated samples; two drops (each about 0.024 ml) are usually collected in each vial from phenolated samples. One aliquot (usually 0.08 ml) from the vial is assayed for radiophosphorus (if present), another aliquot (0.02 ml) is diluted into 0.3 ml of 0.05 M tris, pH 8.1, and assayed for infectious DNA with 0.2 ml of protoplasts and 2.0 ml

of PAM (23). Control experiments indicated that the dilution away from the CsCl and coli DNA was sufficient to allow a correct assay of the amount of infectivity present.

Presentation of data.—All of the gradients to be described have been formed by combining 2.0 ml samples (phenolated or unphenolated) with 2.52 g of CsCl to yield a final volume of 2.6 ml at a density of approximately 1.705 g/ml. Under the conditions described this produces a gradient of 0.233 g/ml from the top of the gradient to the bottom (measured by collecting fractions under oil and weighing them). Infectivity (as single strand (SS) DNA equivalents per ml) and radioactivity (as cpm per ml, background subtracted out) are expressed relative to the 2.0 ml volume. The abscissa is normalized by dividing the fraction number by the total number of fractions. The density decreases from the first fraction to the last, left to right. All densities are determined relative to a density of 1.725 g/ml for SS ϕ X DNA (20) (the single strand DNA reference in most of the experiments was ϕ h DNA). Coli DNA was observed in purified samples to band at a density of 1.712 g/ml; in banded unphenolated lysates the peak of coli DNA is broad and viscous.

Quantitation.—All of the P^{32} label in experiments performed with labeled phage is usually accounted for with results which vary for different phage preparations

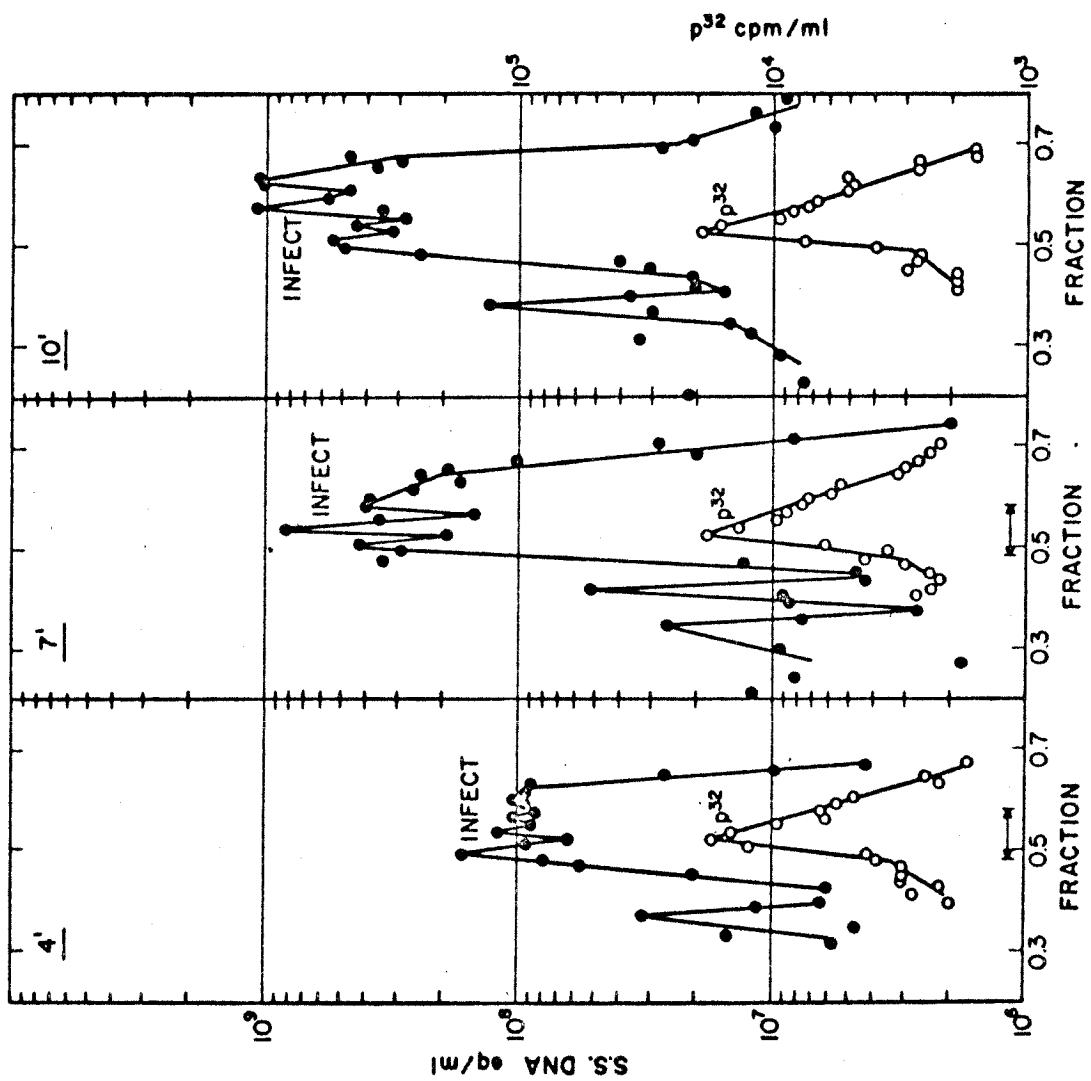
and their age: 20 - 90% of the P^{32} is found in the supernatant after pelleting the infective centers; 10 - 80% of the P^{32} may be trapped in the interface during phenolation or in the pellicle (the scum at the top of the centrifuge tube) of banded raw lysates. Whenever it is desired to compare gradients they are prepared and developed in parallel. All of the ϕX DNA in the cell is not recovered into bands. The determination of the percentage recovery in terms of infectivity is complicated by the differences in the efficiency with which different kinds of DNA infect protoplasts. In terms of radioactivity 30 - 90% of the intracellular ϕX DNA can be recovered into bands. On the low background Nuclear Chicago Counter used in these experiments 1 cpm equals about $10^5 P^{32}$ atoms.

Results

The Formation of RF and the Kinetics of RF Replication

The state of the intracellular ϕ X DNA as seen in an equilibrium CsCl density gradient at various times after a starvation synchronized infection is illustrated in figure 2. Samples were taken from a culture infected with P^{32} labeled ϕ X ρ^- (about 3 P^{32} atoms per phage) at the times indicated, chilled, lysed, and banded. Lysed samples banded without further purification exhibit complex patterns: several small peaks of unknown nature are often seen (see, for example, figure 11 of Sinsheimer et al. (20)); the coli DNA produces a very viscous region of the gradient around its density of 1.71 g/ml with a concomitant perturbation in the banding patterns of other components in that region; a large amount of radioactivity and infectivity usually appears in the pellicle at the top of the tube. Native RF molecules should band at a density of 1.701 g/ml (20), and SS ϕ X DNA bands at a density of 1.725 g/ml. Partially denatured and partially completed RF molecules would thus band right in the region of the coli, as would SS DNA attached to a protein of the right size. All of these species could quite reasonably exist in the infected cell.

From 0 to about 3 - 4 minutes after infection the amount of radioactivity and infectivity banding broadly in



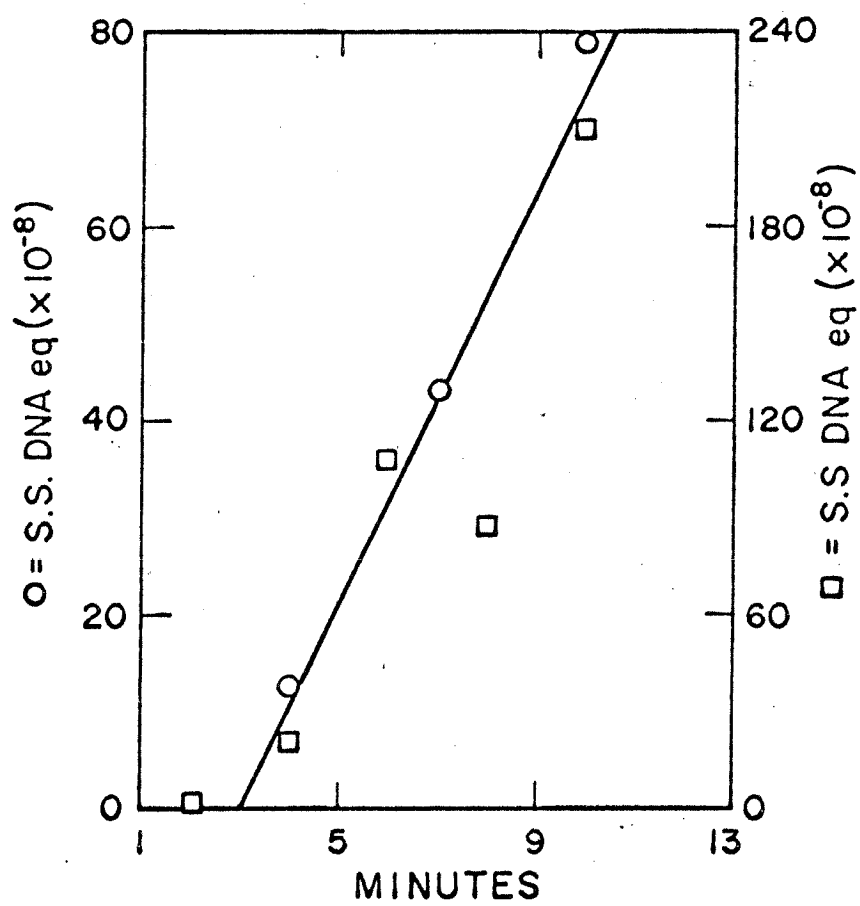
Legend to figure 2

The banding patterns of the unphenolated intracellular ϕ X DNA of 4', 7' and 10' starvation synchronized complexes. The coli DNA was detected by its viscosity at fractions 0.52 to about 0.54. Starved cells at 3.5×10^8 /ml were infected with 3×10^7 /ml P^{32} labeled $\phi X\phi^-$ in SB. An equal volume of TKB was added at 0' (see Part II); 5 ml of the infected culture was diluted into 5 ml of cold SB at the times indicated, lysed, and banded as described in Materials and Methods. The growth curve was normal with an eclipse period of 11'. The banding patterns of the DNA isolated from ϕ X and $\phi X\phi^-$ infected cells have not been observed to differ significantly.

the region of the coli DNA in unphenolated samples increase approximately in parallel. Counts and infectivity are sometimes seen in the SS DNA position, always seen in the pellicle. The ϕ X DNA in the pellicle is trapped there as the result of either a non-specific interaction with a protein or the formation of a specific enzyme-DNA complex during the conversion of the infecting SS DNA to RF.

From about 4' on the amount of P^{32} banding in the coli band remains constant while the amount of infectivity increases approximately at a constant rate (fig. 3). One objection that could be raised to the experiment described in fig. 2 is that many of the counts could be in non-infective DNA molecules since the original phage were so heavily labeled. However, the ratio of infectivity to radioactivity at 4' is what it should be (1 P^{32} atom = approximately 10^{-5} cpm) if there were 2-3 P^{32} atoms per RF molecule and if the efficiency of RF in the protoplast system were 1/10 that of SS DNA as is believed (see below, fig. 5).

There is a discrepancy between the position at which the radiophosphorus is banding (the phage were not density labeled) and the position at which the increase in infectivity occurs. Whether this result (which is reproducible, e.g. figure 6) is an artifact caused by the presence of the coli DNA or represents a real difference between the parental RF and many of the progeny RF is uncertain. The parental RF seems to band in unphenolated



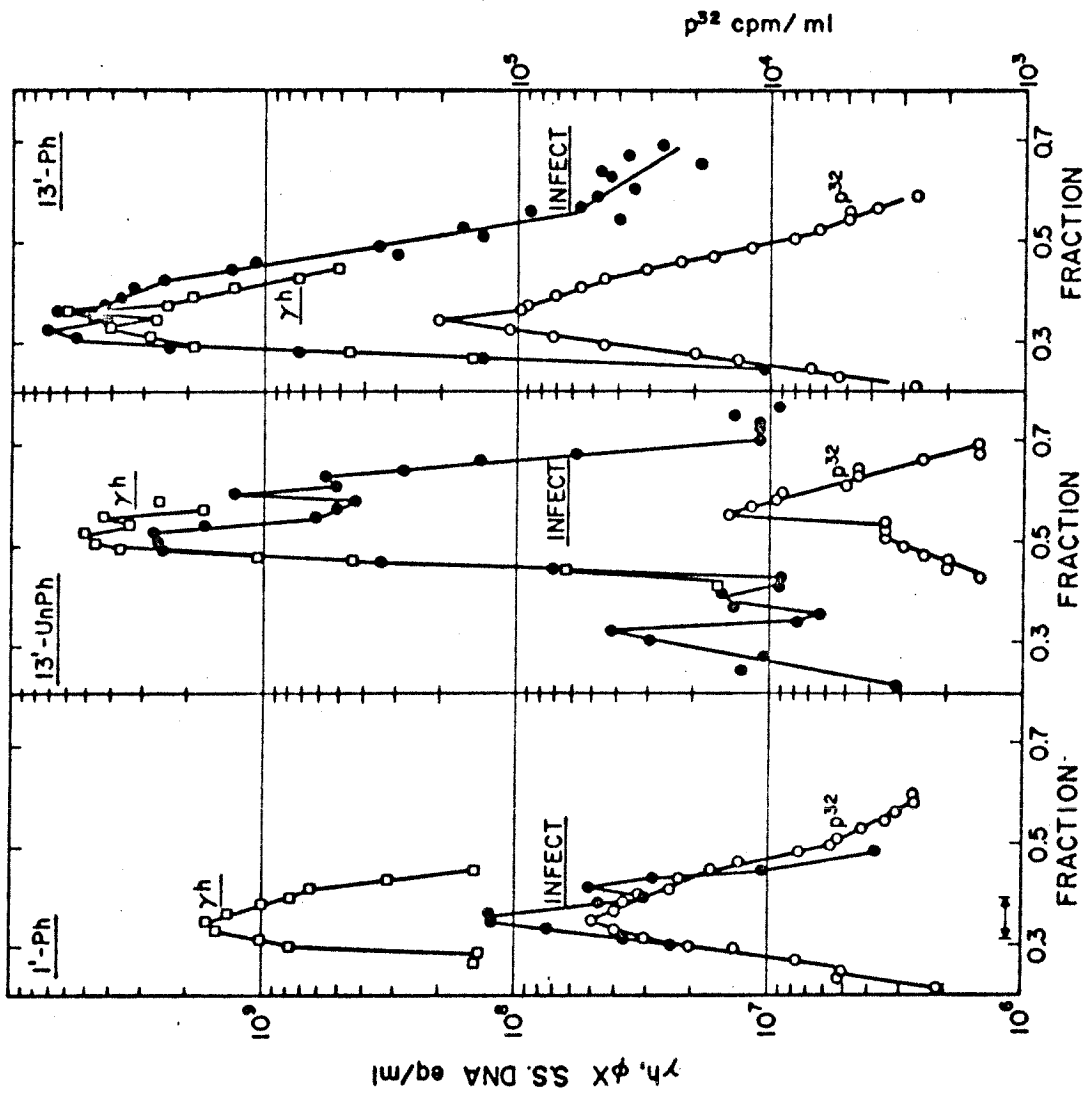
Legend to figure 3

The constant rate of synthesis of RF in ϕ X infected cells as measured by equilibrium CsCl density gradient centrifugation. The circles represent the total amount of single strand DNA equivalents present in the peaks of the 4' (0.47 - 0.63), 7' (0.47 - 0.67) and 10' (0.48 - 0.63) unphenolated gradients shown in figure 2; 6.5×10^7 infective centers were banded. The squares represent data from a separate experiment that has not been discussed, but was done similarly; 1×10^8 infective centers were banded. Both experiments were done at low multiplicities. Two separate ordinates were used in order to simplify the presentation of the data.

samples at a position heavier than expected for a native duplex RF.

Phenol treated lysates give rise to simpler and cleaner banding patterns. The coli DNA usually produces (in the concentrations used in these experiments, from about 10^9 cells) little or no perturbation in the banding patterns of the other components. Heat and phenol release DNA complexed with protein or contained in phage and may permit partially denatured DNA to renature. The banding patterns of phenolated 1' and 13' samples and of a 13' unphenolated sample that are all from the same experiment as described in figure 2 are presented in figure 4. At 1' there is predominantly SS DNA; small RF peaks are sometimes detectable. In the 13' samples, both phenolated and unphenolated, both SS DNA and RF DNA are present. In this particular experiment the fact that much P^{32} is in inactive phage shows up in the large peak of radioactivity in the single strand region of the phenolated patterns.

The amount of infectivity banding in the RF region of the 13' unphenolated sample in figure 4 is not significantly larger than the amount banding there in the gradient of the 10' sample shown in figure 2. After 13' the amount of infectivity banding at the position of the SS DNA masks any infectivity of the RF DNA. Since most of the cells begin synthesizing single strand DNA between 9' and 12', it may be that RF DNA synthesis and SS DNA

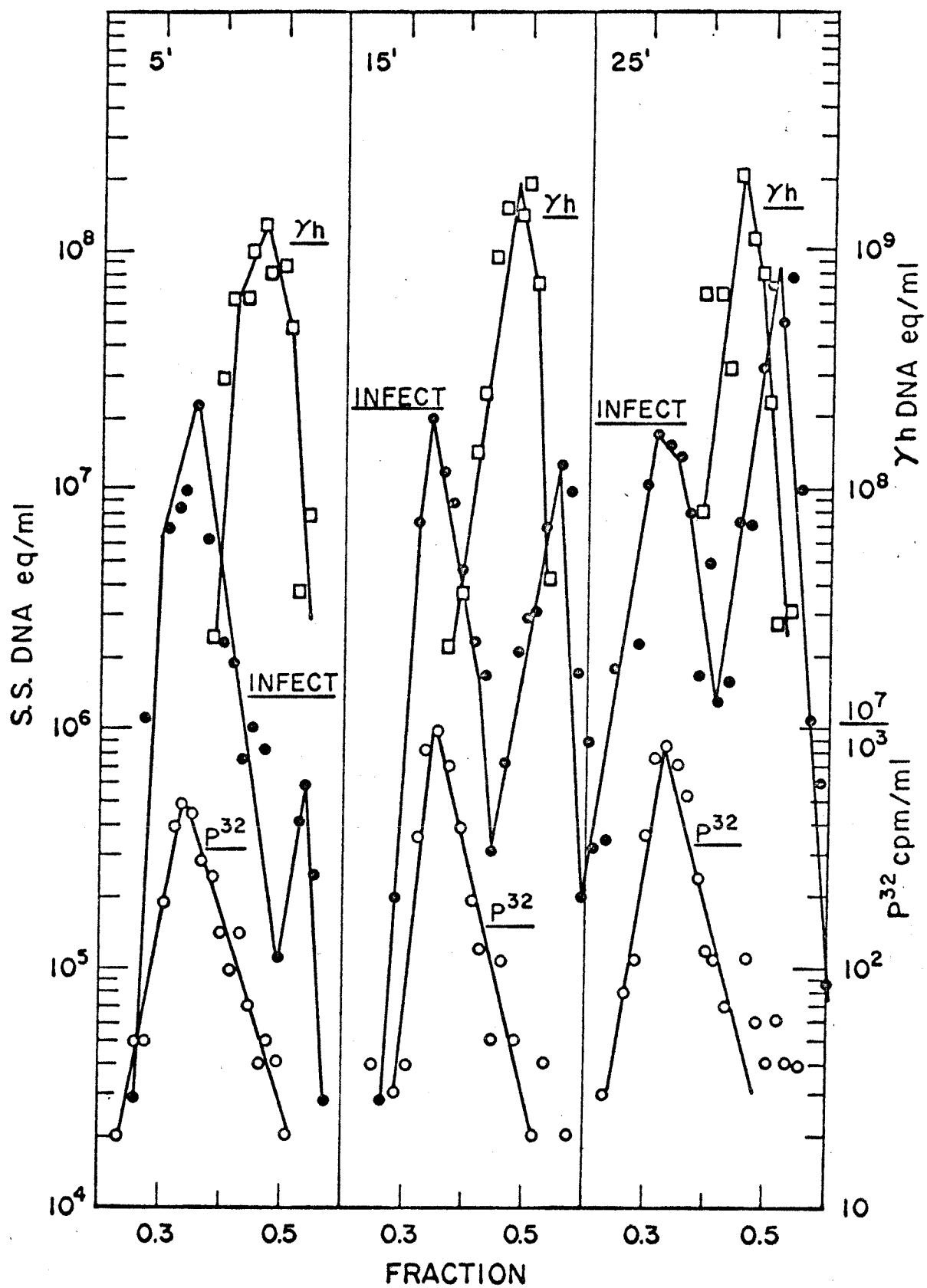


Legend to figure 4

Banding patterns of the intracellular DNA from phenolated 1' and 13' complexes and from an unphenolated 13' complex. γ h DNA (single stranded) was added as a density marker to these gradients. The DNA is from the complexes whose preparation was described in the legend to figure 2. The banding pattern of the counts in the 13' phenolated sample is atypical in that there is no distinct RF peak (a typical pattern is shown in figure 6). The specific activity of the phage preparation used was such (2-3 P^{32} atoms/phage) that there were many labeled inactive phage present; the P^{32} suicide of ϕ X is one, and it is assumed that the inactivated SS DNA is unable to form RF.

synthesis are mutually exclusive. Two problems cloud this interpretation: 1) the separation between SS DNA and RF DNA in the equilibrium CsCl density gradient is so small that if one form is in considerable excess the other cannot be accurately measured; and 2) ϕ X infected cells begin to lyse shortly after the end of the eclipse period (see Part II) with the result that the RF DNA contained in them is lost.

Figure 5 shows the banding patterns of the phenolated intracellular ϕ X DNA isolated from cells infected with Bu- ϕ X in the presence of chloramphenicol. In this case the parental RF (containing Bu in one strand) is separated from the progeny RF and is banding at a density of 1.755 g/ml. The phage used in this experiment carried about 0.3 p^{32} atoms per particle; therefore 6×10^2 cpm/ml correspond to 2×10^8 RF molecules/ml. Since only about 2×10^7 SS DNA equivalents of infectivity are observed to band at the hybrid position it is concluded that RF DNA is only about 1/10 as efficient at infecting protoplasts as SS DNA. This result also obtains in the 4' sample of figure 2, the 5' sample of figure 7, and the 8' sample of figure 8. Another conclusion to be drawn is that fully light progeny RF molecules can be synthesized in cells infected in light medium with Bu substituted ϕ X, even in the presence of chloramphenicol.



Legend to figure 5

The formation and replication of RF in cells infected with P^{32} -Bu- ϕ X in the presence of chloramphenicol.

