

STUDIES ON THE BIOSYNTHESIS OF ϕ X174 COAT PROTEIN

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ABSTRACT

A serum blocking power (SBP) assay for ϕ X174 coat protein, which depends on the ability of whole phage or certain phage components to combine with antiphage serum, was developed. This assay registers complete ϕ X, both viable and nonviable, and 70S particles, but not chemically prepared 5S subunits.

Studies on the growth of wild type ϕ X, at 37°C, showed that SBP synthesis begins at about 5 min, under conditions such that the eclipse ends at 6 to 8 min. Although the curves for SBP and intracellular phage growth have the same shape, the titer of SBP phage equivalents exceeds that of infectious progeny particles at all times. The excess SBP is found in the form of 70S particles, complete but noninfectious phage, and subunits that sediment at about 15S.

Chloramphenicol, 5-fluorouracil deoxyriboside, and pleomycin when present at the time of infection each produce essentially complete inhibition of SBP synthesis. These results suggest that at least one complete RF molecule (the double stranded form of ϕ X DNA) must be made in order for SBP synthesis to occur.

Several temperature sensitive (ts) mutants were studied for their ability to produce SBP at 40°C (nonpermissive conditions for production of infectious phage). Most of the twenty-six mutants examined did make SBP at 40°C; however, mutant ts 79 definitely did not.

Studies were made on the physical state of the SBP synthesized, at 40°C, by the mutants ts γ , ts 9, and ts 4 by the technique of sucrose gradient velocity centrifugation. The results showed that

ts γ , and ts 9 make their SBP principally in the form of subunits that sediment at approximately 15S. The mutant ts 4 makes its SBP principally in the form of particles that sediment at 71S. The ts 4 71S particles may contain both DNA and protein.

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INTRODUCTION

INTRODUCTION

In 1959 Sinsheimer (1) discovered that the DNA of coliphage ϕ X174 consists of 5500 nucleotides arranged in a single stranded structure. Since that time ϕ X has been the subject of numerous experiments directed principally toward elucidating its replicative processes, and details of structure. No doubt its unusual DNA spurred interest in ϕ X, but its rather small information content has, perhaps, been an even more compelling reason for arousing molecular biologists. The rationale is that so small a genome (ϕ X has 1/80th the DNA content of the T-even coliphages) could specify only a few gene products, and thus that its reproduction might be relatively easy to study. Assuming a non-overlapping triplet code (2) ϕ X DNA would carry the information for perhaps ten different polypeptide chains, if each contained 180 amino acid residues.

This thesis deals with ϕ X coat protein biosynthesis. Part A of the Introduction is a brief, overall survey of ϕ X structure and replication. Part B describes the assay for ϕ X coat protein used in this work.

Part A - ϕ X Structure and Replication

General Structure

ϕ X is composed of 25% DNA, 75% protein, has a molecular weight (M. W.) of 6.2×10^6 and a sedimentation constant, $S_{20, w}$ of 114S (3). In electron micrographs it is roughly spherical, with a

diameter of about 250\AA , and appears to be made up of twelve "knobs" (morphological subunits) situated as though on the faces of a dodecahedron, or, equivalently, at the vertices of an icosahedron (4).

Protein Structure

The chemical subunit from which the ϕX protein coat is composed contains 225 amino acids, and has a M. W. of 2.5×10^4 (5). The coat can be dissociated into a subunit of 5S and M. W. about 10^5 , by exposure to sodium dodecyl sulfate at pH 10.5, and 40°C for 20 hrs (5). The 5S subunit can be further degraded to a 2.5×10^4 M. W. product, which is apparently the chemical subunit.

About ten years ago Crick and Watson (6) suggested that the coats of small spherical viruses are built up of sixty, or a multiple of sixty, protein subunits arranged with icosahedral symmetry. Their argument was based on geometrical considerations, and the idea that repeated use of many copies of the same protein subunit, to construct a virus coat, would be a very efficient way to utilize a limited amount of genetic information. Since then a wide variety of viruses, among them ϕX , poliovirus, tomato bushy stunt virus, Tipula iridescent virus, have been shown to possess icosahedral symmetry. This in itself, of course, does not prove these viruses have but one type of protein subunit, nor that they are made of multiples of sixty subunits. In fact, it is now known that poliovirus contains at least three electrophoretically separable protein components (7). Mouse-Elberfeld virus, encephelomyocarditis virus, and Mengovirus, all (like poliovirus) small RNA animal viruses, appear to have at least two electrophoretically different proteins (8).

Applying these concepts to the data on ϕX leads to the following numerology.