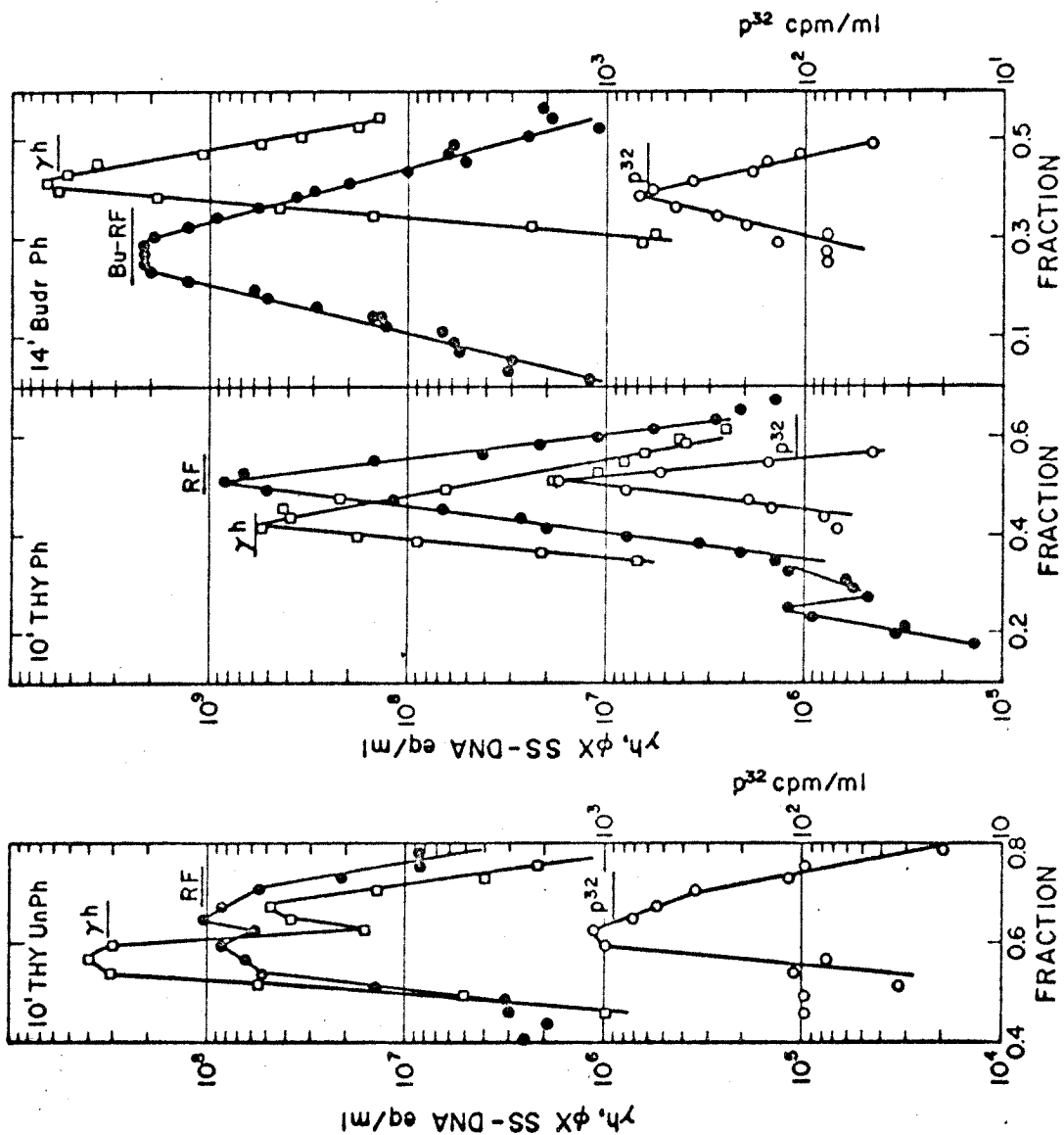
Chloramphenicol (30 mg/l) was added to a log phase culture of E. coli CR (4.5×10^8 cells/ml) growing in DDM plus 20 mg/l thymidine. Five minutes later 1×10^8 P^{32} -Bu- ϕ X/ml were added. At 5', 15', and 25' after adding the phage, aliquots of the culture were lysed, phenolated, and banded. A total of 2.5×10^8 infective centers were banded. The phage used were the same as described in figure 1. Neither mature intracellular phage nor SS DNA were made. The replication of RF in this experiment occurs more slowly than under normal conditions because of both the presence of Bu in the infecting phage and, more importantly, the presence of chloramphenicol in the culture.

The Origin of the Material Composing the RF

The data presented in figure 6 were derived from an experiment in which two aliquots of a culture were infected with P^{32} labeled ϕX , one in the presence of thymidine, the other, in Budr. A comparison of the banding patterns of the phenolated and unphenolated samples of the 10' thymidine culture supports the suggestion made above (page 50) that in raw lysates some of the RF (particularly the parental RF) bands at a denser position than expected for a native duplex structure. The perturbation in the banding pattern of the γh marker is caused by the viscous coli DNA. The infectivity and the counts in the 10' phenolated sample are both banding at a density of 1.704 g/ml, as expected for RF DNA (20).

It was surprising that there was no evidence of any SS DNA in the 10' phenolated sample since mature phage were appearing by 11'; apparently there is only a very short interval between the synthesis of SS DNA molecules and their incorporation into mature phage particles. Although Sinsheimer et al. (20) did not find any free SS DNA in 14' complexes in their system, there is free SS DNA in the 13' unphenolated starvation synchronized complex illustrated in figure 4.

The phenolated DNA from the 14' Budr culture indicates (fig. 6) that considerable replication of the RF has occurred in the presence of Budr to yield Bu-RF banding



Legend to figure 6

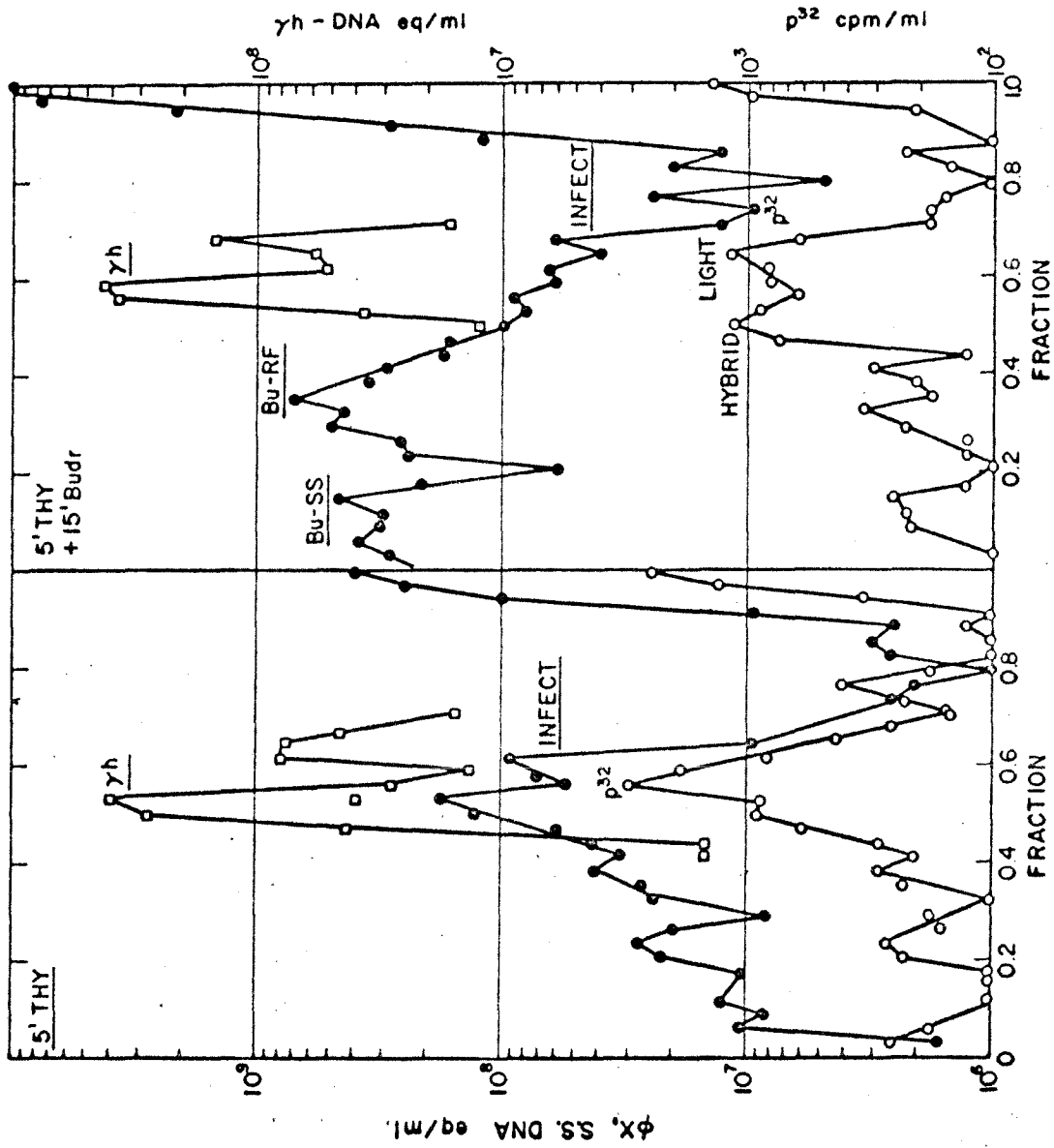
Banding patterns of the unphenolated intracellular DNA from a 10' thymidine culture and of the phenolated intracellular DNA from both a 10' thymidine culture and a 14' Budr culture. E. coli CR was grown in DDM + 10 mg/l thymidine, pelleted, washed, starved in DDM 15' for thymidine, and divided into two portions; 5 mg/l Budr was added to one aliquot, 5 mg/l thymidine, to the other. Five minutes later P^{32} labeled ϕX (about 0.3 P^{32} atoms per phage) at a multiplicity of 0.5 was added to each aliquot. At intervals samples were taken for measurement of infective centers and intracellular phage, for determination of the L-L curves (see Part V), and for an analysis of the intracellular DNA. The eclipse period of the thymidine culture was 10.5', of the Budr culture, 16'; the average burst sizes were about 100 and 14 respectively. The L-L curves indicated a sensitized target in the Budr culture at all times. Samples for the intracellular DNA were lysed and banded as described in Materials and Methods.

at a density around 1.76 g/ml. The hybrid RF containing a P^{32} labeled light parental strand is banding at a density of 1.732 g/ml. Since the bacterial DNA was not Bu substituted it is deduced that the material constituting the complementary single strand with which the parental single strand associates is derived from the medium and does not preexist in the cell. This result also obtains at earlier times before any RF replication has occurred (e. g. fig. 8).

Evidence will be presented below (page 65) that fully substituted Bu-RF does not replicate extensively in the presence of Budr. Hence most of the fully Bu substituted RF observed in the 14' phenolated Budr sample of figure 6 must have derived from the replication, presumably at a constant rate, of only the parental RF, which contains a thymine strand. Because there is considerably more Bu-RF at 14' than thymine RF at 10', it is argued that under normal conditions (thymine present) in this system RF DNA is synthesized at a constant rate. If the rate of RF synthesis in the 10' Th sample had increased with time (e. g. exponentially) then there would have been more RF at 10' in Th than at 14' in Budr.

The Mode of Replication of RF

Figure 7 shows a density gradient analysis of the unphenolated contents of cells infected with P^{32} labeled ϕX after 5' in thymine and after 5' in thymine plus 15' in Budr. About half of the radiophosphorus has shifted



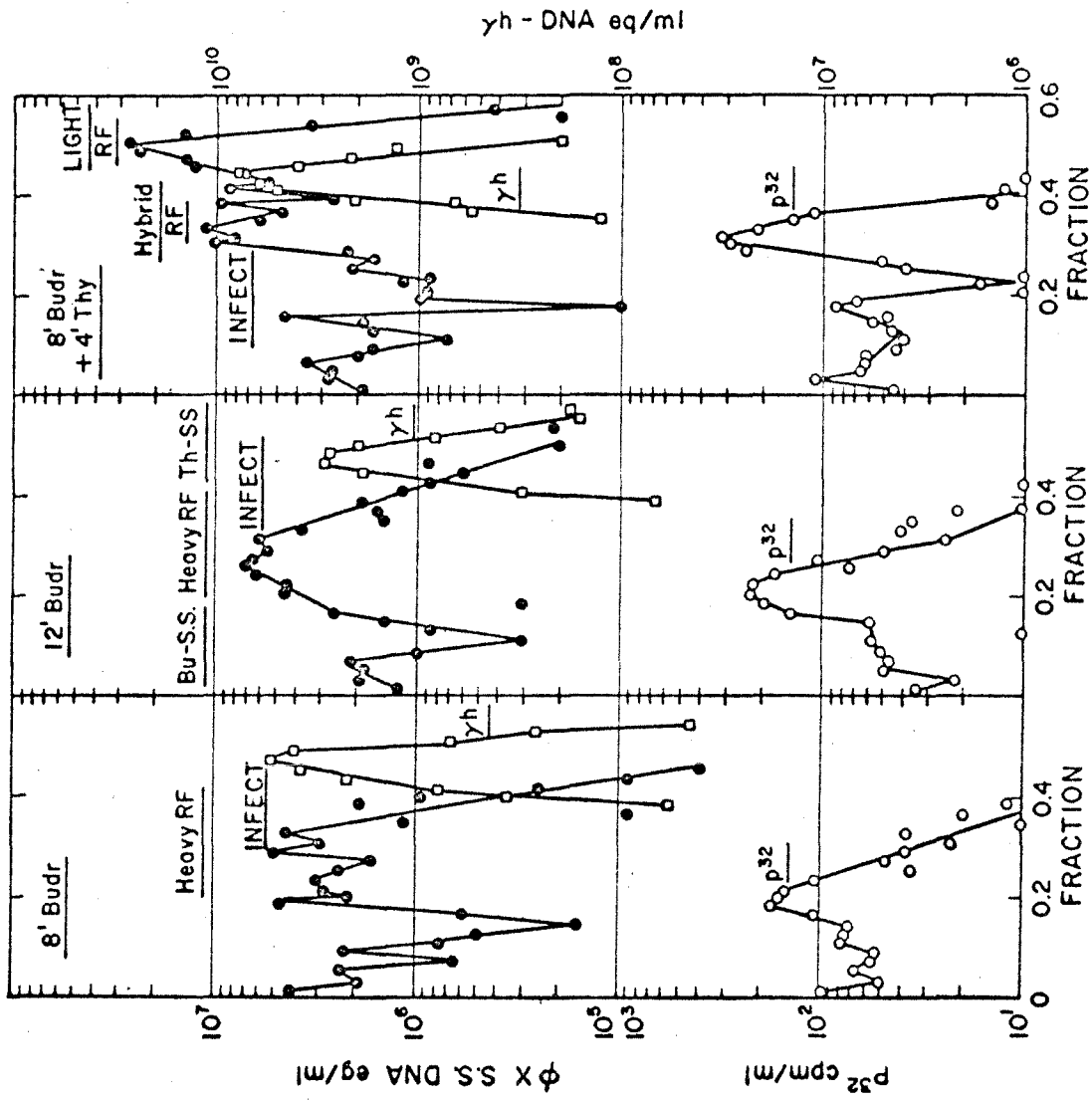
Legend to figure 7

Banding patterns of the unphenolated intracellular DNA isolated from infected cells grown for 5' in the presence of thymine and for 5' in the presence of thymine followed by 15' growth in Budr. The transfer was accomplished by filtration as described in Materials and Methods. A log phase culture of E. coli CR in thymine supplemented DDM was pelleted, washed, and starved in SB for 70'. Phage in buffer at a multiplicity of 1 were added; 5' later thymine supplemented DDM was added. After 5' of development the infected cells were transferred to Budr medium. Aliquots for DNA were taken immediately and 15' after the transfer. The eclipse period ended 11' after the transfer and the average burst size was 13. The uv sensitivity of these complexes is discussed in Part V. γ h single strand marker is present as a reference; the smaller lighter peak of γ h-DNA is an artifact caused by the coli DNA. Relative to the major peak of γ h-DNA the fully light RF (peak of radioactivity) is banding at a density of 1.716 g/ml (5') and 1.709 g/ml (5' + 15'); the hybrid RF is banding at a density of 1.743 g/ml.

to a denser position after the transfer, and fully substituted Bu-RF has been formed. The uv experiments described in Part V imply that the material which remains light does not represent an inactive fraction.

A similar experiment in which thymidine was added to a culture infected for 8' in the presence of Budr with P^{32} -Bu- ϕ X produced the results shown in figure 8. The samples were phenolated prior to banding. The ratio of infectivity to radioactivity in the 8' and 12' Budr samples indicates that no extensive replication of the Bu-RF in Budr has occurred. The reason that the infectivity is skewed toward a lighter position than the counts could be either that the most heavily substituted DNA is impaired in its ability to infect protoplasts or that a few progeny RF molecules somewhat less dense than the parental RF (see figure 2) have been synthesized. After the addition of thymidine most of the radiophosphorus and infectivity shifted from the heavy position, 1.787 g/ml, to the hybrid position, 1.758 g/ml, and fully light RF, 1.71 g/ml, appeared. The uv analysis presented in Part V implies that the material which shifts in density does not represent an inactive fraction.

A variety of experiments like those described in figures 7 and 8 have been performed. In all cases at least half of the radioactivity appeared to shift in density as if it were contained in an RF molecule which replicated



Legend to figure 8

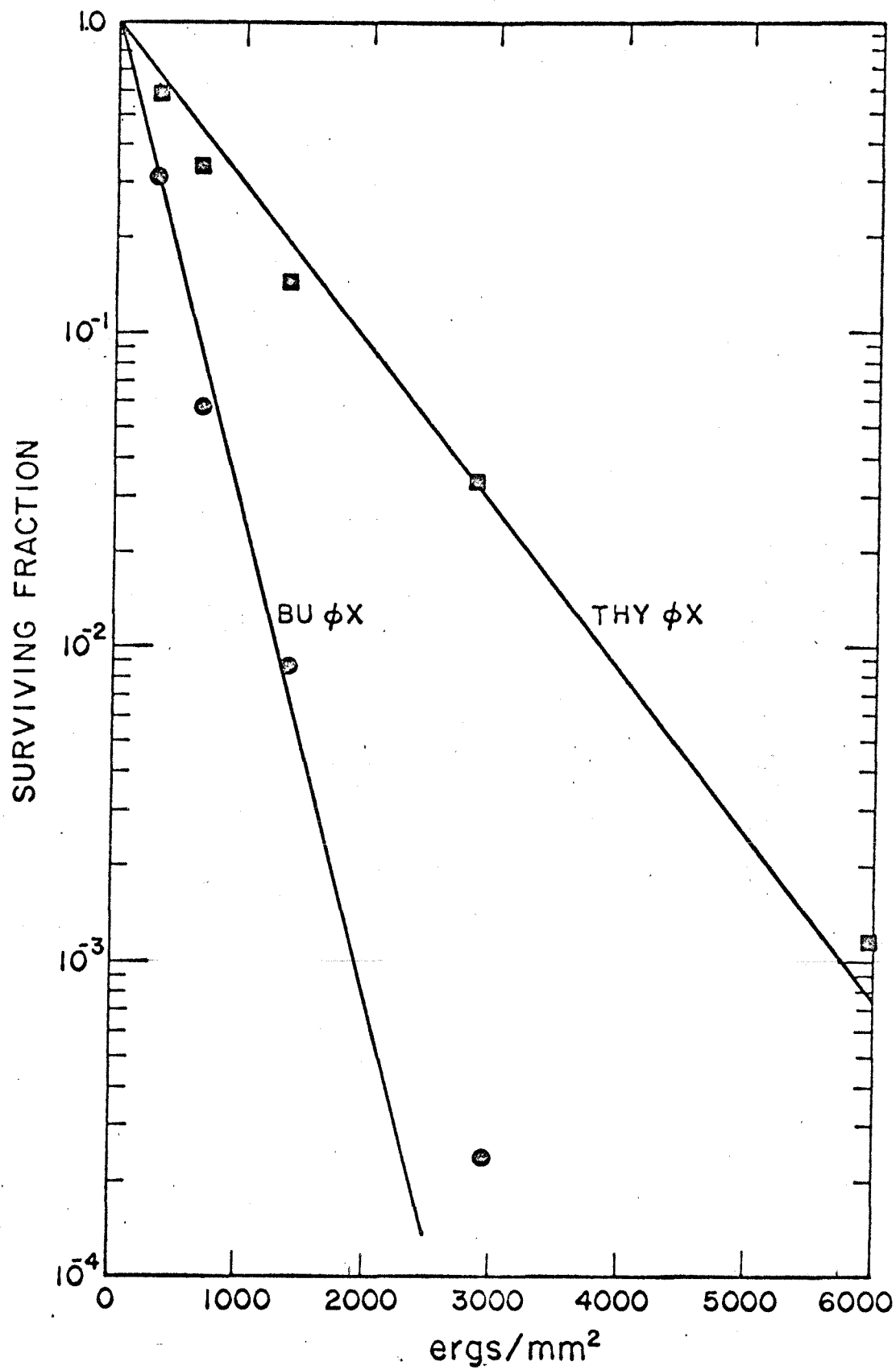
Banding patterns of phenolated intracellular ϕ X DNA isolated from cells infected with P^{32} -Bu- ϕ X (0.3 P^{32} per particle) for 8' and 12' in the presence of Budr and for 8' in Budr plus 4' in thymidine. Log phase E. coli CR grown in thymidine supplemented DDM were starved for 60' in SB at 4.8×10^8 cells/ml and infected with 5×10^7 P^{32} -labeled-Bu-substituted ϕ X (the same phage preparation as described in figure 1). Budr medium was added after 5'; the eclipse period was 18', the average burst size, about 5. Thymidine was added to one aliquot of the infected culture at 8'; the eclipse period ended at 15' in this aliquot, and the burst size was 150. The uv sensitivity of these complexes is discussed in Part V.

semi-conservatively. It is not possible to quantitatively account for the results by assuming that a fraction of the original parental RF is destroyed and that originally inactive parental single strands suddenly become active after the transfer and form RF, because neither of these assumptions is valid in the low multiplicity experiments described here.

The Origin of Progeny Single Strands

A culture of E. coli CR was infected with ϕX in the presence of thymidine and chloramphenicol. Under these conditions RF is formed and replicated (at least 10 fold), but no SS progeny DNA is made. After 50' the infected cells were transferred to Budr medium, and the earliest intracellular phage appearing after the transfer were examined for their uv sensitivity. Since Bu substituted ϕX are more sensitive to uv than thymine ϕX , a few thymine ϕX can be detected among many Bu- ϕX . The inactivation curves shown in figure 9 indicate that RF does not serve as a material precursor to the SS DNA appearing in progeny.

A similar result has also been obtained without using chloramphenicol. In this case the infected cells must be transferred from thymidine to Budr before progeny SS DNA is synthesized (i.e. before 6'-8'); by 6'-8' RF has replicated at least 5x. In an experiment where the infected cells were transferred at 7' an average burst size of 20 was obtained, and more than 99% of the phage were fully sensitized.



Legend to figure 9

The uv inactivation curves of ϕ X and Bu- ϕ X at 297 m μ . The Bu- ϕ X were obtained from cells infected for 50' in thymidine (20 mg/l) and chloramphenicol (30 mg/l) and then transferred by filtration to chloramphenicol free Budr medium. A sample taken 3' after the end of the 17' eclipse period when there were about 5 phage per infective center was lysed prematurely as described in Part II, and the uv sensitivity of the phage present was determined. The line drawn through the points for the Bu- ϕ X has approximately the cross section observed for what are believed to be fully Bu substituted ϕ X (cf. figure 9, Part V).

Discussion

The observation that the material making up the complementary strand of DNA associated with the infecting parental single strand is derived from the medium after infection excludes the possibility that RF is formed by the association of the infecting SS with coli DNA (16). Very little free parental SS DNA is observed inside infected cells, even in starved cells in buffer after the phage have gone into eclipse; it appears in the pellicle, presumably complexed with protein. Not until conditions that permit the synthesis of cellular material are restored is RF formed. Progeny SS DNA is not made until practically the end of the eclipse period (usually 10 - 12 minutes after infection) and is very rapidly incorporated into mature phage particles.

The parental RF replicates semi-conservatively, and the conserved subunit would seem to be a single polynucleotide strand from the agreement between the theoretical and experimental hybrid densities presented in table 1. The theoretical densities are calculated by assuming: 1) the addition of 65 daltons for each thymine replaced; 2) no change in the molar volume of the DNA; 3) the DNA is present entirely as the anhydrous Cs^+ salt; 4) the A:T ratio of ϕX DNA is 0.76 (22); 5) 1790 of the 5500 nucleotides in ϕX are thymidylic residues (24); and 6) the RF DNA is a duplex molecule of the Watson-Crick type.

Table 1: The Densities of Various Forms of Bu Substituted ØX DNA

species	theoretical density	experimental density	experiment
Th-Th RF	1.701 g/ml (20)	1.704 g/ml	figure 6
Th-Bu RF*	1.736	1.732	figure 6
Bu-Th RF**	1.747	1.755	figure 5
Bu-Bu RF	1.780	1.787	figure 8
Bu SS	1.81	1.83	figure 1

* viral strand contains thymine

** viral strand contains bromo-uracil

The experimental densities are calculated using SS DNA as a reference at a density of 1.725 g/ml (20) and are based only on the peak of radioactivity observed in phenol treated samples because: 1) the reference density in banding patterns of unphenolated lysates is not well determined; 2) peaks of infectivity are in general not as well defined as peaks of radioactivity; and 3) the P^{32} is in all cases contained in a parental RF.