	Morphological Subunit	5S Subunit	Chemical Subunit (M. W. = 2.5×10^4)
Entire Coat (M. W. = 4.5×10^6)	12	45	180
Morphological Subunit *(M. W. = 3.8×10^5)	-	4	15
5S Subunit (M. W. = 10^5)	-	-	4

* Calculated assuming each is 1/12th M. W. of entire coat

This is a useful but, for several reasons, tenuous and over-simplified model of ϕX coat morphology. First, it is not at all clear that 5S resembles any real subunit formed during the course of infection; second, the 5S material represented only 60% recovery of the total protein used in the dissociation procedure - thus, there may be other as yet undetected proteins; third, some electron micrographs show, in addition to the morphological subunits, small projections as if at the apexes of an icosahedron (9); and finally, no X-ray diffraction studies on ϕX have been reported.

Serological experiments employing the Ouchterlony technique of agar double diffusion revealed that ϕX has three antigenic sites (10).

One of these sites is not accessible in complete virus, but is exposed in both heated virus and the 70S component (vide infra). Neutralizing antibodies are elicited in response to whole virus, heated virus, and the 70S component. Neutralization of viable ϕ X is a first order process, and leaves phage unable to adsorb to host bacteria (10). First order kinetics is characteristic of single "hit" phenomena, and suggests, in this case, that combination of one anti- ϕ X antibody molecule with a ϕ X particle produces neutralization. In view of the apparent identity of the twelve morphological subunits, the neutralization kinetics imply that either one of the morphological subunits is responsible for adsorption and penetration, or attachment of one antibody molecule sufficiently distorts the whole phage to prevent adsorption.

The existence of three antigenic sites might mean there are three different types of protein in ϕ X. It does not, however, have to mean this. These sites could be separate determinant groups on the same protein. Or, perhaps, one type of site occurs at the corners formed where two morphological subunits meet, a second site might be on the surface of the morphological subunit; the third site is apparently located on the inside of the virus. An antigenically distinct internal protein also exists in phage T2 (11).

It is of interest to note that, by the criterion of complement fixation, ϕ X DNA reacts with sera from some humans with lupus erythematosus (12). Native double stranded DNAs, from other sources, are not reactive in this system, although heat denaturation renders them active. Hence, that ϕ X DNA reacts with these sera is simply a reflection of its single stranded nature. The reaction between denatured DNA and lupus sera can be inhibited by purines or purine containing polymers (13).

It is also true that purines and pyrimidines when linked to protein carriers can function as antigenic determinants (14). However, it seems unlikely that antibodies directed against ϕ X DNA, per se, exist in anti- ϕ X sera. In the serological assay used in this thesis (vide infra) ϕ X DNA is not reactive.

DNA Structure and Replication

The single strand of ϕ X DNA has a M. W. of 1.7×10^6 , and is a covalently linked ring structure containing one E. coli phosphodiesterase-resistant discontinuity (15, 16). Electron micrographs of the DNA also show a ring structure (17).

Within three minutes after infecting E. coli C, the single stranded ring is converted to a double stranded replicative form (RF), which also is a ring (18, 19). Although many copies of the RF are formed none of it serves as a material precursor to virus DNA (20). It is thought that the single stranded DNA, destined for mature virus, must somehow be synthesized off an RF template; but at no time after infection is a pool of free single stranded DNA demonstrable (18). Neither is there any transfer of parental DNA to progeny virus. That RF is formed and replicates even in the presence of chloramphenicol proves that its synthesis is directed exclusively by host cell enzymes (18). Chloramphenicol does, however, prevent the production of ϕ X induced proteins and single stranded DNA. The latter result suggests that a ϕ X specific enzyme makes the single stranded DNA off the RF template.

Both single stranded ϕ X DNA and RF are infective to properly prepared protoplasts of E. coli (21).

Messenger RNA

Pulse labeled RNA extracted from ϕ X infected cells hybridizes with RF, but not with mature virus DNA (22). The base ratios of mature virus DNA and of the RNA hybridizable to RF are essentially identical. These facts are taken to mean that ϕ X messenger RNA is synthesized off RF using only the strand complementary to virus DNA as template. Furthermore, it seems that intact circular RF is needed if only the ϕ X complementary strand is to function in the transcription process, for sonicated RF will transcribe both strands into RNA, in vitro (23).

70S Particles

In addition to complete virus, a second type of particle, the 70S component, appears in ϕ X lysates (3, 24). The 70S particles are composed of approximately 95% protein and 5% DNA; their morphology, as revealed by electron microscopy, is very similar to that of intact ϕ X (25). Although 70S adsorbs to bacteria which can serve as host to ϕ X neither cell lysis nor replication of 70S follows (24). 70S particles are thought to be ϕ X coat protein still associated with small pieces of ϕ X DNA; how they arise is, however, unknown.