If the conserved subunit were, on the other hand, a duplex molecule (3-6), the "hybrid" formed with a light parent should band at a density of 1.758 g/ml (Th:Bu:Bu:Bu) and the "hybrid" formed with a Bu substituted parental infecting phage should band at a density of 1.725 g/ml (Bu:Th:Th:Th). The results described in table 1 and in figures 7 and 8 are clearly quite incompatible with this possibility.

The results that the rate of RF replication appears

constant and that the replication of only the parental RF can account for all the RF synthesized suggest the possibility that most of the RF is derived only from the replication of the RF molecule containing the infecting SS. It was shown that the parental RF molecule does replicate semi-conservatively; similar experiments to determine whether or not progeny RF molecules also replicate semi-conservatively have not been successful. It is insinuated that only the parental RF can replicate in this system. Evidence will be presented in Parts IV and V that the RF molecule containing the infecting SS performs a function that the progeny RF molecules cannot late in the eclipse period; a function that is necessary if the infected cell is to form a plaque.

The discrepancy between the position at which the parental RF molecule (containing radiophosphorus bands and the position at which the progeny RF molecules band in raw lysates, but not in phenolated lysates, hints that they differ. Rosenberg and Cavalieri (25) found that part of the coli DNA was in a metastable state and banded under certain conditions at a density about 0.016 g/ml heavier than native coli DNA, approximately at the position of denatured coli. Sinsheimer (26) has discovered that the parental RF molecule differs from many of the progeny RF molecules in its sedimentation behaviour in alkaline CsCl.

The special function mentioned above (see Parts IV and

V) of the parental RF is most reasonably the production of progeny SS DNA. If so, and if also the parental RF is the principal source of progeny RF, then the observation that RF replication seemed to stop (page 53) after SS DNA synthesis began would suggest that the two functions are mutually exclusive. However, this suggestion must be supported by a direct experiment before it can be accepted.

The function of the progeny RF molecules may be the synthesis of messenger RNA. The progeny RF molecules are not material precursors to the progeny SS DNA; if they are informational precursors then another unique function must be hypothesized for the parental RF.

Because 1) RF is formed and replicated in the presence of chloramphenicol (20, also fig. 5) and 2) ϕ X infection does not detectably affect host cell DNA, RNA, and protein synthesis during most of the eclipse period (27), it can be concluded that the enzyme or enzymes that form and replicate RF DNA preexist in the cell and are not those immediately involved in coli DNA synthesis. A further conclusion that follows from the result obtained in chloramphenicol is that ϕ X RF DNA is formed, recognized, and replicated by a coli enzyme (or enzymes) independently of the control mechanism (28, 29) regulating the synthesis of coli DNA. This conclusion may be relevant to schemes proposed for the regulation of DNA synthesis in E. coli (30).

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PART IV

THE EFFECT ON THE INFECTED CELL OF THE DECAY OF
RADIOPHOSPHORUS INCORPORATED IN THE INFECTING PHAGE

Introduction

Hershey, Kennedy, Kamen, and Gest (1) discovered that radiophosphorus atoms incorporated into phage particles inactivated the phage at a rate proportional to the specific activity of the medium in which the phage were grown. Stent and Fuerst (2) examined T1, T2, T3, T5, T7 and λ and found that the efficiency with which the disintegration of a P^{32} atom inactivated the phage containing it was about 0.1 for all the T phages at 4°C. The calculation of the efficiency of P^{32} suicide requires knowledge of the amount of DNA in the phage, knowledge which until recently has been rather imprecise. Values given by Stent and Fuerst (3) for the number of lethal atoms per phage particle are combined with recent data for the DNA content of each phage to yield the efficiencies, α , of suicide contained in table 1. All of the efficiencies, particularly that for T7, are higher than those calculated by Stent and Fuerst (3). Graziosi et al. (cited in Cordes et al. (9)) have found the P^{32} suicide efficiency for the double stranded DNA virus alpha of Bacillus megatherium to be close to 1. The efficiency of killing of viruses containing single stranded DNA by radiophosphorus decay has been shown by Tessman (10) to be 1.

When a P^{32} atom disintegrates a beta electron of maximum energy 1.7 MEV is emitted and the nucleus of the

Table 1: The Efficiencies of P^{32} Suicide of Several Phages

Phage	$N\alpha$ at 4°C (3)	Molecular Weight*	α
T2	4.5×10^4	130×10^6 (4)	0.125
T3	1.3×10^4	31×10^6 (4)**	0.130
T4	4.3×10^4	130×10^6 (4)	0.122
T5	4.2×10^4	84×10^6 (4)	0.155
T7	1.6×10^4	26×10^6 (5)***	0.191
λ	1.5×10^4	31×10^6 (6)	0.150

* The data for the molecular weights of the DNA are converted to phosphorus atoms per molecule of phage DNA by dividing them by 310 for all but T2 and T4; there are 3.6×10^5 nucleotides per molecule of T2-T4 DNA (4).

** Bendet et al. (7) derived a molecular weight of 23 megadaltons for T3, but for reasons stated by Thomas et al. (4) the value of 31 megadaltons has been used.

*** Davison and Freifelder (8) obtained a value of 19 megadaltons; Thomas et al. (4) observed that the molecular weight was 22 megadaltons.

resulting sulfur atom sustains a recoil which may amount to as much as 80 ev (3). Hershey et al. (1) have shown that the beta particle emitted is not the principal cause of inactivation. Stent and Fuerst (3) have concluded that the transmutation of the P^{32} atom will break the polynucleotide strand containing it, but that this by itself is not lethal for phages whose DNA is double stranded; the lethal event occurring in only a fraction of the decays is postulated to be the breakage of both strands of DNA in the double helix by a single P^{32} decay.

Thomas (11) examined the effect of P^{32} decays on the integrity of a large piece of DNA isolated from T2. He found that about 1 in 5 disintegrations resulted in a

complete break in the double stranded molecule, whereas every decay resulted in a break in the single polynucleotide chains.

The sensitivity of phage-bacterium complexes to inactivation by the decay of radiophosphorus atoms incorporated into the DNA of the parental phage decreases during the intracellular growth of the coliphages T1 (12), T2 (12, 13), T3 (3) and λ (14). In all cases (3), at least under certain conditions (15), there is a slow stabilization during the first 3 to 6 minutes after infection at 37°C followed by a rapid stabilization which becomes complete by about 8 to 15 minutes depending upon the phage. T2 complexes become refractory to inactivation even if the infection is carried out in the presence of sufficient P^{32} to label the intracellular replicas of the parental DNA (13).

Chloramphenicol added prior to infection blocks the stabilization of both T1 and T2 complexes; added a few minutes after infection it interrupts the stabilization process at an intermediate stage (12). The stabilization has been postulated to result from the replication of the phage DNA inside the cell (16) and the ability of the replicas to recombine with each other (15, 17). Uchida and Stent (18) found that if cells in which a primary phage (T2) had already developed and become stabilized to P^{32} decay were superinfected with a second phage in the

presence of chloramphenicol that this genetically distinct secondary phage did not become stabilized to P^{32} decay. Fermi and Stent (19) observed that the DNA of this second phage (in this case T4) was, however, apparently able to replicate since it was susceptible to bromo-uracil mutagenesis during the incubation in chloramphenicol. It would seem that chloramphenicol blocks an individually specific function necessary for P^{32} suicide stabilization, and that this function is not just the replication of the phage DNA.

Henry and Youngner (20) observed that poliovirus was inactivated with an efficiency of about 1, suggesting that only a single strand of RNA is present in this virus. They also obtained evidence that the plaque forming ability of Hela cells infected with labeled poliovirus RNA remained sensitive to inactivation by radiophosphorus decay up to the end of the eclipse period.

The P^{32} suicide experiments described below were undertaken in order to learn more about both the nature of the ϕX infection process and the nature of the mechanism of P^{32} suicide. Because of the single strandedness of ϕX DNA it is possible to label the duplex RF specifically in one strand; the inactivation of a double stranded DNA labeled in only one strand is relevant to the proposed mechanism for the lethal action of decaying P^{32} . The kinetics of stabilization of complexes formed from labeled

phage, if such stabilization occurs, should yield information about the nature of the ϕ X replication process.

Materials and Methods

P³²-ØX.—Labeled phage were prepared and purified as described in Part III.

Freezing media.—Freezing medium for complexes and phage contained 30% TKB (Part II), 30% SB (Part II), 30% PA (22), 0.2% BSA (Armour, 30% solution), 0.05% gelatin, and 10% glycerol. Freezing medium for DNA was 0.05 M tris, pH 8.1.

Preparation of Complexes.—The techniques of synchronization by starvation and by cyanide have been described (Part II). At intervals, aliquots were taken from the growth tube and diluted into ice cold freezing medium. As soon as possible each diluted aliquot was distributed in 0.5 ml portions into a set of vials kept cold in ice water. The vials were corked, placed in metal trays constructed to fit in the cannisters of a Linde liquid nitrogen refrigerator, quickly frozen in liquid N₂, and stored in liquid nitrogen. At appropriate times one vial from each set was slowly thawed and assayed immediately. More than half of the infective centers were recoverable after freeze-thawing in practically all cases; occasionally somewhat more than half of the very young complexes were killed, whereas free phage and mature complexes were usually recoverable with about 90% efficiency. All assays were done on freshly grown log phase E. coli C seed bacteria under the standard conditions described in Part II,

and essentially all of the data points are based on a count of between 100 and 400 plaques.

Calculations and Presentation of Data.—The primary observation is that the rate of inactivation of labeled phage is proportional to the number of incorporated radio-phosphorus atoms.

1. $\frac{dS}{dt} = -\alpha N^* S$ where S is the number of infective phage, t is the number of days, α is the proportionality constant (the efficiency of killing), σ is the fractional decay of P^{32} per day (0.0486), and N^* is the number of radioactive atoms per particle. N^* decreases with time according to

2. $N^* = N_0^* e^{-\sigma t} = A_0 N e^{-\sigma t}$ where N_0^* is the number of radioactive atoms in the phage at time zero, A_0 is the ratio of P^{32} atoms to P^{31} atoms at time zero, and N is the total number of phosphorus atoms in the DNA of the phage. Substitution for N^* in equation 1 and integration yields

3. $\log_e(S/S_0) = -\alpha A_0 N (1 - e^{-\sigma t})$ since when $t = 0$ $\log_e S = \log_e S_0$, where S_0 is the titer of phage at $t = 0$. Replacement of A_0 by the specific activity, A , in mc P^{32} per mg P and conversion to common logarithms yields

4. $\log_{10}(S/S_0) = -1.48 \times 10^{-6} \alpha AN (1 - e^{-\sigma t})$. Hence from the slope of a plot of $\log_{10}(S/S_0)$ vs. $(1 - e^{-\sigma t})$ or from the surviving fraction at $t = \infty$ the value of αAN is obtained; if any two of these parameters are known

the third may be calculated. The specific activity (A) is calculated from the amount of P^{32} added (carrier free radiophosphorus of known activity was obtained from Nuclear Consultants Corporation). N for ϕX is 5500 (21).

Whenever a straight line is obtained on a semi-log plot of surviving fraction vs. $(1-e^{-\sigma t})$ from 0 to 1, it may reasonably be concluded that the bulk of the population is homogeneously labeled and sensitive to radiophosphorus decay with an efficiency α . Inactivation curves concave upwards indicate population heterogeneity with respect to sensitivity to P^{32} decay inactivation; inactivation curves concave downwards indicate that something besides P^{32} disintegrations is inactivating the population or that more than 1 transmutation is required for each individual inactivation.

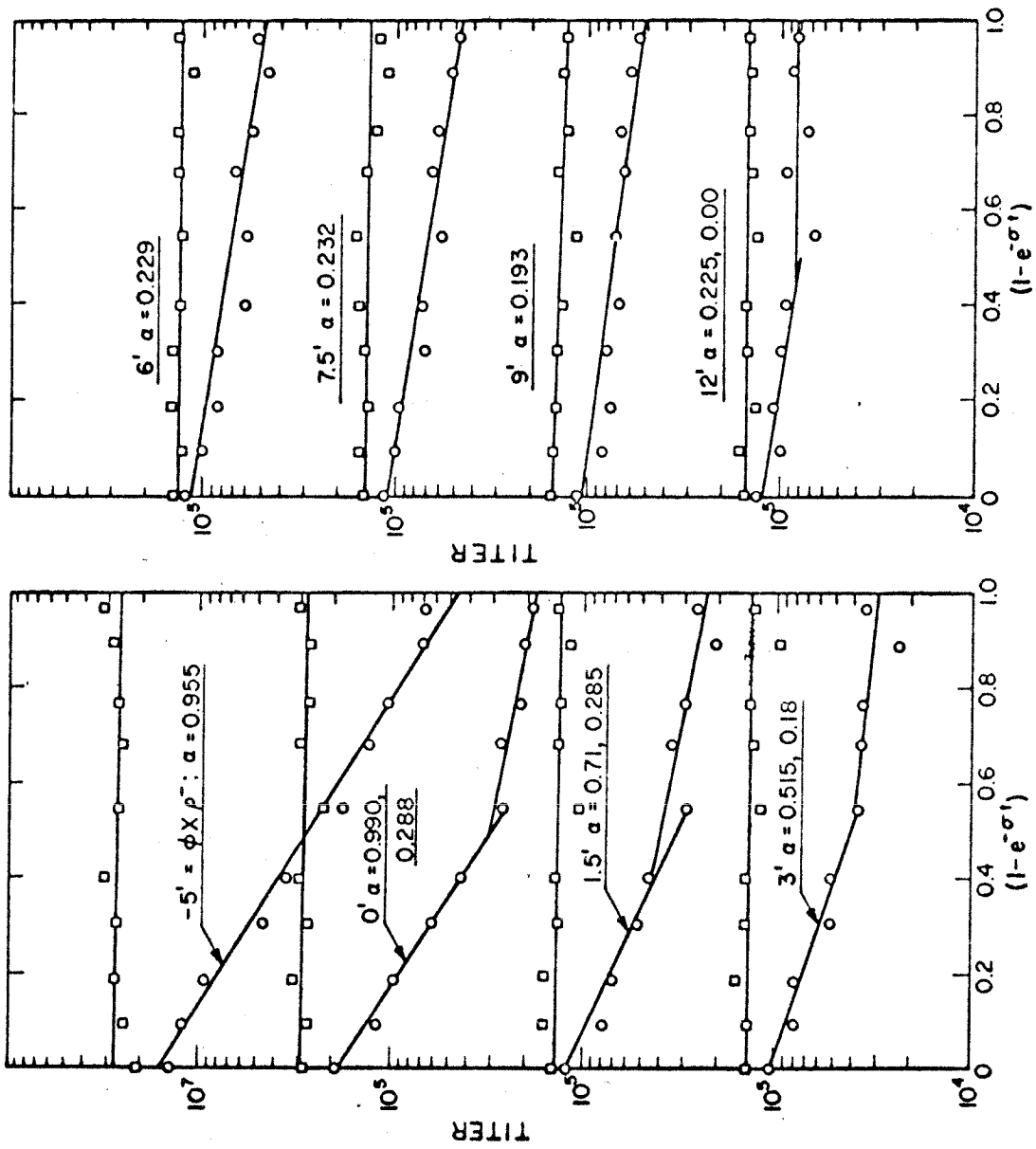
In some experiments, α for ϕX was observed to be 1, in others it deviated from 1 significantly. Deviations from 1 are presumed to result from an error in the determination of A. When the labeled SS ϕX DNA is incorporated in cold medium into a duplex RF DNA N is doubled, but, since A must then be halved, the product AN remains unchanged. For convenience, the actual titer rather than the surviving fraction is plotted vs. $(1-e^{-\sigma t})$; the product αAN is determined from the ratio of the titers at $t = \infty$ to the titer at $t = 0$.

Results

Suicide of Starvation Synchronized Complexes

The radiophosphorus inactivation curves of starvation synchronized E. coli C complexes formed with P^{32} labeled $\phi X-174p^-$ in cold medium are shown in figure 1, and the ages of the complexes are indicated together with values for α determined from the straight lines drawn through the points. Simultaneously obtained control inactivation curves of complexes formed with unlabeled phage are also shown; in practically all cases complexes formed with cold phage were stable when stored at -196°C in liquid nitrogen. Since purified phage preparations were used, the amount of beta radiation the cells received from radiophosphorus atoms was negligible.

Similar experiments done with wild type ϕX in E. coli C, E. coli C_s (see Part V), and E. coli CR (see Part III) have furnished equivalent results. Because mature ϕX complexes release phage more readily than ϕXp^- complexes, it has not been possible to determine the sensitivity of ϕX complexes to P^{32} suicide near the end of the eclipse period (9'-11') under normal conditions. Growth curves for all the P^{32} suicide experiments were determined and in all cases were normal; the heavily labeled phage did not contain sublethal damages that retarded development.



Legend to figure 1

P^{32} suicide inactivation curves of $\phi X-174\phi^-$ -E. coli C complexes at various stages of development. The ordinates for the complexes at each stage of development are displaced 1 log cycle, and the titers indicated refer only to the initial portion of the inactivation curve which originates in that cycle. The squares and circles represent the titers in the vials containing complexes formed with cold and hot phage respectively. Log phase E. coli C was starved for 75' in SB at 3×10^8 cells/ml and then divided into two portions. One portion was infected with 2×10^7 /ml purified P^{32} labeled $\phi X\phi^-$ in buffer, the other, with 2×10^7 /ml unlabeled $\phi X\phi^-$. After 5' prewarmed TKB was added; the eclipse period was 11' for both cultures.

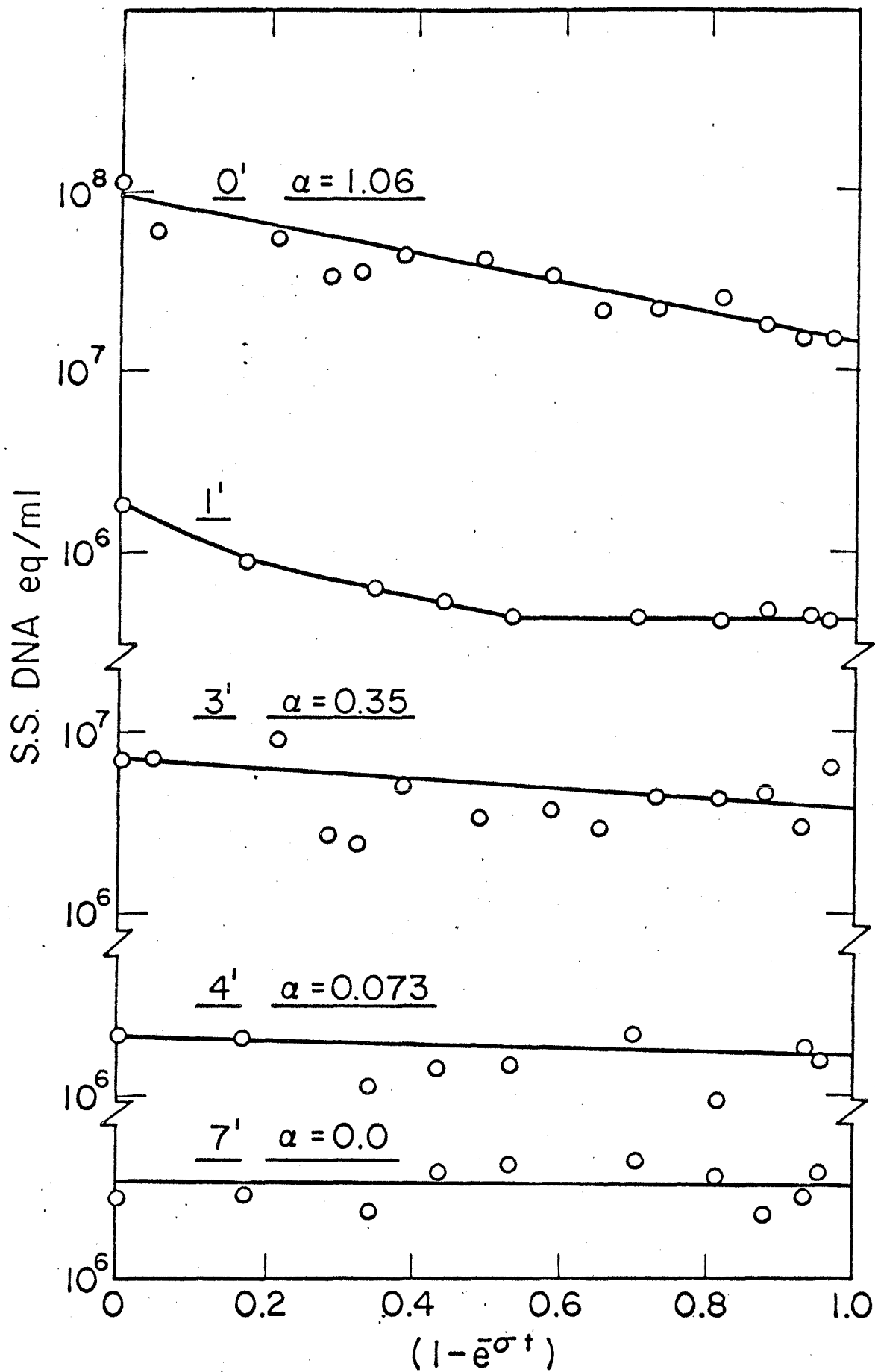
It might be argued that in a fraction of the infected cells RF has not replicated, and that it is the suicide of this fraction that is observed. If this is true, α for the sensitive fraction will be larger than that calculated by assuming an homogeneously sensitive population. The 6'-9' curves show that at least 50% of the population is sensitive to suicide and that this fraction remains fairly constant from 6'-9'; α for the sensitive fraction would be about 0.4 - much higher than any of the values shown in table 1. Furthermore, the experiments described in figures 7 and, especially, 8 of Part III indicate that more than half of the infected cells clearly do replicate their RF.

Suicide of ϕ X DNA in vitro

The complexes used in the suicide experiments shown in figure 1 were also used to provide the banding patterns for the intracellular DNA shown in figures 2 and 4 of Part III. The fractions indicated by the arrows in the 1', 4', and 7' patterns were pooled, dialyzed against 0.05 M tris, pH 8.1, distributed into vials, and frozen and stored in liquid nitrogen. DNA was similarly isolated from 0' and 3' complexes formed with heavily labeled wild type ϕ X, frozen, and stored at -20°C . The inactivation curves for all of these DNA samples are shown in figure 2. The important conclusions are that the DNA isolated from phage and early complexes is sensitive to suicide to about the same extent as the phage and the very early complexes, and that after 3-4 minutes of development the isolated, intracellular DNA is more resistant to inactivation than the complex from which it was isolated. The straight line drawn through the data for the 3' and 4' DNA's are to illustrate the amount of inactivation expected for particular values of α ; clearly, the data can also be fit in other ways. In all cases DNA isolated from complexes older than about 4' has not been detectably ($\alpha < 0.1$) sensitive to suicide.

Suicide of Cyanide Synchronized Complexes

The suicide of E. coli C- ϕ X-174 complexes synchronized using the cyanide method (Part II) is demonstrated



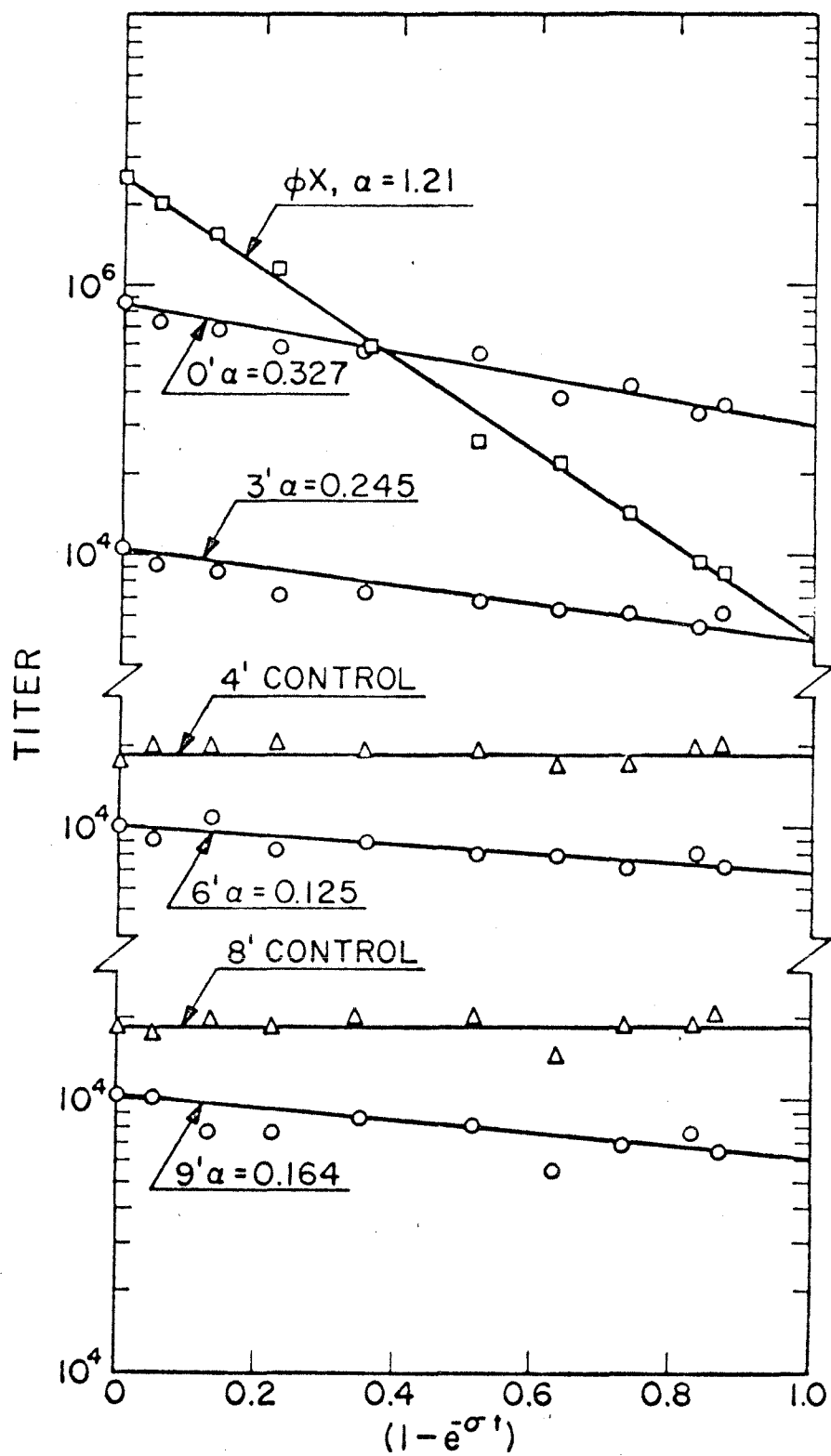
Legend to figure 2

The P^{32} suicide sensitivity of DNA isolated from cells at various times after infection with heavily labeled ϕX . The DNA was purified by banding in CsCl and stored frozen in small aliquots. Quantitation of the protoplast assay technique for ϕX -DNA (22) requires standardization against a known amount of purified single strand DNA; in all cases a sample of unlabeled ϕX DNA was stored with the P^{32} labeled DNA and assayed simultaneously with it as a standard. Although the efficiency of infection in the protoplast system is somewhat variable, there was no general trend up or down, indicating that the unlabeled DNA was stable during storage. The 0' and 3' DNA's from one experiment were stored at -20°C . The 1', 4', and 7' DNA's from another experiment were stored at -196°C . The efficiencies of P^{32} suicide indicated in the figure refer to the lines drawn rather arbitrarily through the points. The scatter in the points reflects the inability of the protoplast system to assay accurately the small amount of DNA present. Each data point is based on the average of duplicate assays at 2 or 3 dilutions of the DNA.

in figure 3. The 0' complex is more resistant than the 0' starvation synchronized complex, almost as resistant as the mature complexes. Independent evidence that development sufficient to form at least partly the first RF molecule can occur slowly in cyanide has been obtained from experiments of the type described in Parts III and V. The 9' complex is sensitive to inactivation by decaying radiophosphorus atoms, though less so than the corresponding starvation synchronized complex, especially when the free phage sensitivity is normalized to 1.0.

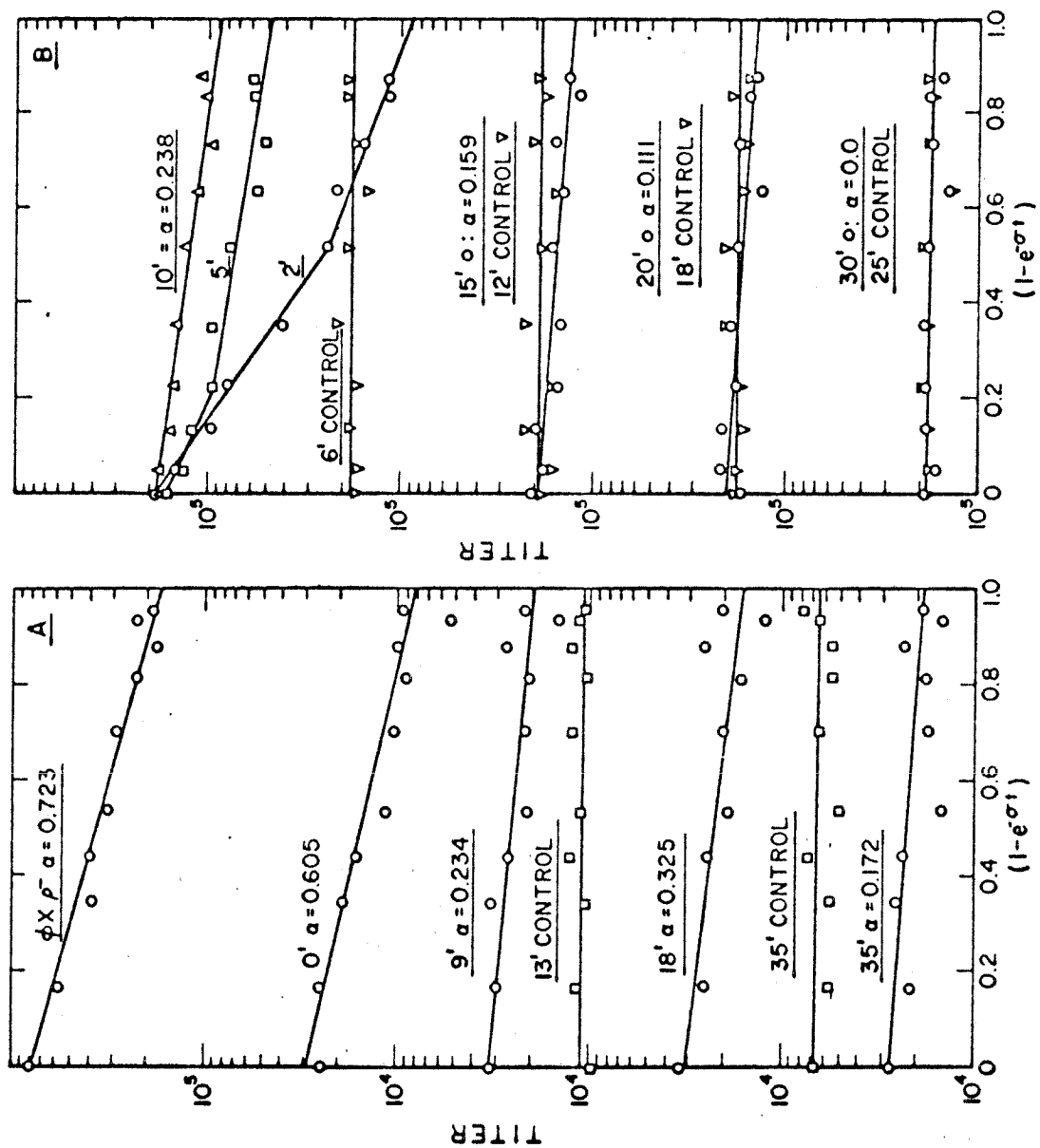
Development of Resistance to Suicide in Chloramphenicol

The development of resistance to P^{32} suicide can also occur in chloramphenicol. Figure 4 shows the results of two experiments, one done with $\phi X-174e^-$ in starved E. coli C (A), the other done with $\phi X-174$ in unstarved, log phase E. coli C (B). In the former many of the complexes appear to have remained sensitive to suicide with an efficiency of about 0.17 even after 35' in broth plus chloramphenicol. In the latter the complexes have become very resistant to inactivation by 20' and completely refractory to P^{32} suicide by 30'. The "growth curves" in both experiments were similar; the phage went into eclipse (better than 90% by 5') normally and intracellular phage were not made.



Legend to figure 3

Inactivation by radiophosphorus decay of cyanide synchronized ϕ X-E. coli C complexes. The ordinates have been displaced as in figure 1. The circles and triangles represent the inactivation of complexes formed with hot and cold phage respectively, and the squares indicate the suicide of the phage used in this experiment. Cyanide was added to a log phase culture of E. coli C at 1.5×10^8 /ml to give a final concentration of 0.003 M. The culture was divided into two parts; to one part P^{32} labeled ϕ X was added, to the other, unlabeled ϕ X. The multiplicities were about 0.07 for both. Five minutes after adding the phage, both cultures were filtered and resuspended in fresh TKB. Aliquots from both cultures were taken at various times for suicide and for determination of the growth curve; the eclipse period for both cultures was 10 minutes.



Legend to figure 4

A. P^{32} suicide of $\phi X \rho^-$ complexes formed in starvation synchronized E. coli C in the presence of chloramphenicol. The squares and circles refer to samples taken from cultures infected with cold and hot phage respectively. TKB grown log phase cells were starved for 60' in SB. Chloramphenicol was added to 30 mg/l to the starved cells 3' before the phage; broth was added 5' after the phage. P^{32} labeled $\phi X \rho^-$ and unlabeled $\phi X \rho^-$ were used in parallel aliquots at multiplicities of about 0.05. The ages of the labeled and control complexes are indicated. The ordinates are displaced as described in figure 1.

B. P^{32} suicide of ϕX complexes formed with log phase E. coli C in chloramphenicol. The downward pointing triangles refer to samples from the control culture infected with cold phage; all other symbols refer to the culture infected with P^{32} labeled phage. Chloramphenicol was added to a log phase culture of bacteria at 1.4×10^8 /ml in TKB to a concentration of 30 mg/l. The culture was divided into two parts; one part was infected with P^{32} labeled ϕX , the other, with unlabeled ϕX , both at multiplicities of about 0.08.

Discussion

The most important conclusion from these experiments is that the complex is sensitive to the decay of radio-phosphorus atoms carried only in the infecting single strand in spite of the fact that considerable replication of the RF has occurred. The replication of the RF is well established (23, Part III) and is supported by the observation that the RF DNA isolated from mature complexes near the end of the eclipse period is insensitive to P^{32} suicide. Evidence was cited in Part III that many of the supposed replicas of the parental RF differ physically from it in some way. It would seem that although the progeny RF DNA molecules are able to infect protoplasts and cause the production of mature ϕX , they are unable to perform a necessary late function in the infected cell - a function that only the parental RF can perform. A similar situation may also obtain for superinfecting T2 DNA synthesized in the presence of chloramphenicol (18, 19) and for the RNA of poliovirus (20). In the normal ϕX infection the production of progeny single strands seems to correlate closely with the onset of complete refractoriness to P^{32} suicide.

At least one of the progeny RF's can under certain circumstances achieve the status of the parental RF; this result was only observed in unstarved cells in chloramphenicol after 15-20 minutes of development. Why starved

cells infected with $\phi X \rho^-$ in chloramphenicol did not also become refractory to P^{32} suicide is not known. Evidence does exist that starved cells have a lower participation number than unstarved cells (24, 25), and this observation can be interpreted as indicating a fewer number of "centers" (see Part V) for phage synthesis in starved cells as compared with unstarved cells.

The efficiency of inactivation, α , of the mature complexes, although variable, is similar to the efficiencies listed in table 1. Thomas (11) observed that double strand breaks were produced in T2 DNA with an efficiency of about 0.2. That the reduced efficiency of suicide of most of the double stranded DNA phages as compared with the single stranded DNA viruses may not be the result of the host cell reactivation system (discussed in detail in Part V) that is present in E. coli C and absent in E. coli C_s is suggested by the observation that complexes made with E. coli C_s become as resistant (at least within 10-20%) to suicide as complexes made with E. coli C; however, it is possible that in this system single strand breaks in the ϕX strand of the parental RF are not lethal anyway. Rörsch et al. (26) found that the non host cell reactivating strain E. coli B_{syn}⁻ did not have a reduced ability to plate T1 undergoing P^{32} suicide. Although there is a repair mechanism present in T4 (u gene (27) or v gene (28)) and absent in T2, both T2 and T4 are inactivated

similarly (3). However, neither a small reduction in α by the host cell reactivating mechanism nor other types of repair mechanisms active on P^{32} damages in double stranded DNA are excluded.

Matheson and Thomas (29) discovered that at 4°C 2.8% AET (a sulfhydryl radical trapping agent described in Part V) reduced the efficiency of P^{32} suicide of T4D by about 40%. Stent and Fuerst (2) showed that the temperature at which the double stranded DNA phages were stored during inactivation affected the value of α ; α for T5_{st} (a heat stable mutant of T5) at 65°C was about 0.3, α 's measured at -196°C were about 40% smaller than at 4°C .

There is excellent evidence (23, Part III) that the radiophosphorus atoms brought into the mature ϕX complex by the infecting phage are contained in a single polynucleotide strand associated with a complementary strand of DNA to form a duplex RF molecule; that this DNA is apparently inactivated with an efficiency similar to double stranded DNA's randomly labeled with P^{32} lends support to Stent and Fuerst's (2, 3) model. Since both the temperature at which the duplex DNA is stored and the medium in which the duplex DNA reposes can affect in unknown ways the probability that a phosphorus transmutation is lethal, it is not yet possible to attach any significance to the small differences in the values of α observed. Two other points to be considered in any

detailed interpretation of P^{32} suicide efficiencies are the possible existence of mechanisms that can repair P^{32} damages and the fact that transcription occurs off only one of the strands of a duplex molecule.

The behaviour of ϕX complexes in P^{32} suicide experiments is very different from that of the other virus-cell complexes (except possibly for poliovirus (20)) studied so far. The sensitivity to P^{32} suicide of ϕX complexes begins to decrease immediately after infection, even in the presence of chloramphenicol. The sensitivity to P^{32} suicide of the T phage complexes begins to decrease significantly only after a lag of 3-6 minutes (2, 3); no decrease is observed in the presence of chloramphenicol (12). Furthermore, the degree of stabilization observed in the T phage system is very much larger than that observed in the ϕX system. These differences are probably intimately connected with the single vs. double stranded nature of the DNA of these viruses.

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PART V

AN ANALYSIS OF \emptyset X DEVELOPMENT WITH
MONOCHROMATIC ULTRAVIOLET LIGHT

Introduction

The goal of radiobiological studies on phage infected cells has long been the elucidation of the mechanism or mechanisms of virus replication. Unfortunately, for the most part, this goal has been thwarted by the complexity of the effects of radiation on the cell. If virus reproduction were simply the multiplication of virus particles, for example by binary fission, then target theory predicted that an initially single hit curve representing the inactivation of the infected cell's ability to produce virus progeny would develop a shoulder with an ever increasing back extrapolate as the complex developed. Such results were not in general obtained (2, 3). The experiment of irradiating the infected cell at different stages of development was conceived by T. Anderson (4) and performed by S. Luria and R. Latarjet (2), and consequently curves so obtained are called Luria-Latarjet (L-L) curves. Up to the present the L-L curves obtained for virus systems have not been particularly informative because they were not amenable to any simple explanation; they seemed instead to represent the summation of a complex set of events whose nature was mysterious. In a review written in 1959 (5) Stahl characterized the subject of phage radiobiology as "leading no one knows where."

Recently several advances in our knowledge about the

action of ultraviolet light on DNA, and of the cell's response to irradiation and to irradiated DNA, have renewed hope that significant information could be obtained from the L-L curves. One breakthrough was the finding by Beukers and Behrends (6, 7) that thymine was dimerized by ultraviolet light under certain conditions; one such condition was its incorporation into DNA. Wacker et al. (8) isolated thymine dimers from DNA irradiated in vivo and showed (9) a correlation between thymine dimerization and bacterial survival. Rupert (10, 11) discovered a photoreactivating enzyme that in the presence of light could nullify a fraction of the uv lesions in transforming DNA. Wulff and Rupert (12) showed that the enzyme could probably split thymine dimers. Using the fact that the dimerization of thymine by uv is a photochemically reversible reaction (13) which favors the dimer (80%) at 275 mμ and the monomer (90%) at 235 mμ, Setlow and Setlow obtained evidence that thymine dimers were a cause of uv damage in cells (14) and, furthermore, that the photoreactivable lesions in DNA were predominantly thymine dimers (15). There are, however, still some difficulties with the in vitro identification of the thymine dimer with the substrate for Rupert's photoreactivating enzyme (16).

While the thymine dimer was being discovered and its role in uv photobiology unraveled another inquiry was also proving very informative. This research stemmed both from

the observations by Greer and Zamenhof (17) and Greer (18) that forcing cells to use 5-bromouracil (Bu) instead of thymine increased their uv sensitivity and from the discovery by Hill (19) of an uv sensitive mutant of E. coli B, E. coli B_s. Opara-Kubinska et al. (20) found that Bu substitution sensitized both the bacterial cells and the DNA isolated from them (both before and after irradiation), to about the same extent. Stahl et al. (21) found that T2 substituted with Bu was sensitized to uv, especially at longer wavelengths extending even into the region emitted by daylight fluorescent lights. They also observed that irradiated Bu substituted DNA was neither photoreactivable nor u-gene reactivable (Streisinger (22) showed that the difference in uv sensitivity between T2 and T4 resulted from the presence in T4 of a gene, the u gene, that could cancel a fraction of the uv lesions). Ellison, Feiner, and Hill (23) demonstrated that when T1, T3, and T7 (but not T2, T4, and T5) were assayed on E. coli B_s they appeared to be more sensitive to uv than when they were assayed on E. coli B.

Harm (24), Howard-Flanders et al. (25, 26), Rörsch et al. (27, 28), and Sauerbier (29-31) investigated the nature of the sensitization resulting from both Bu incorporation into DNA and genetic mutation; the result is that there are enzyme systems able to repair a large fraction of the uv damages in normal DNA, but only a small fraction, if

any, under most conditions in Bu substituted DNA. This repair phenomenon is called host cell reactivation (HCR). There are at least two different reactivating systems in both T4 (32) and E. coli (27) that act additively to varying extents. Different phage host systems behave in different ways: irradiated T1 is reactivated by only a fraction of the population of log phase E. coli K12 or E. coli C, whereas irradiated lambda is reactivated by all of the cells (24). Harm (33) has written a review on the repair of uv damages. Evidence has accrued recently that thymine dimers are excised from DNA by at least one of the HCR systems (34, 35). The finding by Haug (36) that TpBu dimerizes by forming a cyclobutene ring in contrast to the cyclobutane ring formed by TpT may explain why Bu substituted DNA is not subject to much HCR after uv irradiation.

Concurrently with the enlightenment occurring in the field of phage radiobiology, knowledge was accumulating concerning the events taking place during bacteriophage replication. The following summary of T2 replication is abstracted from Stent (37). Soon after the phage DNA has entered the cell, leaving its protein coat behind, part or all of it is read by host cell enzymes to make messenger RNA, which then synthesizes, again using the host cell system, T2 specific "early proteins." This takes about 5 minutes at 37°C and is followed by the synthesis of T2 DNA. More T2 messenger RNA, distinct from that made

earlier, appears and codes for the synthesis of "late" T2 specific proteins. Beginning around the 10th minute T2 DNA and protein condense to form mature T2 particles.

The uv sensitivity of T2 complexes, studied by Luria and Latarjet (2) and Benzer (3), is approximately constant and single hit for about the first 5' after infection and then decreases rapidly from 6'-12'. Elegant experiments by Symonds and McCloy (38) showed that during the first 5' after T2 infection an increase in uv resistance occurred if photoreactivation was prevented, but not if it was permitted. Thymine dimers located in a critical cistron (one that must function before DNA replication can ensue) presumably can prevent transcription, and hence function, of that cistron; photoreactivation would revert these dimers.

Symonds and McCloy (38) defined two types of lesions: "functional" damages that occurred only in critical cistrons and prevented their function, but not replication; "genetic" damages that occurred anywhere and blocked DNA replication. Reactivable thymine dimers could be a cause of functional damage. Genetic damages can be functional (Krieg, 39) and may result from non-reactivable uv lesions.

Simon (40) discovered that the onset of DNA synthesis was directly correlated with the increase in resistance occurring after 5'. Symonds (41) obtained evidence that the increase in resistance which accompanies the synthesis of T2 DNA results from the ability of the T2 DNA molecules

to recombine with each other, at least once the necessary enzymes have been formed.

Many other virus-cell systems have been studied radiobiologically, but in no case have the inactivation curves been explained. Benzer's (3) L-L curves for T7 and Matsubara et al.'s (42) L-L curves for ϕ X are remarkably similar; both sets of curves are characterized by the development of a shoulder on an initially single hit curve with only a small (1x-3x) decrease in the limiting slope. Friesen et al. (43) found a like result with vaccinia virus. Powell (44) investigated the resistance of L cells infected with Herpes Simplex virus to monochromatic uv and found that the complex initially became more sensitive to uv than the free virus and then more resistant. The inactivation curves in all cases appeared single hit and the action spectra derived were nucleic acid like. Kaplan (45) found single hit inactivation curves for ERK cells infected with Pseudorabies virus; however, he did not follow the inactivation curves for the complexes at later stages of development beyond about 0.5 survival. Bertani (46) has studied the L-L curves of the temperate phage P2, and virulent mutants of it, in Shigella dysenteriae; the system appears to be very complicated. Tessman and Ozaki (47) investigated the interaction of S13 with irradiated host cells.

Among the RNA viruses, Rous Sarcoma virus (48),

Newcastle Disease Virus (48), and Poliovirus (49-51) have been studied radiobiologically. For the last, single hit curves of gradually decreasing slope are observed. The results of the Poliovirus studies were interpreted by Dulbecco (49) as indicating one or two "centers" of virus development. Fenwick and Pelling (50) showed that the increase in uv resistance of Poliovirus infected cells occurred when viral RNA synthesis was blocked with high temperature, but did not occur when RNA and protein synthesis were blocked with puromycin. Tershak (51) found that prior infection with one strain of Poliovirus did not confer increased uv resistance upon a second superinfecting strain whose synthetic abilities were held in abeyance by puromycin.

Epstein (52), like Dulbecco, has postulated the existence of cellular phage synthesizing centers to explain his results with T1, T2, and T3. He provided evidence that the centers contained nucleic acid and that there were 3 to 5 for T2 (depending upon the host), but only 1 for T1 and T3.

Setlow (53) observed that the minimum in the absorption spectrum of double stranded DNA (T2, thymus DNA) was near 230 m μ , whereas the minimum in the absorption spectrum of the pyrimidines and ϕ X virus was nearer to 240 m μ . Action spectra for T2 inactivation, ϕ X inactivation, and thymus DNA gelling had minima similar to their absorption minima

(53, 54). The action spectrum for the inactivation of native transforming DNA appeared to have a minimum near 238 m μ and for denatured transforming DNA, near 232 m μ (55). From the minima observed in action spectra for T2 complexes (56) and ϕ X complexes (57) Setlow inferred that T2 DNA went through a "disordered" state during part of the eclipse period, while ϕ X DNA went through an "ordered" state. Two serious objections to the reasoning used by Setlow to arrive at his conclusions about events occurring during ϕ X infection are: 1) The minimum in the absorption spectrum of ϕ X DNA is at 230 m μ (58). 2) The amount of data published (57) is sparse and does not unambiguously support his interpretation.