Some General Characteristics of the Infection

ϕ X infected cells continue to divide, and synthesize cellular DNA, RNA, and protein at rates equal to those of uninfected cells, up until the time of lysis (26).

Infection can be synchronized by using either starved cells or cells treated with cyanide (27). Phage development, under these conditions, is initiated by adding nutrients or removing cyanide, respectively. Synchronized infections of *E. coli* C by wild type ϕ X at 37°C have a minimum latent period of fourteen minutes, an eclipse time of about ten minutes, and a burst size ranging from 100 to 300 (27).

Lysis

A single ϕ X infected cell liberates its yield of progeny phage in a discrete burst (28). However, all efforts at detecting a lytic enzyme in mature virus or infected cells (analogous to the lysozyme formed in T4 infected cells, and present in T4 itself) have been negative (24, 29). ϕ X infected cells do synthesize a factor, probably a protein, required for lysis, whose production is sensitive to chloramphenicol (29). This factor can first be detected around twelve minutes after infection and is distinct from a soluble antigen precipitable by anti- ϕ X serum (29).

There is an amber (suppressor-sensitive) mutant of ϕ X that makes large numbers of complete intracellular phage, in the non-permissive host, but fails to lyse these cells; this means the lytic factor, in normal infections, is specified by ϕ X - not the host (30).

Temperature Sensitive Mutants

Over 100 nitrous acid-induced temperature sensitive (ts) mutants of ϕ X have been isolated by Hutchison (30). These are conditional lethal mutants in that they grow normally at 30°C, but, unlike wild type ϕ X, are unable to produce plaques when grown at

40°C. Edgar, Epstein and their associates (31, 32, 33, 34) have used ts (and amber) mutants in their physiological genetic studies on T4 with great success. They were able to obtain ts mutations widely distributed over the genetic map, and thus investigate several different gene functions. Hutchison (30) has found that the ϕ X ts mutants fall into three complementation groups. Dowell and Sinsheimer (35) studied representative mutants from these groups with regard to their ability to make infectious DNA (assayed in the protoplast system) at 40°C. All the mutants studied synthesized infectious RF, but only one made infectious single stranded DNA. Results on ϕ X protein synthesis, at 40°C, by some of these same mutants will be presented here.

Protein Biosynthesis

Rueckert and Zillig (26) have determined the time course of ϕ X protein synthesis, within infected E. coli C, by pulse labeling with a radioactive amino acid and isolating ϕ X protein by immunological precipitation. They first detected ϕ X antigen at about eight minutes after infection, and the rate of synthesis reached a maximum at fifteen minutes. At the maximum rate ϕ X protein accounted for only 10% of the total protein synthesized in the cell. The in vitro synthesis of ϕ X protein has also been detected, using unfractionated cell free extracts derived from ϕ X infected cells, with the immunological precipitation assay (36). Although direct evidence is lacking it is assumed ϕ X protein synthesis takes place upon ribosomes and proceeds by the same mechanism that accounts for bacterial protein synthesis.

Part B - The Serum Blocking Power Assay for ϕ X Protein

The experiments in this thesis are concerned with various aspects of the in vivo synthesis of ϕ X protein. The assay used throughout is the serum blocking power (SBP) test, similar to the one devised by DeMars (37) for phage T2. The assay consists of incubating dilute anti- ϕ X serum with the material thought to contain ϕ X protein for a length of time sufficient to allow the adsorption of ϕ X antibodies to reach completion (16 hrs, 48°C). The residual neutralizing activity is inversely related to the concentration of ϕ X antigen in the incubation mixture; and can be titered by addition of a ϕ X host range mutant (tester phage) whose inactivation, after two hours, 48°C, is then measured. A standard curve can be constructed by correlating tester survival with particle concentration of purified wild type ϕ X used in the sixteen hour incubation mixture. This assay is then specific for the ϕ X antigen(s) capable of adsorbing neutralizing antibodies.

Some comments about the relative merits of the SBP versus the precipitation assay are in order here. The SBP assay may be more sensitive and hence offers the advantage of allowing one to work on a smaller scale. At least it appears from the precipitation data of Rueckert and Zillig (8) that about 10^{10} phage equivalents per ml was the least amount of ϕ X protein detectable; 5×10^7 phage equivalents per ml is the lower limit of the SBP assay used in this thesis. The SBP assay is specific for antigens of the phage concerned with adsorption to bacteria, whereas precipitation probably measures all types of antigen. Non-specific precipitation of labeled host proteins is sometimes a problem with the precipitation assay. A serious drawback of the SBP assay (which is avoided in the radio-

active precipitation assay) is that it records a background of phage protein added with the infecting inoculum. In crude ϕ X stocks there can be considerable amounts of protein in the form of 70S particles, and inactive phage. Even purified ϕ X stocks contain large numbers of inactive phage particles. The assay of choice should depend on the experiment in question.

This work will first show that, under the conditions employed, the SBP assay registers complete ϕ X, both viable and nonviable, and 70S particles, but not the 5S subunit. Results on SBP synthesis by wild type ϕ X and several ts mutants will be presented. Analysis of the physical state of the SBP produced by wild type, and ts mutants, grown at 40°C, will reveal that a subunit of approximately 15S exists in lysates, and is detected in the assay. The ts mutants examined could be grouped into three categories with respect to SBP produced at 40°C; 1) make SBP principally in the form of 15S subunits, 2) make SBP principally in the form of particles that sediment at 71S, 3) do not make SBP.

MATERIALS AND METHODS

MATERIALSPhage

ϕX^+ : The wild type $\phi X174$ described by Sinsheimer (3).

$\phi XH_A H_B$: A host range mutant of $\phi X174$ obtained from D. Pfeiffer (38).

ϕX Temperature Sensitive Mutants: These mutants form plaques on E. coli C at 30°C but not at 40°C, and were obtained from C. A. Hutchison.

Bacteria

Escherichia coli C: BTCC # 122.

Escherichia coli C_{ab}: A mutant of E. coli C resistant to ϕX^+ but sensitive to $\phi XH_A H_B$. Obtained from D. Pfeiffer (38).

Escherichia coli C T⁻: A thymine requiring strain obtained from Dr. P. Hanawalt.

Escherichia coli C₄₀₆: This strain is ϕX^S , F⁻, arg⁻, T1^R, try⁻, Sm^R, gal⁻, Pur I⁻, and was obtained from Dr. B. Kelly.

Purified 70S and 5S

These were gifts from E. Carusi. The methods of preparation and purification have been described (5).

Anti- ϕ X Serum

This was a gift from U. Rolfe. The immunization schedule was described by Rolfe and Sinsheimer (10). The serum used in all the experiments here was prepared in October 1960, and has been stored at -20°C .

Media

KC Broth: 1 liter H_2O , 10g Bacto-tryptone (Difco), 5g KCl, and 0.5 ml 1M- CaCl_2 . This is a good adsorption medium for ϕ X.

Tryptone Broth (TB): 1 liter H_2O , 10g Bacto-tryptone, 5g NaCl. This is a poor adsorption medium for ϕ X.

Tryptone Bottom Agar: 1 liter H_2O , 10g Bacto-agar (Difco), 10g Bacto-tryptone, 2.5g NaCl, 2.5g KCl, 1ml 1M- CaCl_2 .

Tryptone Top Agar: 1 liter H_2O , 8g Bacto-agar, 10g Bacto-tryptone, 5g NaCl.

Bresch Bottom Agar: 890ml H_2O , 15g Bacto-agar, 4g Bacto-tryptone, 2g Bacto-yeast extract (Difco), 2g NaCl. After autoclaving 120ml of a sterile 10% glucose (w/v), in H_2O solution, and 6ml of a sterile Na_2CO_3 solution (7.4g anhydrous Na_2CO_3 per 100ml H_2O) are added.

Bresch Top Agar: 890ml H_2O , 8g Bacto-agar, 4g Bacto-tryptone, 2g Bacto-yeast extract, 2g NaCl. After autoclaving 120ml of a sterile 10% glucose solution is added.

TPG Medium: 1 liter H_2O , 0.5g NaCl, 8.0g KCl, 1.1g NH_4Cl , 1ml 0.1 M- CaCl_2 , 0.2g $\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$, 12.1g Tris (2-amino-2-hydroxymethylpropane-1:3diol, Sigma Chemical Co., St. Louis,

Missouri), 0.023g KH_2PO_4 , 0.8g sodium pyruvate, 1ml 0.16 M- Na_2SO_4 . After dissolving the salts, the pH is adjusted to 7.4 with HCl. The solution is then autoclaved. After autoclaving, 0.1ml of a sterile solution containing $1\mu\text{g/ml}$ $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and 2ml of a sterile 10% glucose solution are added to each 100ml of medium.