The investigation described below was undertaken primarily to deduce, hopefully, some facts about ϕ X replication, and, secondarily, to learn more about the effects of uv on cells. A study of this type on a phage containing a single strand of DNA has not been reported, and, because of the single strand nature of the viral DNA, it is possible to do some types of experiments that cannot be done with the double stranded DNA viruses. The plan of the work was to perform a variety of different kinds of experiments such that only a single simple interpretation consistent with all of the known effects of ultraviolet light on cells and DNA was possible. Preliminary results of this work have been reported (59, 60).

Materials and Methods

ØX-174, E. coli C, synchronization techniques, and general materials and methods were described in Part II. Bu-ØX, γ h-ØX, E. coli C₁, E. coli CR, and the methods for handling them were described in Part III.

Escherichia coli C_s is a mutant of E. coli C isolated by Dr. R. P. Boyce that will not host cell reactivate T1. It was obtained through the generosity of Dr. P. Howard-Flanders.

T7 was kindly provided by Dr. Robert Edgar.

Lambda and Bu-Lambda (fully substituted) were generous gifts from Dr. Jean Weigle.

Cysteamine.—2-Mercaptoethylamine·HCL, B grade, was obtained from Cal. Biochem. It was freshly prepared in SB before use.

Ultraviolet Irradiation.—The quartz prism monochromator used in these experiments has been described in detail by Rapaport (61, 62) and Winkler, Johns, and Kellenberger (63). Ultraviolet light from an air cooled General Electric high pressure mercury vapor lamp (BH6) was focused on the entrance slit by a quartz lens. The monochromator made use of two crystalline quartz lenses and prisms in a Thollon-Young mounting (64). Table 1 of the paper by Winkler et al. (63) lists the characteristics of this instrument; since a precise determination of absolute cross sections was not the aim of this work,

slit widths slightly larger than those described were used when convenient. The error introduced in this way was partly offset in most cases by doing the irradiations at wavelengths corresponding to mercury lines.

A volume between 1.9 and 3.9 ml was irradiated in a standard 1x1x4.4 cm Beckman quartz cuvette situated immediately behind an 0.5 cm^2 aperture placed 4 cm behind the exit slit so that none of the radiation struck the sides of the cuvette. A stirring paddle in the solution, but out of the beam, continually mixed the sample. The radiation was monitored with a calibrated (61-63) S-5 RCA photocell in conjunction with a model PH-200 Eldorado photometer (Eldorado Electronics Co., Berkeley, Calif.). At the completion of all of the experiments reported here, the calibration was checked (using a malachite green leucocyanide actinometer generously provided by Dr. Heinrich Peter) and found to be within 10% of the original calibration. A further check on the technical aspects is contained in the agreement between ϕX cross sections obtained in this work and ϕX cross sections determined independently (54, 65). Corrections for the optical density of the solution were made as described by Morowitz (66). Corrections for the volume removed for sampling were routinely made.

The dose in ergs/mm^2 delivered to the sample was calculated by multiplying together the following terms:

1) the average of the photometer readings taken immediately before and after the irradiation; 2) the number of seconds of irradiation; 3) the correction factor for the optical density (in all cases these corrections were small enough so that they were accurately approximated by $\frac{1}{2}(1.0 + \text{observed transmittance})$); 4) the reciprocal of the total volume of solution in the cuvette; 5) a calibration number relating the photometer reading to the light intensity.

It was assumed that over the range of intensities used in these experiments there was no dependence of the inactivation kinetics upon the intensity of the radiation; a variation in the intensity by a factor of 2 at 231 m μ and 265 m μ did not affect the observed cross section. Some typical intensities in ergs/mm²/sec delivered to the solution were: 231 m μ , 2.5-5.0; 240 m μ , 5.5-7.5; 248 m μ , 6.0-7.5; 258 m μ , 7.5-11.5; 265 m μ , 4-11; 280 m μ , 8-10; 289 m μ , 10-12; 297 m μ , 11-14. In some experiments, where the absolute value of the cross section was not of interest, wider slits, with concomitant higher intensities, were used. In particular, at 297 m μ the intensity was increased to 30-45 ergs/mm²/sec, and, in order to measure this intensity, the sensitivity of the photometer was reduced by $\frac{1}{2}$ and a neutral density filter (a piece of ordinary screen blackened by heating and provided by Michael Yarus) was inserted over the photocell surface causing a further twofold reduction in the sensitivity.

When irradiating infected bacteria, the plating for the surviving infective centers was performed immediately after the irradiation. In all cases precautions were taken to prevent photoreactivation. Complexes at various stages of development were prepared by starvation synchronization; growth curves were routinely determined, and samples from the infected culture were diluted 1/50 or 1/100 into SB for irradiation.

Capacity Determinations.—The bacteria were suspended in SB at a concentration of 2×10^8 /ml together with MS-2 bacteriophage at about 10^8 particles/ml. MS-2 and its host, E. coli C₃₀₀₀, were generously supplied by Mr. James Strauss; MS-2 neither plates on nor adsorbs to E. coli C, C_s, or CR, and ϕ X does not plate on E. coli C₃₀₀₀. Irradiations were performed as described and an "uncorrected dose" calculated by omitting the optical density correction factor. The correction factor was determined from a comparison of the MS-2 survival in the bacterial suspension with the survival determined in buffer; it ranged from 0.4 to 0.6.

The capacity of the irradiated cells to support ϕ X growth was measured in the following way: An 0.1 ml aliquot from the cuvette was placed in a 1.0 ml volumetric kept in ice. After completion of all the irradiations, 0.1 ml of an appropriate phage stock in SB was added to each aliquot, still on ice. The entire set of volumetrics was then

incubated in a 37°C water bath for 5' (to allow phage adsorption) and returned to the ice bath. Forthwith, 0.2 ml of TKB containing 0.006 M KCN and ØX antiserum with a K of 6 (to inactivate unadsorbed phage) was added to each volumetric. The set of volumetrics was incubated for another 5' at 37° and again returned to the ice bath. The assays for infective centers in the volumetrics were performed as rapidly as possible under yellow lights.

Calculations and Presentation of Data.—The inactivation curves are plotted semi-logarithmically as surviving fraction vs. dose in ergs/mm². Data that can be fit by a straight line on such a plot are compatible with the hypothesis that there is a single structure, the target, that can be inactivated (hit) by a single photon (1, 67). Although the simplest situation occurs when the straight line includes the origin, linear portions of more complicated curves (concave upwards or downwards) may also be interpreted in terms of target theory (1), particularly if the reason for the shape is known. Curves with a shoulder followed by a linear portion ("multi-hit curves") result when several hits are required to inactivate the target or when the radiation alters the environment of the target in such a way as to increase its sensitivity; the linear portion following the shoulder may be considered a simple single hit type of curve.

The statistics of the distribution of hits in a

population can be described by the Poisson approximation (68); in particular, the zero term, e^{-H} , yields the surviving fraction. The average number of hits, H , is related to the dose delivered and the probability that the target be hit. If the dose is expressed in terms of the number of photons/cm² then the probability that the target be hit must be expressed (in order to keep the exponent dimensionless) in units of cm², the cross section. When an average of one hit per target has been delivered to the population, 0.37 (e^{-1}) of the targets survive. Hence the cross section of the target can be simply calculated as the reciprocal of the dose (in photons/cm²) required to inactivate 63% of the population. The "one hit dose," D_0 , in ergs/mm² is converted to cm²/photon by dividing $h\nu$ by $100D_0$; h is Planck's constant, and ν is the frequency of the radiation employed.

The probability that a target be hit (e. g. inactivated) is not only a function of the physical size of the target but also a function of the ease with which the photon interacts (absorbed, usually) with the target. As the energy of the bombarding photons varies, the probability of interaction varies in a manner described by the absorption spectrum. If it is assumed that the probability that an absorbed photon inactivates the target (quantum yield) does not vary strongly with wave length, then a plot of the observed cross section vs. wavelength (an action spectrum)

should resemble the absorption spectrum of the target.

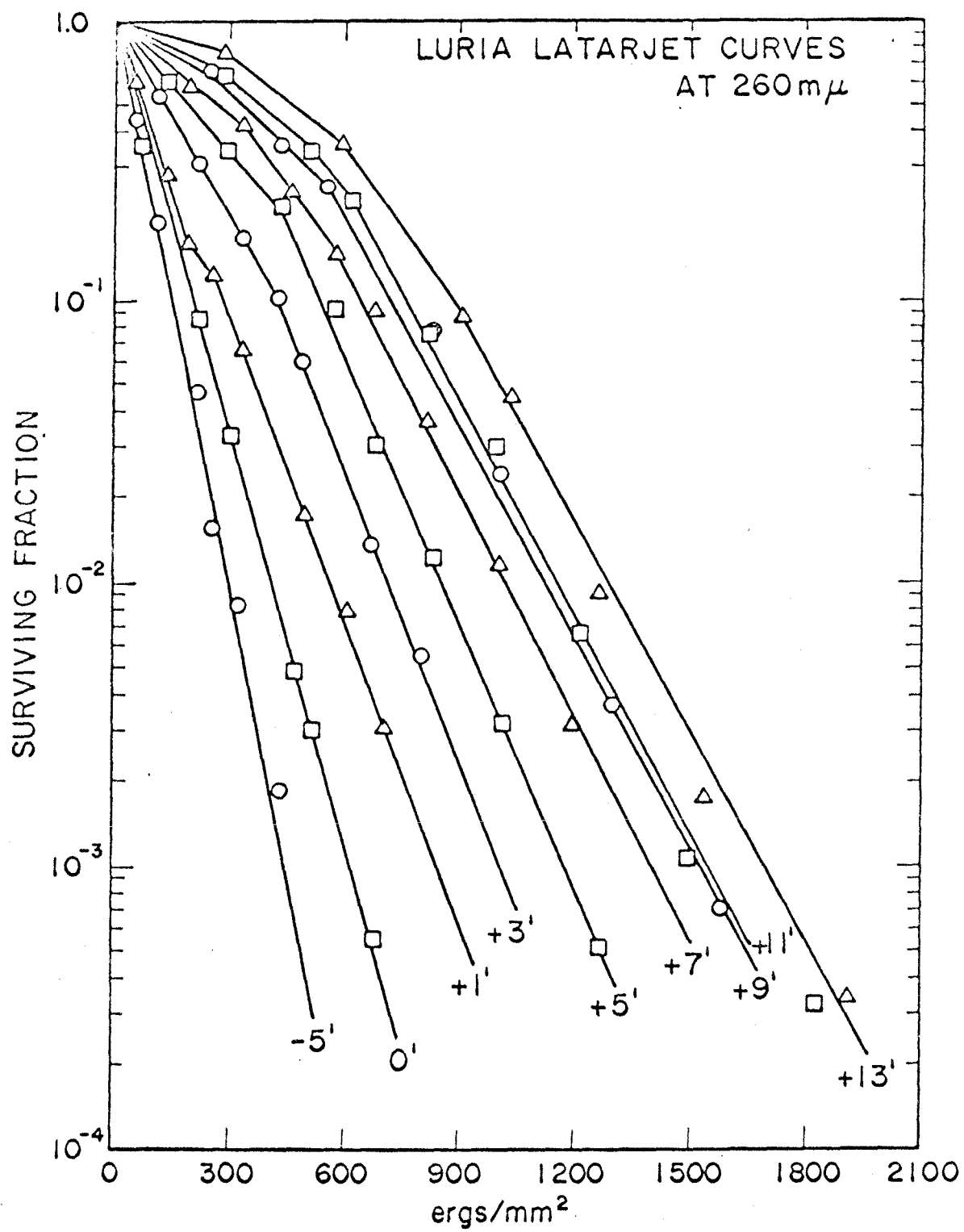
The above assumption, approximate invariance of the quantum yield with wavelength, is usually good (67).

Results

Luria-Latarjet Curves in E. coli C and E. coli C_s

A set of Luria-Latarjet (L-L) curves at 260 mμ obtained for ØX-174 in E. coli C is shown in figure 1. There are two important aspects (59) of these curves: 1) the large shoulder, which develops very rapidly and may be characterized by the number obtained by extrapolating the linear portion observed at higher doses back to zero dose; 2) the limiting slope, from which a cross section for the hypothetical target may be calculated. Representative back extrapolates and cross sections obtained at several wavelengths for complexes at various stages of development are listed in table 1. The significance of the (somewhat erratic) dependence of the back extrapolate on the wavelength of the radiation and the age of the complex is not understood. The cross sections calculated from the limiting slopes of the inactivation curves of complexes at various stages of development decrease during the first three to eight minutes of development and then remain approximately constant. Complexes which have reached this stage of development are called mature. The L-L curves develop in an identical fashion in the presence of chloramphenicol and remain constant after 5-10 minutes, even though RF is still replicating (fig. 5, Part III).

When E. coli C_s is used as the host, the set of L-L



Legend to figure 1

Inactivation curves at 260 m μ of starvation synchronized ϕ X-E. coli C complexes. The age of each complex is indicated on the curves. The data of several experiments are plotted, and the multiplicities of infecting phage were in all cases less than 0.1. These experiments were done in collaboration with Michael Yarus.

Table 1: The Characteristics of the L-L Curves of Low Multiplicity Starvation Synchronized Complexes Formed with E. coli C and E. coli C_S.

age in min.	back extra- polate	cross section x 10 ¹⁶ cm ²	age in min.	back extra- polate	cross section x 10 ¹⁶ cm ²
<u>235 mμ - C - 7/20/62</u>			<u>240 mμ - C_S - 9/29/63</u>		
0	1	4.50	0	1.0	3.89
3	4	3.25	6	3.0	1.73
7	45	3.00	12	9.5	1.96
11	160	3.20			
<u>258 mμ - C - 8/26/63</u>			<u>254 mμ - C_S - 9/29/63</u>		
0	1	5.80	0	1.0	7.84
5	5	4.20	4	1.0	2.67
10	14	4.10	8	2.6	2.82
			12	9.0	3.15
<u>260 mμ - C - 7/19/62</u>			<u>258 mμ - C_S - 8/26/63</u>		
0	1	7.30	0	1.0	9.40
5	6	5.50	5	1.0	4.00
9	13	4.70	10	4.5	4.00
13	18	4.40			
<u>265 mμ - C - 2/24/64</u>			<u>265 mμ - C_S - 10/5/63</u>		
0	1	7.60	0	1.0	9.00
2	18	5.80	1.5	1.0	6.40
4	10	4.30	3	1.0	4.80
6	14	4.20	4.5	1.0	4.10
8	24	4.60	6	3.0	4.30
10	30	4.40	9	5.5	4.30
			12	2.8	4.30
<u>289 mμ - C - 1/19/63</u>			<u>270 mμ - C_S - 9/29/63</u>		
0	1	2.36	0	1.0	7.93
5	6	1.76	6	2.4	3.88
10	20	1.56	12	3.0	3.82

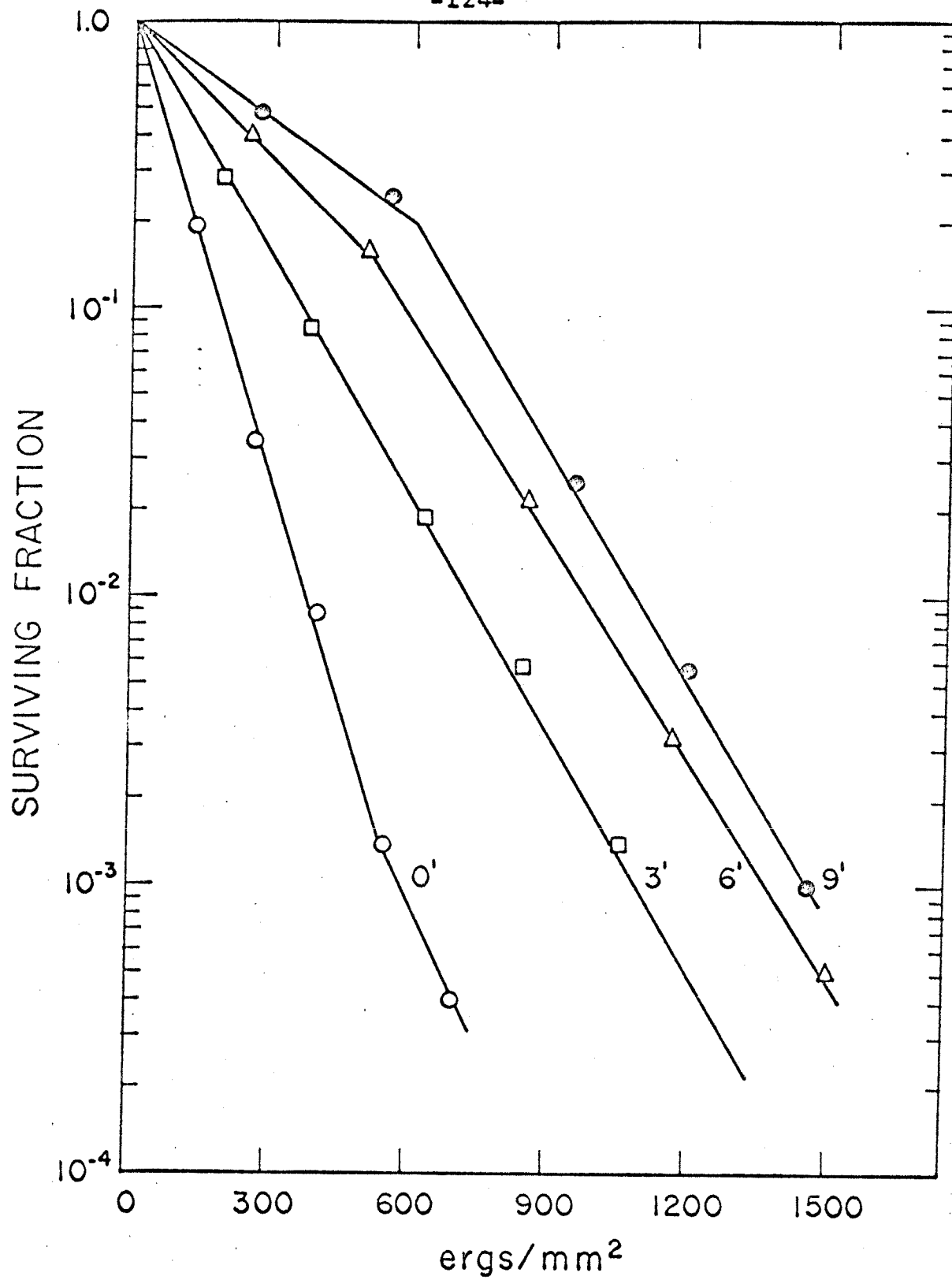
Legend To Table 1

Representative back extrapolates and cross sections derived from the limiting slopes of the L-L curves.

curves shown in figure 2 is obtained. At all wavelengths the shoulder is much reduced and does not appear until after 5-7 minutes of development. Cross sections calculated from the limiting slopes of the inactivation curves of mature \underline{C}_s complexes are similar to those derived from \underline{C} complexes, and the growth curves of ϕX in \underline{C} and \underline{C}_s are identical. The 0' \underline{C}_s complexes are usually a little more sensitive than 0' \underline{C} complexes. Typical cross sections and back extrapolates are listed in table 1.

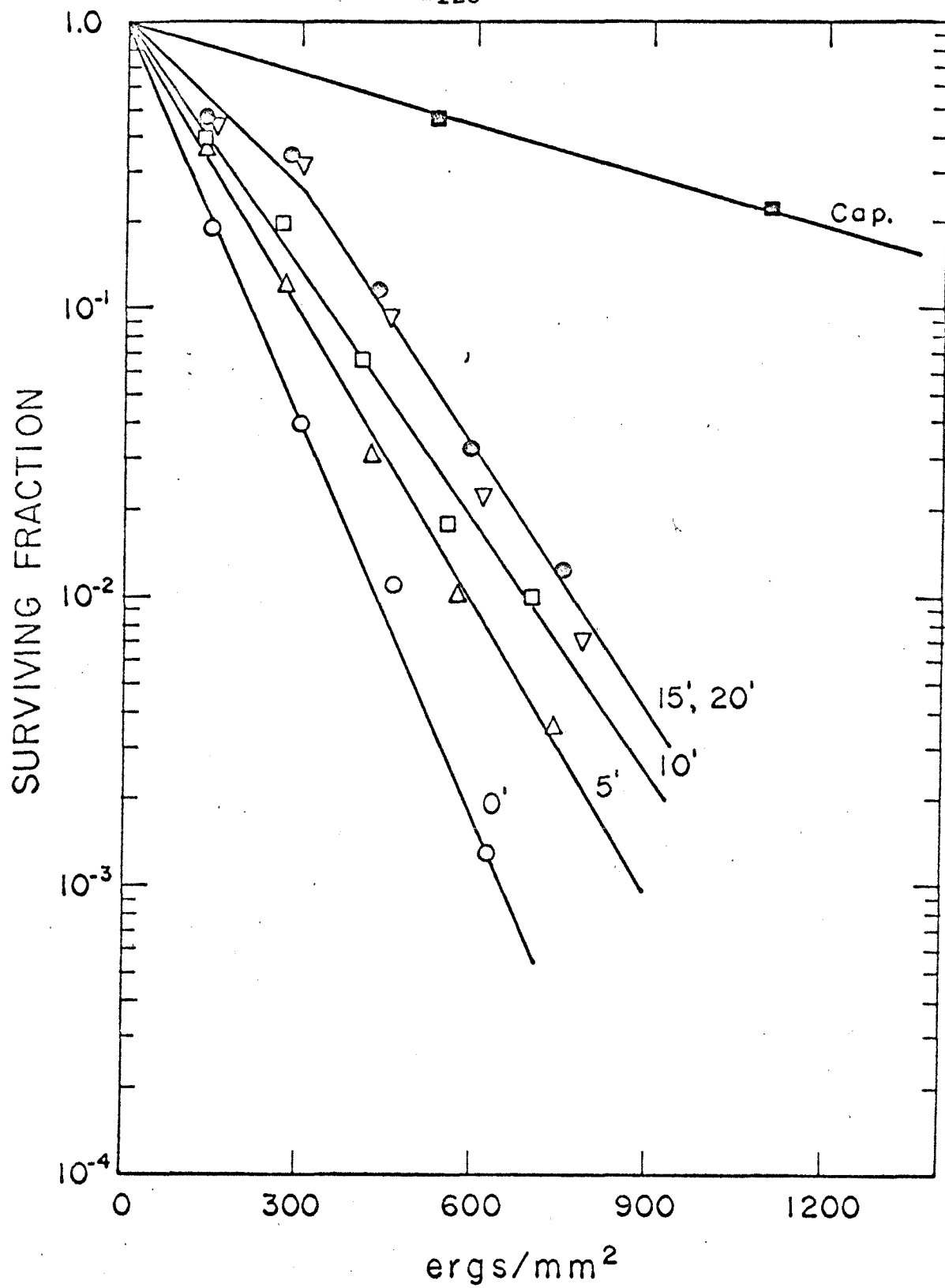
When irradiated E. coli \underline{C} is used as the host the L-L curves plotted in figure 3 result. Approximately 93% of the irradiated cells were unable to support ϕX growth; hence the L-L curves derived only from the remaining 7% of cells. These cells were unable to form colonies, and the growth of ϕX in them was delayed; the eclipse period was 18', the minimum latent period was 22', and the average burst size was 10. Here again the shoulder is greatly reduced while the limiting slope is not affected.

An experiment was designed to determine whether or not the uv dose necessary to attain the limiting slope of the L-L curves was necessary and sufficient to block host cell reactivation of lambda (24). Starved E. coli \underline{C} were irradiated with several doses of 254 m μ uv from a germicidal lamp and used as host cells for the assay of irradiated λ . It was observed that a dose of about 1000-1200 ergs/mm² was necessary and sufficient to prevent host cell



Legend to figure 2

Inactivation curves at 265 mμ of starvation synchronized ØX-E. coli C_s complexes. The ages of the complexes are indicated on the curves; the multiplicity of infection was 0.12.



Legend to figure 3

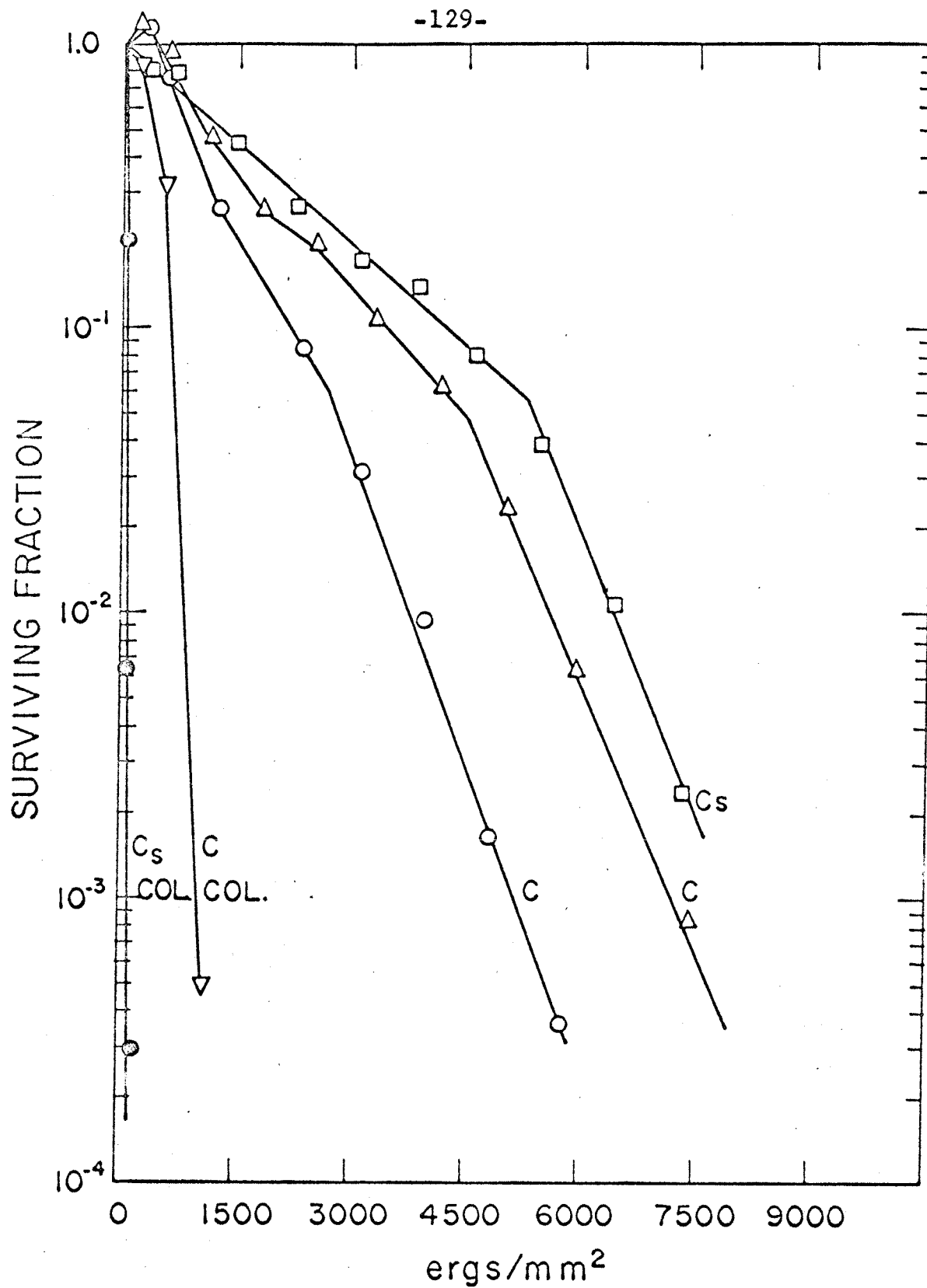
Inactivation curves at 265 mμ of starvation synchronized ØX complexes formed with irradiated E. coli C. The cells were given 2930 ergs/mm² to reduce their capacity (see below) to 0.072 survival, and then infected with a multiplicity of 0.5 phage per pre-irradiation colony former. The characteristics of the growth curve of the phage in the surviving cells are described in the text. The ages of the complexes are indicated on the curves. The uv sensitivity of the capacity of the surviving cells to support phage growth is also shown; the two points plotted plus several lying off the graph all fell on a good straight line through the origin.

reactivation of uv damages in λ ; at 254 m μ a dose of about 1150-1350 ergs/mm² is required to get off the shoulder of the L-L curves of the mature complex.

The UV Sensitivity of the Capacity of E. coli C and C_s

The ability, or capacity, of a cell to support phage growth can be destroyed by uv irradiation of the cell prior to infection; inactivation curves of this type will be called "capacity curves." Capacity curves obtained at 265 m μ for ϕ X in both E. coli C and C_s are shown in figure 4 together with inactivation curves of the colony forming ability of these strains. Although the colony forming ability of E. coli C_s is very much more sensitive to uv than the colony forming ability of E. coli C, the capacity curves are not very dissimilar. Two independently obtained capacity curves for E. coli C are shown to illustrate the amount of variation between experiments. The limiting slopes of all three capacity curves are, within experimental error, identical. The apparent activation of the capacity of E. coli C at low doses and the shoulder at around the 15% survival level obtain in all experiments done at wavelengths above 240 m μ .

Most of the capacity curves of E. coli C_s determined at wavelengths above 240 m μ can be described by two straight lines as indicated in figure 4. At wavelengths below 240 m μ the nature of the inactivation curves,



Legend to figure 4

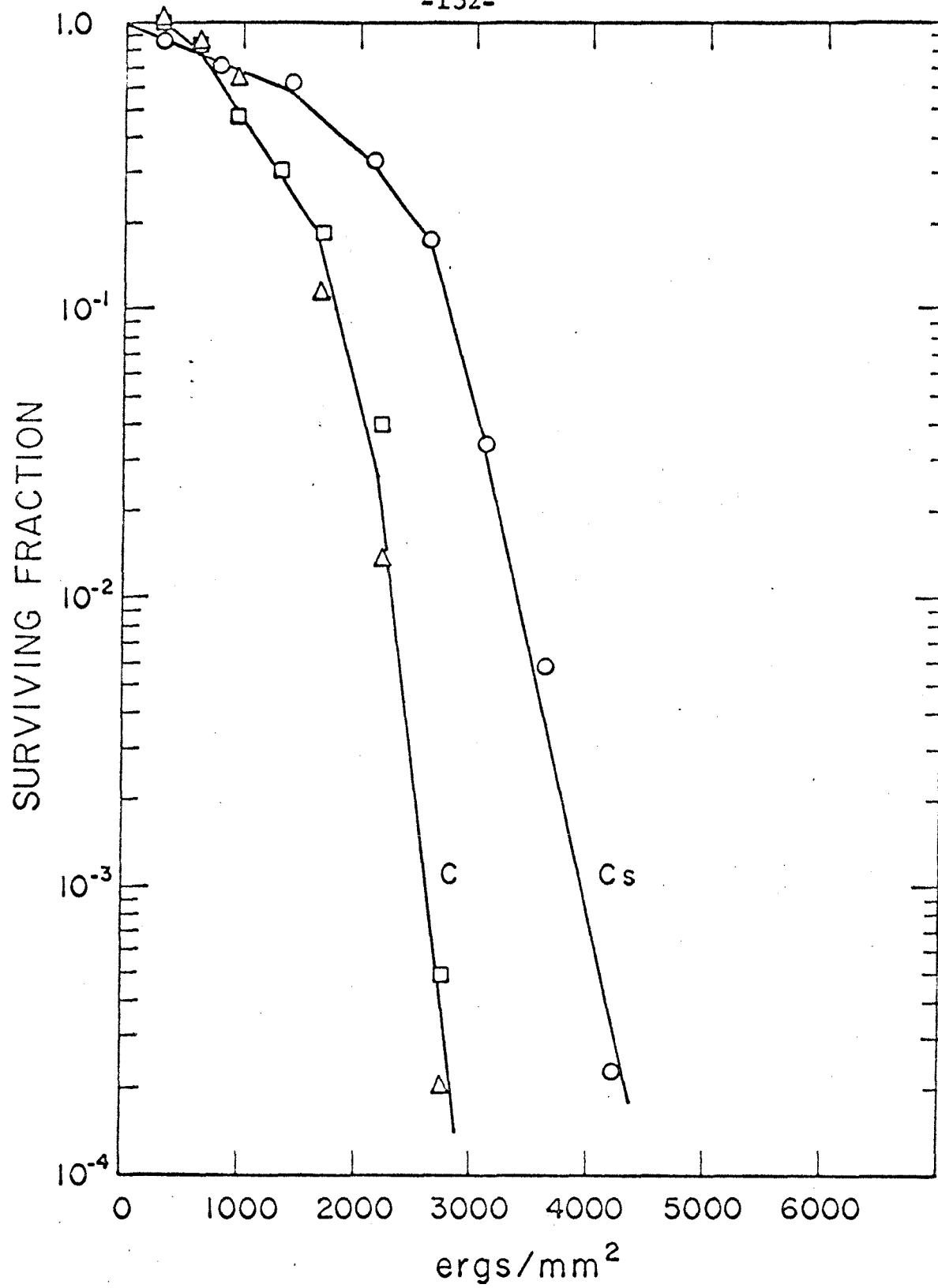
Inactivation curves of the colony forming ability and capacity of starved E. coli C and starved E. coli C_s at 265 mμ. The irradiated cells were infected with ØX-174 at a low multiplicity as described in Materials and Methods. The two curves shown for E. coli C were determined independently. The number of colony formers was determined by plating the irradiated cells in top agar and incubating them in the dark for about 36 hours. The capacity of unstarved cells has not been examined; however, addition of broth to starved cells did not change the capacity curves for at least 12'.

figure 5, changes; both \underline{C} and \underline{C}_s give rise to similar curves, curves which have a large shoulder at the origin and a steep limiting slope. The back extrapolate for both \underline{C} and \underline{C}_s varies considerably between experiments. Above 240 mμ it is, within an order of magnitude, 20; below 240 mμ it increases rapidly to 500 and higher.

The capacity curves discussed so far have been measured with a low multiplicity of phage. At 231 mμ capacity curves measured at high and low multiplicities of infection are identical (fig. 5). At 258 mμ the differences are that there is no activation of the capacity at low doses (fig. 6), and the shoulder may be more pronounced.

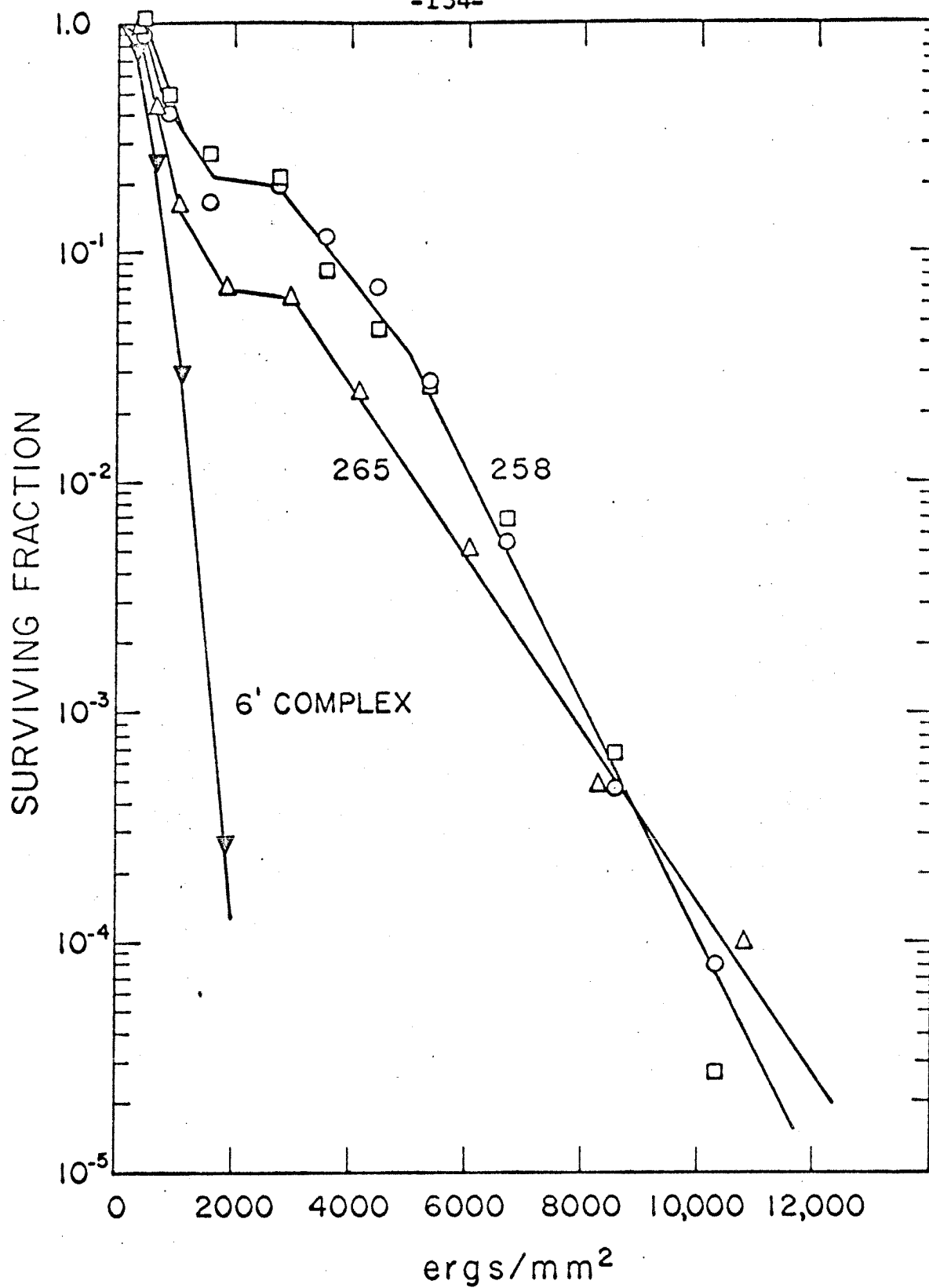
The kinetics of the inactivation at 265 mμ of the ability of infected cells to support phage growth is shown in fig. 6. Evidence is presented in the legend that it was the capacity of the infected cells that was measured. The uv sensitivity of the capacity of the 6' complex to support the growth of a superinfecting phage was essentially identical to the uv sensitivity of the capacity of the uninfected cells. Also shown in fig. 6 is the L-L sensitivity of the 6' complex used.

In a similar experiment using phage of two different genotypes it was possible to confirm that the capacity of infected cells is actually unchanged after infection and to determine the origin of the progeny phage. Starved cells (*E. coli* \underline{C}_s) were infected with wild type ϕX , allowed to develop, irradiated, and superinfected with γh . Since ϕX



Legend to figure 5

Capacity inactivation curves of starved E. coli C_s at 236 mμ (circles) and of starved E. coli C at 231 mμ (squares) measured with low multiplicities (0.1) of phage; the capacity of a second aliquot of the 231 mμ irradiated C suspension was also determined with a high multiplicity (10) of phage (triangles).



Legend to figure 6

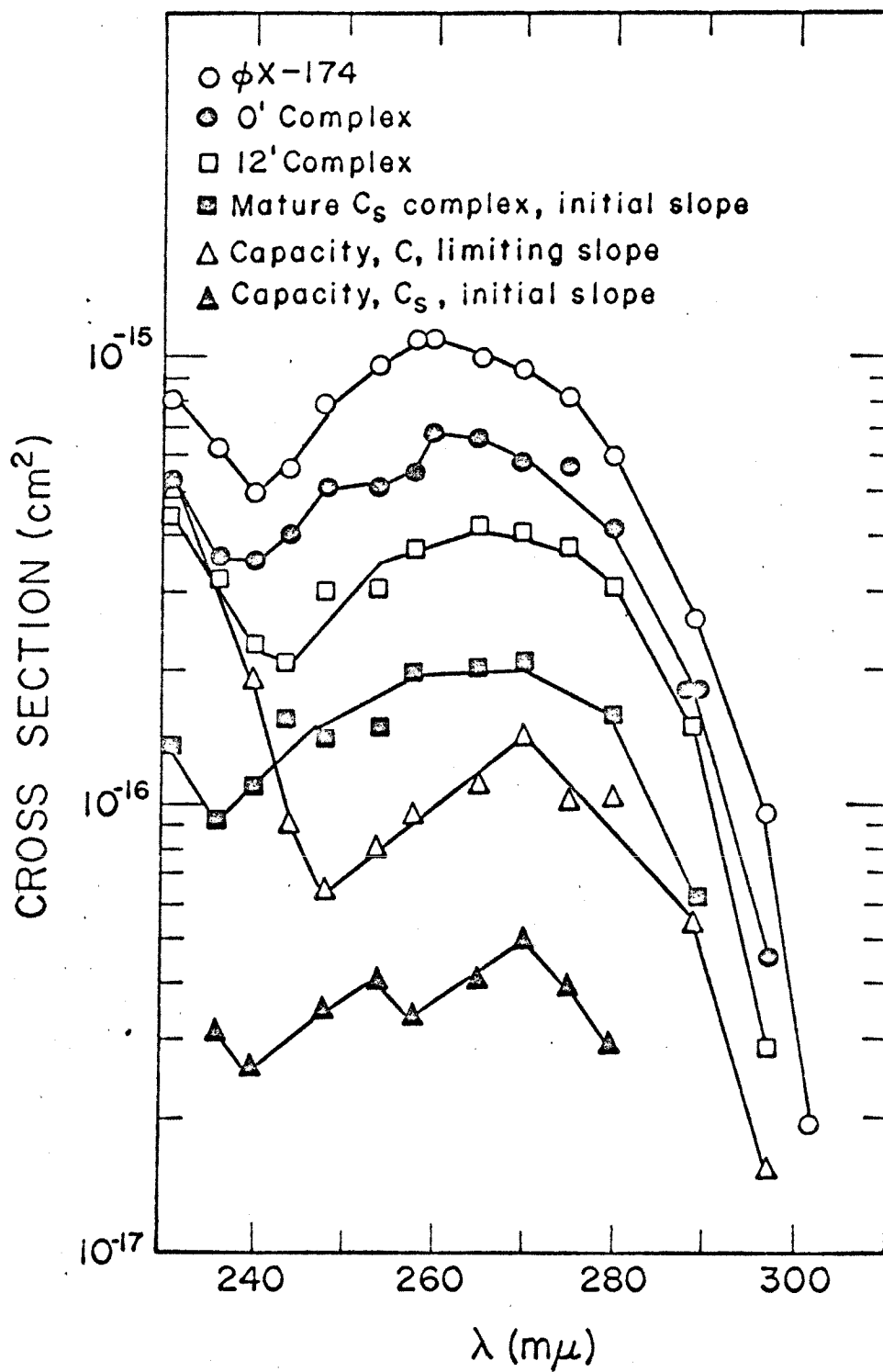
The inactivation curves at 258 mμ of the capacity of E. coli C measured with low (0.06) and high (5.1) multiplicities (squares and circles respectively) of phage; the L-L curve of a 6' E. coli C starvation synchronized complex (solid triangles), and the capacity (to support the growth of a superinfecting phage) curve for that same complex (open triangles), both at 265 mμ. In this latter experiment cells at 3.5×10^8 /ml were infected with 2×10^9 phage/ml. The complexes were allowed to develop for 6', filtered, washed, and resuspended in cold SB. The titers of infective centers and surviving bacteria in the resuspended sample were 1.8×10^8 /ml and 1.9×10^7 /ml respectively. After irradiation and superinfection with 7×10^8 phage/ml the infective titer in the 0 dose sample was 9.4×10^7 /ml. All titers are referred back to the volume in the original infection tube. A loss of infective centers after handling or after high multiplicity infections is routinely observed; what happens to them is not known, except that they do not reappear as colony formers. It cannot be argued that only the capacity of uninfected cells was measured. If only 20% (1.9×10^7 bacteria/ml) of the 9.4×10^7 infective centers/ml (20% is the maximum value, 5% is undoubtedly a more realistic figure) were "superinfectable" then at a dose of 1000 ergs/mm², where 97% of the infective centers have been inactivated, the capacity survival (17%) would have been, but was not, 1/5 the survival (20%) observed at this dose for the uninfected cells.

plates on \underline{C} , but not on \underline{C}_1 , at both 30° and 40° , and γh plates on both \underline{C} and \underline{C}_1 at 30° , but not at 40° , it is possible by incubating the plates for 3 hours at 30° and then for 3 hours at 40° on a mixture of \underline{C} and \underline{C}_1 to determine whether the infected cells release only ϕX , only γh , or both. The decline in the ability of the irradiated superinfected complexes to release ϕX followed normal L-L kinetics, and the results clearly showed that once the ability to release ϕX had been destroyed, γh , and only γh , was produced.

Action Spectra of the UV Sensitive Structures

From the slopes of the inactivation curves at various wavelengths, cross sections have been calculated. The action spectra derived in this way are shown in figure 7. The cross sections for 0' *E. coli* \underline{C} complexes are usually 25-50% smaller than the ϕX cross sections, even though the target is believed to be the SS DNA in both cases. Several possible reasons for this are: 1) shielding; 2) change in the configuration of the DNA; 3) change in the environment of the DNA; and 4) an effect of the uv on the cell that cancels some of the uv lesions in the single stranded DNA.

This last reason is possibly related to the phenomenon of "ultraviolet reactivation" (UVR) (24). UVR refers to the process by which a small dose of uv delivered to the cells often increases their ability to plate irradiated phage. Strains that cannot perform HCR also have



Legend to figure 7

The action spectra of ϕX , ϕX complexes formed with E. coli C and C_s, and the capacity of E. coli C and C_s. Because the C_s complexes varied in age from 8' to 12', the action spectrum is designated as representing mature C_s complexes; although the data is not extensive (see table 3), there did not seem to be any significant change in the cross section between 8' and 12'.

a reduced ability to perform UVR, and it has been suggested (24) that UVR is just an enhancement of HCR. This explanation is not entirely satisfactory, however, because ϕX is subject to UVR, but not HCR (see below). The UVR sector (the fractional reduction in the observed cross section brought about by the reactivation process) of ϕX on \underline{C} is about 0.2 and overlaps with at least half of the photo-reactivable sector, which is about 0.15-0.2; the UVR sector of ϕX on \underline{C}_s is at least 0.13. The fact that cross sections obtained from 0' \underline{C}_s complexes (see table 1) are only about 10-20% smaller than the free virus cross sections may indicate that part of the observed reduction in the cross sections of 0' \underline{C} complexes is the consequence of a reactivation mechanism.

The cross sections obtained from the limiting slopes of the 12' complexes are from 20-40% smaller than the 0' \underline{C} cross sections. Cross sections calculated from the initial slopes of the inactivation curves of both mature (8' or older) \underline{C}_s complexes and the capacity of \underline{C}_s are also plotted in figure 7. Finally, the cross sections derived from the limiting slopes of the capacity curves in E. coli \underline{C} are shown. All of the data from which the action spectra in figure 7 are derived are contained in tables 2 and 3.

Most of the action spectra more or less resemble the absorption spectra of nucleic acid, except that derived from the limiting slope of the capacity curves which has

Table 2: Cross Sections of ϕ X-174 and O' E. coli C Complexes.

Wave-length (m μ)	Values ($\times 10^{16}$ cm 2)	Average ($\times 10^{16}$ cm 2)
<u>ϕX-174</u>		
231	9.1, 7.0, 8.1, 7.7, 7.4, 7.8	8.0
236	6.6, 5.8, 6.7	6.3
240	6.9, 6.4, 5.2, 5.4, 6.1	5.0
244	5.7, 5.4	5.6
248	8.6, 8.2, 8.0, 6.8	7.9
254	13, 11, 9.4, 7.4, 8.9, 8.3	9.6
258	12, 9.3, 9.2, 9.6, 12, 11, 10, 11, 11	11.0
260	11, 11, 9.1	11.0
265	10, 11, 12, 9.6, 9.0	10.0
270	9.2, 9.4, 8.9, 10	9.3
275	8.4, 8.7, 8.4, 8.2, 8.3, 7.4	8.2
280	5.8, 5.6, 6.4, 6.6, 6.4	6.1
289	1.8, 2.5, 3.1, 3.1	2.6
297	0.81, 1.2, 0.82	0.94
302	0.19	0.19
<u>O' Complex - E. coli C</u>		
231	5.1, 5.5, 5.4, 5.3	5.3
236	3.9, 3.0, 4.0	3.6
240	3.1, 3.8, 3.6, 3.0, 4.0	3.5
244	4.0	4.0
248	4.4, 6.2, 4.7	5.1
254	4.9, 5.9, 5.3, 4.3	5.1
258	5.9, 5.8, 4.5, 5.8	5.5
260	8.2, 6.9, 5.3	6.8
265	6.2, 7.6, 6.6, 6.1	6.6
270	5.9, 5.8	5.8
275	4.8, 6.8, 5.7	5.7
280	3.9, 4.1, 4.3, 4.2, 4.4	4.1
289	1.6, 1.5, 2.1, 2.0	1.8
297	0.46, 0.40, 0.49	0.45

Legend to table 2

Cross sections determined for ØX-174 and for O' starvation synchronized E. coli C complexes. Both inactivation curves are simple exponentials. These cross sections were among the first determined and were obtained at a stage when familiarity with the vagaries of the monochromator was still being acquired. Slightly different slit widths and prism settings account for some of the variation observed. In any one experiment when the settings of the monochromator are not altered between irradiations much greater reproducibility is easily achieved.

Table 3: Cross Sections for Mature Complexes and Capacities

mpu	Values (x 10 ¹⁶ cm ²)	Aver- age	mpu	Values (x 10 ¹⁶ cm ²)	Aver- age
<u>Limiting Slope - 12' C</u>			<u>Limiting Slope - C - Capacity</u>		
231	4.1, 4.7	4.4	231	4.4, 5.4, 5.5, (7.0)	5.1
236	3.2, 3.3, 3.2,	3.2	236	4.5, 2.7, (3.4)	3.6
240	2.3, 2.3, 2.3	2.3	240	1.9, (1.3)	1.9
244	2.1	2.1	244	0.90	0.90
248	2.9, 3.0	3.0	248	0.66, 0.61	0.64
254	3.4, 2.8	3.1	254	0.99, 0.61, (0.84)	0.80
258	4.0, 3.5, 4.1	3.8	258	1.0, 0.97, 0.82, (1.2)	0.93
265	3.8, 4.6	4.2	265	1.2, 0.95, (1.1, 0.72)	1.1
270	3.7, 4.6	4.1	270	1.4, 1.3	1.4
275	3.8, 3.8	3.8	275	1.0, 1.0	1.0
280	3.1, 3.4, 2.8	3.1	280	1.0, (0.67)	1.0
289	1.2, 1.8	1.5	289	0.54	0.54
297	0.35, 0.22	0.28	297	0.19, 0.11, 0.15	0.15
<u>Init. Slope-mature C_s</u>			<u>Init. Slope - C_s - Capacity</u>		
231	1.4, 1.3	1.4	236	0.31	0.31
236	0.90, 0.96, 0.89	0.92	240	0.26	0.26
240	1.2, 1.0	1.1	248	0.34	0.34
244	1.6	1.6	254	0.40, 0.40	0.40
248	1.4	1.4	258	0.33	0.33
254	1.5	1.5	265	0.4, 0.38	0.39
258	1.8, 2.2	2.0	270	0.49	0.49
265	1.9, 2.0	1.95	275	0.39	0.39
270	2.1	2.1	280	0.29	0.29
280	1.6	1.6			
289	0.62	0.62			

Legend to table 3

Cross sections obtained from the limiting slopes of the inactivation curves of 12' E. coli C (starvation synchronized) complexes and of the capacity of E. coli C, and cross sections determined from the initial slopes of the inactivation curves of mature (8'-12') starvation synchronized E. coli C_s complexes and of the capacity of E. coli C_s. Some cross sections obtained from the limiting slopes of the inactivation curves of the capacity of E. coli C_s are listed in parenthesis in the list of capacity cross sections for E. coli C.

a distinct maximum at 270 m μ , a minimum at 248 m μ . The action spectrum obtained from the initial slope of the C_s capacity curves is based on scant data (table 3) and is included only to indicate the general sensitivity of the capacity to low doses of uv.

Below 240 m μ the limiting slope cross sections obtained for the inactivation of capacity are identical, within experimental error, to the limiting slope cross sections obtained for the 12' complexes. L-L curves of the mature complex and the capacity curves of the uninfected cell determined at wavelengths below 240 m μ , particularly with E. coli C_s , are virtually superimposable at uv doses sufficient to attain the limiting slopes of these curves; the capacity is more resistant at low doses. If the same target is being detected in the limiting slopes of both L-L and capacity curves at wavelengths below 240, then it follows that the structure (or structures) identified by the action spectrum of the limiting slope of the capacity curves is required in the mature complex and hence is probably not a phage adsorption site. This possibility has not been tested directly; however, in addition, it is known (69) that the SS DNA of an infecting ϕ X particle can be incorporated into RF DNA in cells whose capacity to support ϕ X growth has been inactivated by uv.

The action spectrum derived from the limiting slopes of the inactivation curves of the colony forming ability

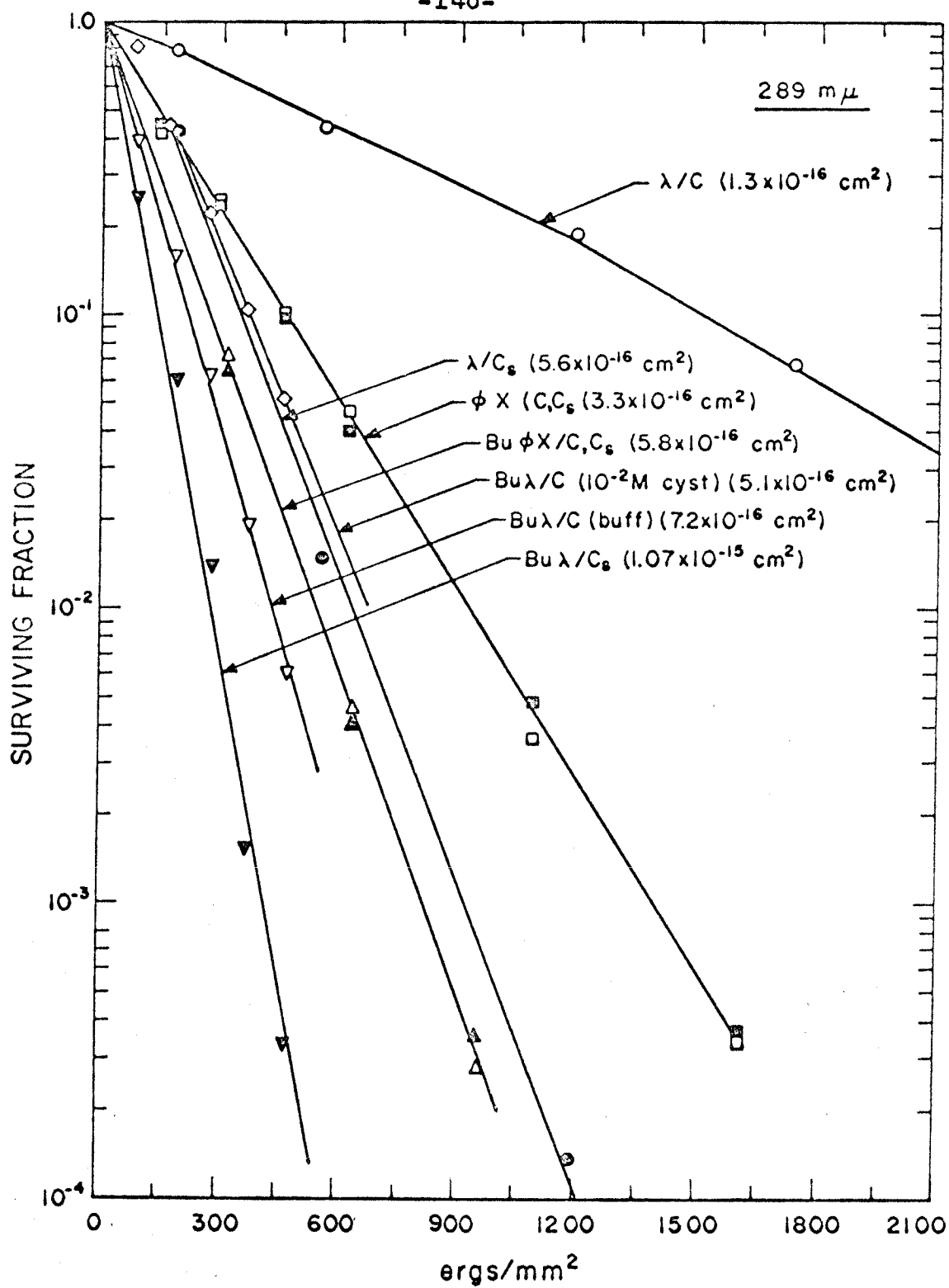
of E. coli C has been roughly determined and is approximately like that of the 0' and 12' complexes.

The action spectrum of MS-2 has been determined from its single hit inactivation curves; the ratio of the MS-2 cross sections to the ϕ X cross sections from 231 m μ to 297 m μ is about 0.11-0.13 and does not seem to vary significantly with wavelength.

The cross sections for Bu- ϕ X have been determined at several wavelengths and are approximately as follows, in units of 10^{-16} cm²: 10 at 235 m μ , 12 at 260 m μ , 13 at 270 m μ , 9 at 280 m μ , 5 at 289 m μ , 2.5 at 297 m μ , and 1.7 at 302 m μ . The action spectrum maximum is shifted to slightly longer wavelengths in comparison with the ϕ X action spectrum, and the sensitization is most pronounced at longer wavelengths. Bu- ϕ X is not photoreactivable.

Sensitization Resulting from Budr Incorporation

The data presented in figure 8 establish the following points: 1) the host cell reactivating system present in E. coli C and absent in E. coli C_s is able to repair a large fraction (HCR sector = 0.74) of the otherwise lethal uv lesions in λ , but not in ϕ X; 2) substitution of the thymine in the DNA by 5-bromouracil increases the sensitivity not only of λ assayed on E. coli C but also of λ assayed on E. coli C_s and ϕ X assayed on both C and C_s; 3) Bu- λ irradiated in the presence of 10^{-2} M cysteamine and



Legend to figure 8

The uv sensitivity at 289 mμ of ØX, Bu-ØX, λ , and Bu-λ (irradiated both in buffer and buffer plus 10⁻² M cysteamine) determined on both E. coli C (open symbols) and E. coli C_s (solid symbols). The curves are labeled in the figure, and the cross sections listed are derived from the limiting slopes.

That λ assayed on C_s is only about 1.7x more sensitive to uv than ØX is intriguing since λ contains 18x as much DNA as ØX. RF assayed on a non host cell reactivating strain (74) is about ½ as sensitive to uv as the SS DNA; hence λ is 3-4x as sensitive to uv as RF even though it contains 9x as much DNA. The resolution of this paradox remains to be discovered.

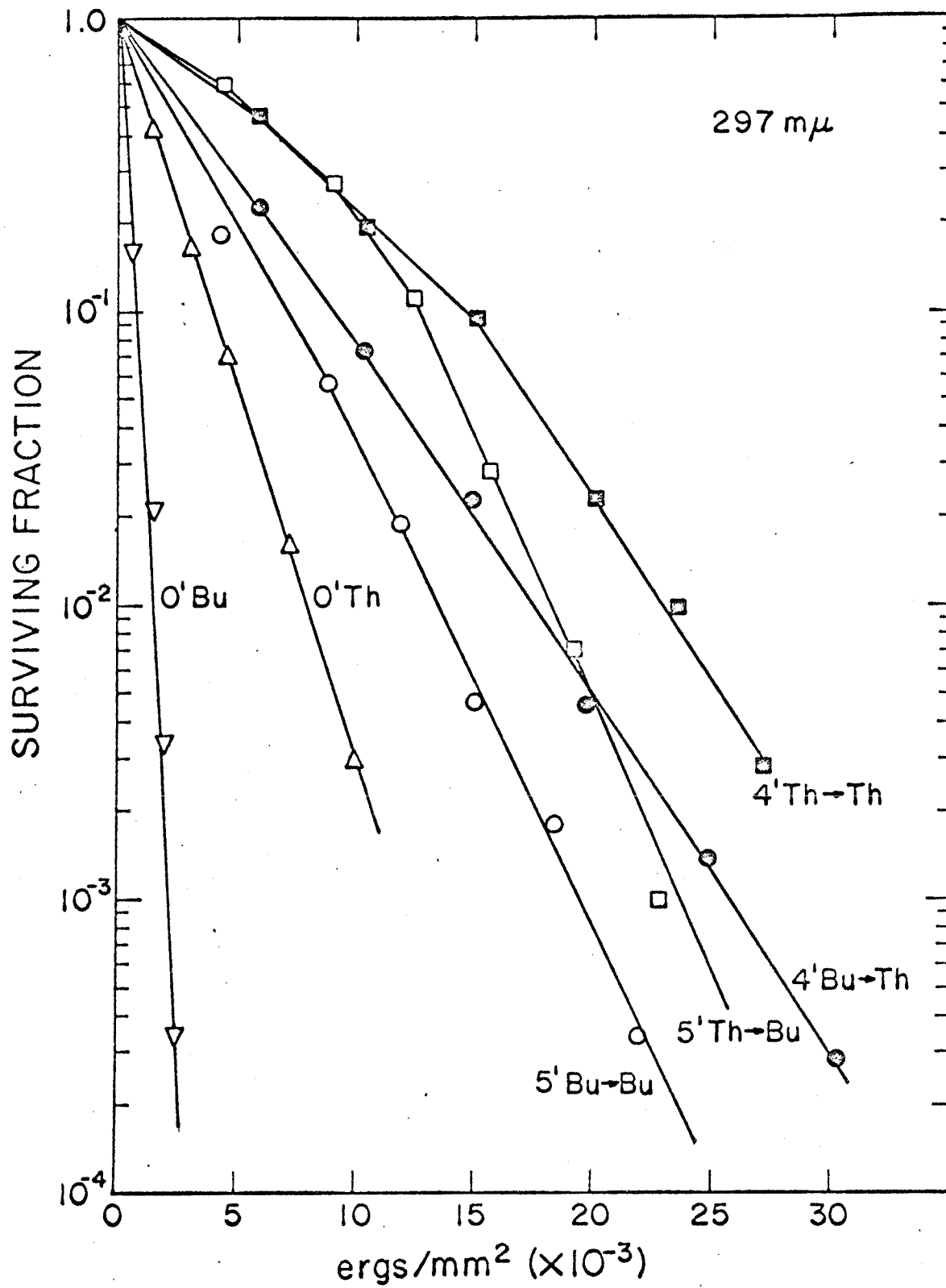
assayed on C has a sensitivity intermediate between Bu- λ irradiated in buffer and normal λ . In this same experiment it was also observed that cysteamine did not decrease the uv sensitivity of either Bu- λ assayed on C_s or λ , ϕ X, and Bu- ϕ X assayed on either host. The inactivation curves of λ assayed on C and C_s have slight shoulders, and the cross sections indicated in the figure are determined from the limiting slopes. The ϕ X and Bu- λ (irradiated in buffer only) inactivation curves are simple exponentials.

The thymine requiring strain, E. coli CR, is like E. coli C in its ability to host cell reactivate T7 (HCR sector of about 0.25). E. coli CR will not plate lambda. Unbalanced growth of CR in the absence of thymine, or growth in the presence of Budr, sensitizes the cells to uv. Both of these results have been previously obtained in other systems (70, 71; see 18 for a contradictory result however). The uv inactivation curves of the colony forming ability of thymine starved bacteria about to undergo thymineless death (72) are single hit with a cross section smaller than that observed for C_s or obtained from the limiting slope of the inactivation curves of the unstarved cells. The effect of thymine starvation does not seem to be simply the loss of the ability of the cells to perform HCR; on the contrary, thymine starved cells were observed to host cell reactivate T7 normally.

The uv sensitivity of the capacity of CR to support

ϕ X growth is increased by both starvation for thymine and growth in Budr. The capacity curves become single hit with a slope very roughly half the slope of the colony inactivation curves. When the cells undergo thymineless death they lose their ability to support ϕ X growth in the same fashion as they lose their ability to form colonies.

Figure 9 illustrates the result of an experiment in which two samples of a starved (in SB) culture of E. coli CR were infected, one with ϕ X, the other with Bu- ϕ X. Each infected culture was divided into two parts; growth in one part was initiated with thymidine containing medium, in the other part, with Budr containing medium. The sensitivity of the O' complex is increased by about the same factor as the free phage when the phage DNA is substituted with Bu. The sensitivity of the complex after development has begun reflects more the nature of the medium in which development is occurring than the nature of the phage initiating the infection. Mature complexes resulting from infection with ϕ X and Bu- ϕ X in thymidine medium usually yield identical L-L curves (60), although the rate at which the Bu- ϕ X complexes develop is sometimes slightly slower than normal. L-L curves of mature complexes formed with Bu- ϕ X in Budr medium sometimes exhibit a slight shoulder, and sometimes lack any shoulder whatsoever. L-L curves of complexes formed with normal ϕ X in Budr develop a shoulder that is smaller than normal; however, the



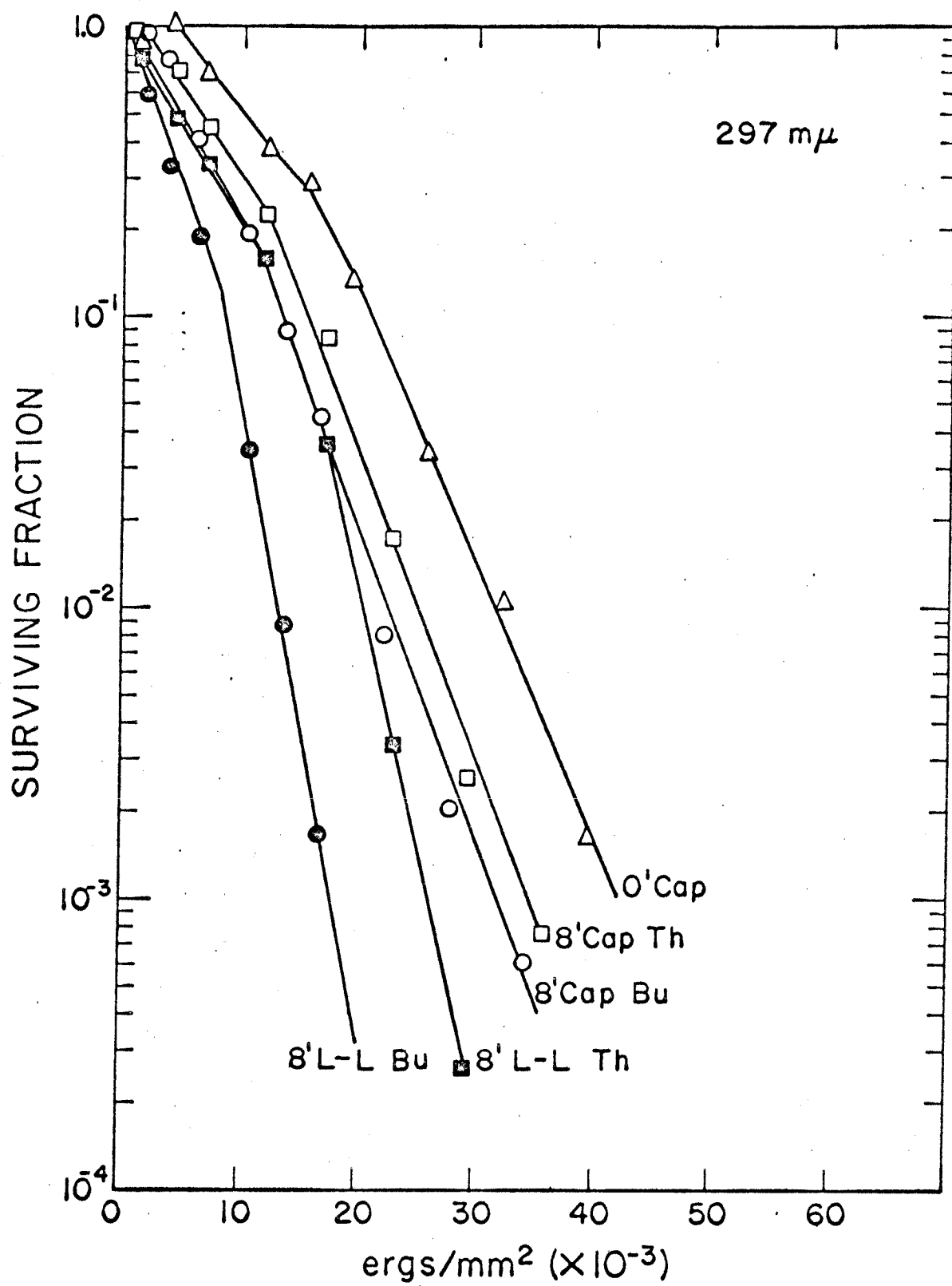
Legend to figure 9

L-L curves at 297 mμ of starvation synchronized CR complexes formed with low multiplicities of ØX (squares) and Bu-ØX (circles) and allowed to develop with either Budr (open symbols) or thymidine (closed symbols). The open triangles refer to the 0' complexes of the two types. The ages of the complexes are indicated on the curves; the eclipse period of the complexes developing with thymidine was 14-15 minutes, with Budr, 16-17 minutes. At least 80% of the phage in the 0' complexes were in eclipse. The cross sections of the limiting slopes of these curves are, in units of 10^{-17} cm^2 : for the 5' Bu to Bu, 2.54; for the 5' Th to Bu, 2.87; for the 4' Th to Th, 1.84; for the 4' Bu to Th, 1.86; for the 0' Bu, 23.1; for the 0' Th, 3.90. The cross sections obtained from the complexes in Th medium are smaller than those shown for the 12' E. coli C complexes at 297 mμ in table 3 because wider slits were used to minimize the irradiation times. The absence of any shoulder on the 4' Bu to Th curve is unusual; usually complexes formed with Bu-ØX in thymidine medium develop very similarly to complexes formed with normal ØX (see figures 10 and 11).

limiting slope is steeper.

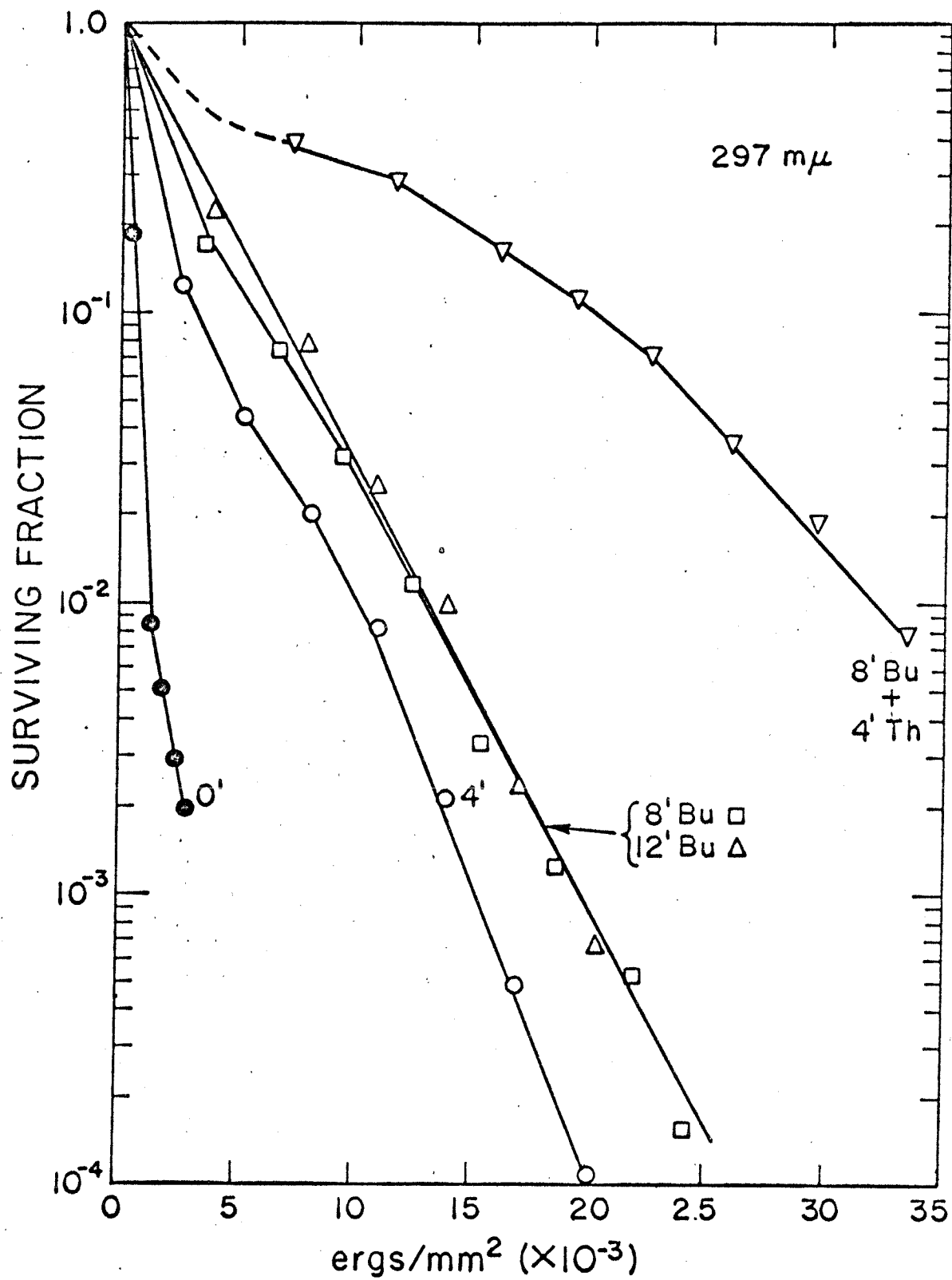
Since at 297 m μ the cross section for capacity is only about 30% smaller than the cross section for the mature complex, the possibility exists that the increased uv sensitivity of the complex formed in Budr medium is really an effect on the capacity of the cell. This problem is made particularly acute because both starvation of the cells for thymine and growth of the cells in Budr increase the uv sensitivity of their capacity as well as their colony forming ability. Fortunately, the rate at which the capacity is affected is slow relative to the rate of ϕ X development, and the data presented in figure 10 show that the capacity curves of 8' complexes formed with Bu- ϕ X in Budr and thymidine medium are more resistant than the corresponding L-L curves determined on the same aliquot of cells. The 8' L-L curve of the Bu- ϕ X complex formed in Budr shows the slight shoulder which sometimes develops. At 265 m μ the capacity is considerably more resistant to uv than the complex (see fig. 6); unfortunately, on the other hand, the difference in sensitivity between normal DNA and Bu substituted DNA is less.

When thymidine is added to an infected culture developing in the presence of Budr, a fraction of the complexes become more resistant to uv than they would if thymidine were not added (figure 11). When an infected culture is allowed to develop in the presence of thymidine,



Legend to figure 10

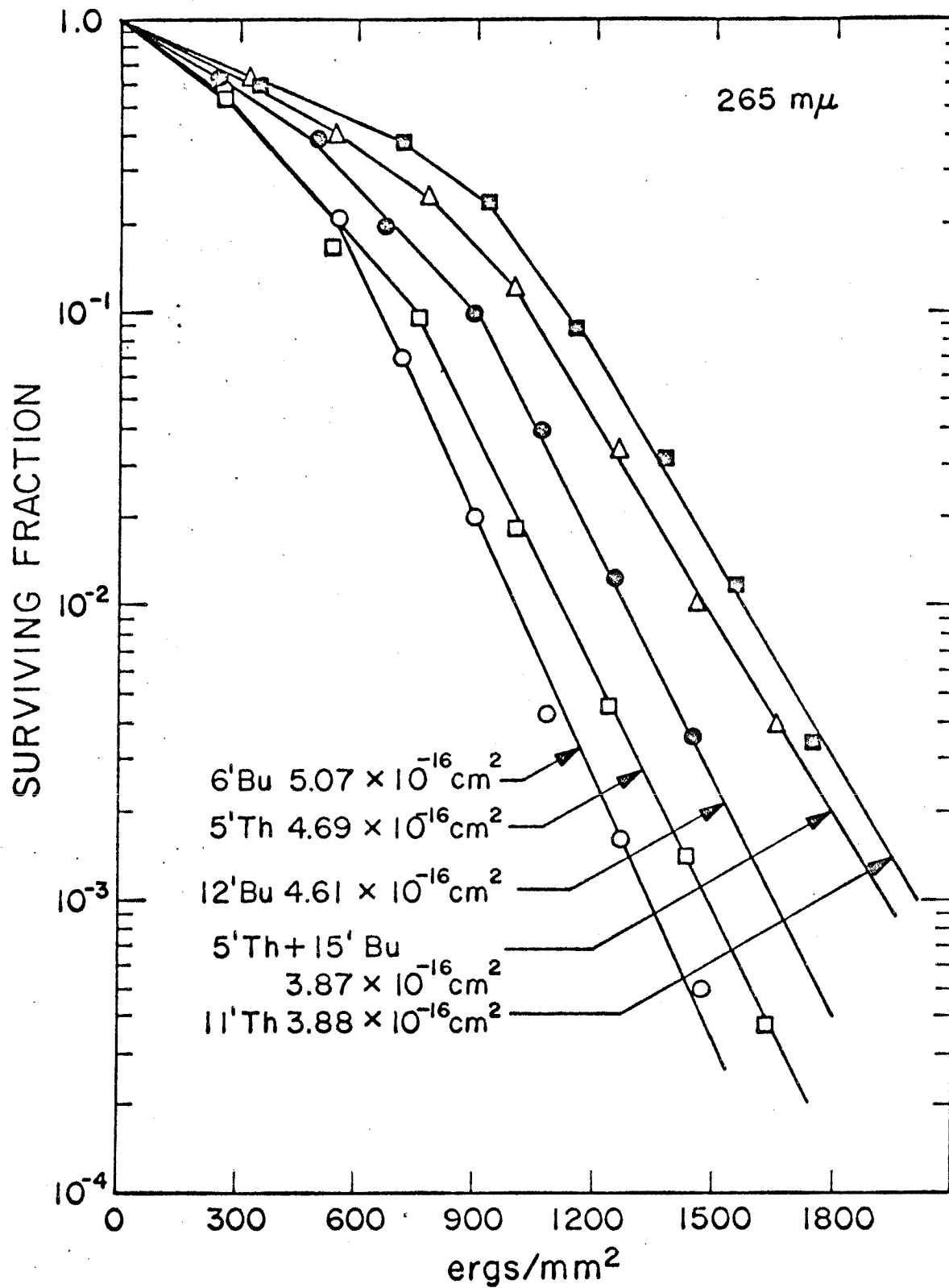
L-L (solid symbols) and capacity (open symbols) curves of complexes allowed to develop for 8' in the presence of Budr (circles) and thymidine (squares); the capacity of the starved uninfected cells is also indicated (triangles). A culture of SB starved E. coli CR at 2.7×10^8 /ml was infected with 6×10^7 /ml Bu-ØX. One aliquot was allowed to develop for 8' with thymidine, another aliquot, with Budr. L-L curves and capacity curves were determined for both aliquots on the same sample; the capacity was measured with a multiplicity of 0.5 phage per bacterium. The cross sections of the limiting slopes of these curves are, in units of 10^{-17} cm^2 : 3.35 for the 8' Bu L-L; 2.61 for the 8' Th L-L; 1.67 for the 8' Bu Cap; 1.67 for the 8' Th Cap; and 1.49 for the 0' Cap. In the concentrated suspension of bacteria (2×10^8 /ml) it was necessary to use an MS-2 dosimeter (see Methods), and this introduces an additional source of error which probably accounts for the somewhat high values observed for the L-L cross sections (see figures 9 and 11).



Legend to figure 11

L-L curves at 297 mμ of starvation synchronized E. coli CR complexes formed with Bu-ØX for 0', 4', 8', and 12' in Budr medium and for 8' in Budr medium plus 4' with added thymidine. The cross section of the 12' Budr complex is $2.33 \times 10^{-17} \text{ cm}^2$, of the 8' Budr plus 4' thymidine complex, $1.49 \times 10^{-17} \text{ cm}^2$ (limiting slope). As in figure 9 this last cross section is smaller than those shown in table 3 because wider slits were used to minimize the irradiation times. The intracellular DNA of these complexes was studied in Part III, figure 8, and further details of the growth are presented there. The broken line in the early portion of the 8' Budr plus 4' thymidine curve denotes the general shape of the curve in this region as observed in other experiments.

filtered, and transferred to Budr medium, a fraction of the complexes retain the uv resistance of thymine complexes (figure 12). These L-L curves are derived from the same complexes whose intracellular ϕ X DNA was examined in Part III. All of the experiments in which the complexes were switched from one medium to another have yielded L-L curves which suggested that some of the complexes retained the uv sensitivity characteristics of the original medium and some of the complexes acquired the uv characteristics of the new medium; when complexes of both characteristics are present the thymine complexes dominate the L-L curves.



Legend to figure 12

L-L curves at 265 mμ of starvation synchronized E. coli CR complexes formed with ØX for: 1) 6' and 12' in Budr medium, 2) 5' and 11' in thymidine medium, and 3) 5' in thymine medium followed by 15' in Budr medium. The limiting slopes of the curves are indicated; and, the differences between the L-L curves in Budr and thymidine, although small, are reproducible within any one experiment where the monochromator settings remain untouched between irradiations. The complexes used in the thymine to Budr transfer are the same ones whose intracellular DNA was studied in Part III, figure 7, and further details of the growth are discussed there.

Discussion

The essentially summarizing statements in this paragraph will be followed by a more detailed discussion of the individual points. The action spectrum of the 12' complex and a comparison of the L-L curves of developed E. coli C complexes with developed E. coli C_s and irradiated E. coli C complexes imply that the target is in part or entirely double stranded DNA. That the uv sensitive component in mature complexes develops after infection can be inferred from a comparison both of the capacity action spectrum with the 12' L-L action spectrum and of the L-L development in thymidine with the development in Budr. The intracellular ϕ X replicative form is the obvious candidate for the most uv sensitive component. More specifically, at least at early times, the target is (with qualifications noted below) the single strand of DNA complementary to the input strand and associated with it in a duplex molecule.

A large part of the shoulder which develops is a manifestation of the phenomenon of host cell reactivation. Theory (34, 35) and experiment (fig. 8) indicate that only double stranded DNA is subject to HCR. As soon as the complementary strand of DNA is formed in the ϕ X complex, most (approximately 90%) of the potentially lethal damages produced by low doses of uv can be repaired in bacterial strains able to perform HCR. The initial sensitivity of

the mature complex to low doses of uv is comparable to the uv sensitivity of RF assayed on bacterial strains that can perform HCR, whereas the final sensitivity at high doses is comparable to the uv sensitivity of RF assayed on HCR⁻ strains (73, 74). The downward concavity of the early part of the 12' L-L curves is a consequence of the fact that the ability of a cell to perform HCR can be destroyed by uv (24); once the uv sensitive HCR system has been put, one way or another, out of commission the limiting slope is attained.

Because of the existence of the HCR system it is difficult to determine whether or not there is an increase in the number of targets characterized by the limiting slope during the infection. During times when RF is known to be replicating inside the cell no change in the back extrapolate of the L-L curves is observed. The explanation of the small shoulder observed in mature C_s complexes and in complexes formed with irradiated E. coli C is not known. The initial portions of the L-L curves of mature C complexes are not as flat as would be expected for true multiple-hit curves, and it is suggested instead that the L-L curves evince the development of an unique target. This conclusion is consistent with the results obtained from the P^{32} suicide experiments described in Part IV, which indicated that the one RF molecule containing the infecting single strand was uniquely responsible (at least in the starvation

synchronized system described here) for one or more necessary late functions.

The effect of Budr substitution on the "host cell reactivability" of DNA is not clear. The enormous increase in uv sensitivity of Budr substituted double stranded DNA compared with normal DNA when irradiated in buffer and assayed on a strain of bacteria capable of performing HCR is well documented (21, 25, 29). Hotz (75) has found that the presence of 10^{-2} M cysteamine in the buffer in which Bu substituted T1 is irradiated nullifies the sensitization caused by the Bu. However, Bu substitution clearly has effects other than preventing HCR (see fig. 8): 1) ϕX , which is not host cell reactivable, is sensitized by Bu substitution; 2) λ assayed on a bacterial strain incapable of performing HCR is sensitized to uv by Bu; 3) the presence of 10^{-2} M cysteamine during the irradiation of Bu- λ reverses only part of the sensitization although the shoulder reappears. At first blush it would appear that if cysteamine were able to reverse the photobiological effects of Bu incorporation then Bu substituted DNA present inside cells should not appear sensitized to uv; however, it is (20). Also the O^+ Bu- ϕX complex (fig. 9) and mature Budr complex (fig. 11) are sensitized. However, it should be noted that the mature Budr complex is not as sensitive as might have been expected.

Fox and Meselson (76) observed that a population of

hybrid Bu-Th λ contained two subpopulations of different sensitivities to radiation from fluorescent fixtures when the phage were irradiated in the presence of 0.2% AET (2-amino-ethyl-isothiuronium bromide, a radical trapping agent believed to act like cysteamine). It would seem that in one of the two subpopulations uv lesions in the Bu strand were lethal, in the other, they were not. This result was only obtained in the presence of AET.

Litman (77) found that the L-L curves of T2 complexes that had developed with Budr were not sensitized to 254 m μ radiation. This result may signify either that T2 resembles T1 in its response to the irradiation of Bu substituted DNA in the presence of sulfhydryl reagents or that at the wavelength used the sensitization was sufficiently small that it was not detected.

It is possible to interpret the results of the uv inactivation of ϕ X complexes in the light of the following considerations:

- 1) The single strand of DNA associated with the infecting parental strand is uniquely responsible in this system for a necessary late function. This hypothesis is supported by the results of the P³² suicide experiments described in Part IV.

- 2) The parental RF replicates semi-conservatively, with the consequence that the material composing the polynucleotide strand combined with the parental ϕ X strand turns

over. This observation was made in Part III.

3) One of the first effects of low doses of uv is the inhibition of DNA synthesis (78).

4) If it is true that thymine dimers are indeed the lethal uv lesions removed by HCR (6-16, 34, 35), then it follows that the principal uv lesions in normal DNA assayed under non host cell reactivating conditions are thymine dimers.

5) Bu present in a strand of DNA sensitizes that strand of DNA to uv and most probably precludes host cell reactivation (assuming that strand is in a duplex molecule) of uv damages in that strand (fig. 8 and reference 76). Attempts to isolate enough hybrid RF molecules in order to study their uv sensitivity in vitro have not been successful.

Some of the results of the L-L experiments that are particularly relevant are:

1) The cross sections derived from the limiting slopes of the L-L curves of mature ϕ X complexes are 20-40% smaller than the 0' cross sections (in E. coli C), and ϕ X DNA contains 31% more thymine than adenine (58). These facts are consistent with the postulate that the target characterized by the action spectrum of the 12' complex is the strand of DNA associated with the infecting single strand and complementary to it.

2) The L-L curves of complexes formed in Budr with Bu- ϕ X are single hit, or nearly so (figures 9-11). In this case neither strand of DNA in the parental strand of RF is

subject to much, if any, HCR. The single hit nature of the curves indicates a single target in the cell, and it was noted in Part III, figure 8, that under these conditions RF may not replicate.

3) The L-L curves of complexes formed with normal ϕ X in Budr (fig. 9) are subject to HCR, but not as much as normal complexes. This result can be interpreted by assuming that HCR of damages in the ϕ X strand of the parental RF followed by semi-conservative replication of the parental RF leads to the production of an active strand associated with the parental ϕ X strand. The fact that not as much HCR occurs would be explained if a dose less than that required to inactivate the HCR system prevented RF replication.

4) The L-L curves of complexes formed with Bu- ϕ X in normal medium are normal. In this case the critical DNA strand is not substituted with Bu and is subject to equal amounts of HCR under both conditions. A corollary of this conclusion is that uv damages in the Bu- ϕ X strand are not lethal to the complex per se; this deduction is consistent with the observation that breaks introduced in this strand by P^{32} decays may not be lethal (Part IV).

5) When the complementary strand of DNA combined with the infecting ϕ X strand is Bu substituted, the limiting slope of the L-L curve for the complex is steeper than when that strand is not Bu substituted. This result is

consistent with the idea that this strand is vital. At doses of uv sufficient to reach the limiting slope of the L-L curve, it has been shown that the cell can no longer perform HCR; and, it has been postulated that RF is no longer capable of replicating.

6) The L-L curves derived from complexes switched from Budr to thymidine (fig. 11) betoken the turnover of the material constituting the target in many of the cells; whereas the behaviour of complexes switched from thymidine to Budr (fig. 12) betokens the absence of turnover in many of the complexes (at least under this particular set of circumstances). These observations can be interpreted consistently with the observations described in Part III on the semi-conservative replication of the parental RF by identifying the target with the strand of DNA conjoint with the infecting single strand.

The results of the capacity inactivation experiments suggest that the ability of the cell to support ϕ X growth can be inactivated in at least two ways. The limiting slopes of the capacity curves of E. coli C and C_s yield an action spectrum more reminiscent of the absorption spectrum of a protein rich in tyrosine than anything else, and the variable and sometimes large back extrapolate indicates that there may be a number of copies of this protein. Its nature, other than that it probably does not represent a ϕ X adsorption site, is not evident.

The action spectrum of the initial slope of the capacity curve of C_g and the small difference in this region between C and C_g are consistent with the possibility that the target is RNA. Hayashi et al. (79) found an RNA complementary to ϕX DNA in the uninfected cell. The linearity of the initial part of the C_g capacity curve implies that there is an unique structure which may perhaps be looked upon as a virus reproduction center (49, 52). It would seem that there is in the cell no host cell reactivable DNA whose activity is required for ϕX development.

The reason for the sensitization of the capacity of E. coli CR to uv after growth in Budr or after thymine starvation is not known. The loss of the ability of thymine starved cells to form colonies and to support ϕX growth could be caused by the induction of a colicinogenic factor as observed in E. coli 15 T⁻ by Mennigmann (80).

Although the question of what happens to the uv sensitivity of the capacity of a cell infected prior to the irradiation to support the growth of a superinfecting phage has not been investigated in detail, the experiments which have been done have not suggested any large increase or decrease in the resistance after infection of the capacity to uv.

The relation of the capacity results presented here to those presented by Tessman and Ozaki (47) on the interaction of S13 with irradiated host cells is not clear; they

found that both the colony forming ability and capacity had about the same uv sensitivity and that multiple infection led to a more resistant capacity curve than did single infection. These results were not obtained with ØX, and this is somewhat surprising in view of the close relationship between these two viruses.

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PART VI

SUMMARY

The following may be considered to have been reasonably well established as facts about the ϕ X replicative process prior to this study. These facts were all stated in the introduction and were referenced there. Very rapidly after the infection of a cell with ϕ X the single strand of DNA is incorporated into a duplex structure, which replicates, even in the presence of chloramphenicol. Inside the infected cell ϕ X messenger RNA is made which has the same base sequence as ϕ X DNA. Single strand progeny DNA is not made until just before the end of the eclipse period and requires the prior synthesis of one or more proteins. There is no parental to progeny transfer of DNA, and (somewhat less well established) RF is not a material precursor to single strand DNA. ϕ X infection does not interfere with any of the synthetic capacities of the cell.

The following may be considered to have been reasonably well established in this study as facts about the ϕ X replication process. Virus maturation is a linear process; cell lysis accompanies phage release and, more so for wild type ϕ X than for the lysis delayed mutant, occurs very soon after the end of the eclipse period. The times of lysis of the individual cells are not normally distributed. In Part III it was shown that: 1) the material combining with the infecting single strand to form the first, parental, RF is derived from the medium after infection; 2) the parental RF replicates semi-conservatively, and the

conserved subunit is a single polynucleotide strand; 3) RF is not a precursor to progeny single strands; rather, the precursor is low molecular weight material derived from the medium. On somewhat less than convincing evidence it was suggested that only the parental RF could replicate.

The experiments discussed in Parts IV and V suggest strongly that in the mature starvation synchronized complex (in which RF has been formed and replicated, and ϕ X messengers and ϕ X specific proteins have been formed, but single strand DNA synthesis has not yet begun) the newly synthesized strand of DNA associated with the input single strand in the parental RF duplex is solely responsible for a function necessary if the infected cell is to form a plaque. It is postulated that this function is the production of progeny SS DNA. There is evidence (Part III, R. L. Sinsheimer, personal communication) that the RF containing the infecting single strand is physically different from many of the progeny RF molecules.

In order to infer that the complementary strand of DNA associated with the infecting single strand was essential, certain assumptions about the nature of P^{32} suicide and the effects of uv on intracellular DNA were made; these assumptions become facts if the above stated inference be true. The decay of a P^{32} atom in one strand of DNA can, with a probability of about 0.2 (\pm 0.1, probably depending intimately on the intracellular state of the DNA

and its local environment), inflict a lesion in the other strand of DNA associated with it in a duplex molecule; a lesion (or at least some manner of interference) that prevents that strand of DNA from forming a viable complementary strand of DNA. A corollary to this is that single strand breaks in one strand of a duplex do not in general interfere with the functioning (in a genetic sense of making DNA) of the other strand, even in cells which can not host cell reactivate uv damages.

Most lesions produced in intracellular DNA by ultraviolet radiation do not damage both strands of DNA in a duplex molecule. A large class of these lesions are reactivable by an uv sensitive enzymatic repair system that is genetically determined by the host cell. Substitution of the thymine in one strand of an intracellular DNA duplex by 5-bromouracil does not enhance the uv sensitivity of the other strand, but it does enhance the uv sensitivity of the strand containing it, particularly at longer wavelengths. Budr substitution in one strand of a duplex DNA impairs the ability of the host cell reactivating system to repair uv lesions in that strand, but not in the other strand.

In summary, the sequence of events occurring in the ϕ X developmental cycle would seem to encompass the following:

- 1) Adsorption of the phage to the cell and release

of the SS DNA into the cell.

2) Formation of a complementary strand of DNA on the infecting SS to produce the parental RF. This process, which takes about 4', is accompanied by a decrease in the sensitivity of the complex to both uv irradiation and P^{32} decays occurring in the parental SS DNA.

3) Replication of the parental RF by a semi-conservative process in which the conserved subunit is a single polynucleotide strand; RF replication may not be an obligatory step in the formation of ϕ X progeny. Progeny RF's seem for the most part to be physically distinct from the parental RF.

4) Formation of messenger RNA of the same base sequence as the ϕ X SS DNA, presumably by both parental and progeny RF DNA.

5) Synthesis of ϕ X specific proteins using the ϕ X messengers. A phage coat protein is definitely formed, and it seems very reasonable to postulate that an enzyme for making single strand DNA and an enzyme for getting progeny phage out of the cell are made.

6) Production of progeny single strand DNA. It has been argued in this thesis that this is done using only the complementary strand of DNA associated with the parental single strand in the parental RF.

7) Immediate incorporation of the SS DNA into phage particles followed shortly by cell lysis.

Events 2-5 may be considered to be performed by the host cell for the phage. Events 3-5 do not seem to affect the uv sensitivity or P^{32} suicide sensitivity of the complex. Event 6 presumably results in complete refractoriness of the complex to P^{32} suicide.

APPENDIX

List of Abbreviations

BU	5-bromouracil
Budr	5-bromodeoxyuridine (page 38)
C	centigrade
CAP	capacity
cm	centimeter
cpm	counts per minute
DCM	drop collecting medium (page 38)
DDM	diluted defined medium (page 38)
DM	defined medium (page 38)
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetracetate (versene)
<u>E. coli</u>	<u>Escherichia coli</u>
eq	equivalents
ev	electron volts
g	grams, or gravity
HCR	host cell reactivation (page 107)
L-L	Luria-Latarjet (page 104)
l	liter
M	molar
mc	millicuries
MEV	million electron volts
mg	milligrams
min	minutes
ml	milliliters
mm	millimeters
mμ	millimicrons
RF	replicative form (page 2)
RNA	ribose nucleic acid
RPM	revolutions per minute
SB	starvation buffer (page 9)
sec	second
SS	single strand (page 45)
T, Th, THY	thymine
TKB	tryptone-KCl broth (page 9)
TPG	tris-pyruvate-glucose (page 38)
uv	ultraviolet
UVR	ultraviolet reactivation (page 136)