TPG + Amino Acids Medium Modified: Exactly like the TPG medium except that 1g of KH_2PO_4 is used. In addition the following are added, per liter, before autoclaving: 20ml of 0.05% adenine (w/v); and 15mg each of the L-amino acids, histidine, isoleucine, lysine, phenylalanine, threonine, valine, alanine, aspartic acid, glutamic acid, glycine, proline, serine, and tyrosine; 150mg each of L-tryptophan and L-arginine (this is the medium in which E. coli C₄₀₆, an auxotroph for try and arg, was grown). Since this is the medium in which sulphur-35 (^{35}S) labeled ~~OX~~ was grown, methionine, cystine and leucine (which is sometimes contaminated with sulfur containing amino acids) were completely omitted. All amino acids were from Calbiochem.

Starvation Buffer: 1 liter H_2O , 1.2g Tris, 7.5g KCl, 0.1g MgSO_4 , 1.5ml 1M- CaCl_2 ; after dissolving the salts the pH is adjusted to 7.1 with HCl.

Chemicals

Chloramphenicol was obtained from Parke, Davis & Co., Detroit, Michigan.

Fluorouracil deoxyriboside was obtained from the Cancer Chemotherapy National Service Center, Bethesda, Maryland.

Phleomycin was obtained from the Bristol Labs., Syracuse, N. Y.

METHODS

Phage Assay

The methods described by Adams (39) were used. Plating bacteria are grown at 37°C in KC broth, with aeration, to a concentration of 2 to 5x10⁸/ml. For each plate 0.3ml of plating bacteria is added to 2.5ml of melted top agar, followed by an aliquot of phage; this is then poured onto about 20 to 30ml of hardened bottom agar contained in a plastic petri dish (Falcon Plastics, Los Angeles). When the incubation temperature is 37 or 40°C, three hours are allowed for plaques to develop, at 30°C five hours are allowed.

Phage Stocks

Crude Stocks: A single plaque of the desired phage type is added to 20 to 40ml of E. coli C grown to 0.5 to 1.0x10⁸/ml in KC broth. Wild type stocks are grown at 37°C, ts mutants at 30°C. Aeration is continued until the culture clears (about three to five hours). The lysed culture is centrifuged at 10,000 rpm for ten minutes. The supernatant is discarded and the pellet resuspended in 2 to 5ml of 0.05M sodium tetraborate (borate). This is allowed to sit at 4°C for several hours, in order to allow phage to elute from the bacterial debris which composed the pellet, and is then centrifuged as before. The supernatant is the phage stock and usually has a titer in the range of 10¹⁰/ml. Stocks prepared in this way are crude in that they contain inactive phage and 70S particles in addition to viable phage.

The crude stocks of ts mutants had titers at 40°C about 10⁻⁶ times those at 30°C.

Purified Wild Type Stocks: In most of the experiments employing ϕX^+ the stocks were aliquots taken from large preparations made by the method of Sinsheimer (3). Phage prepared in this way are devoid of 70S, because they have been purified by CsCl density gradient centrifugation. Furthermore these are high titer stocks ($> 10^{12}$ /ml) and contain enough material to measurably absorb ultraviolet light at $260m\mu$. Since the specific absorption of ϕX at $260m\mu$ has been determined (3) it is possible to calculate the ratio of active phage to total phage (the fraction of plaque formers) in these stocks.

Purified ts Mutant Stocks: These were grown in E. coli C and purified as described below for ^{35}S labeled phage.

Growth and Purification of ^{35}S - ϕX^+ : Ten millicuries (mc) of carrier free ^{35}S as H_2SO_4 in 0.3ml of a 0.1N-HCl solution (obtained from Oak Ridge) are neutralized with 0.3ml of 0.1N-KOH. The resultant 0.6ml is combined with 9ml of the modified TPG + amino acids medium, and to this 1ml of E. coli C₄₀₆, previously grown to 10^8 /ml in modified TPG + amino acids is added (this strain is used because it gives good yields of phage in the purification procedure used here). The culture is aerated at 37°C and upon reaching 10^8 /ml is infected with 0.1ml of purified ϕX^+ , diluted in modified TPG + amino acids, sufficient to produce a multiplicity of 3.

Aeration is continued for about four hours and then the following solutions are added: 0.1ml-0.1M-EDTA (disodium salt) adjusted to pH 7.0 with HCl, 2.5ml 44% (w/v) of a non-sterile polyethylene glycol solution (carbowax 6000, Union Carbide Chemical Co.), 0.85ml of a non-sterile 20% (w/v) dextran sulfate (500) solution (Pharmacia, Uppsala, Sweden), and 0.55ml 5M-NaCl.

This mixture is shaken vigorously by hand, transferred to a conical centrifuge tube and spun in a clinical centrifuge at maximum speed for 20 minutes. Two phases separated by a sharp interface, composed of bacterial debris with adsorbed phage, are seen near the bottom of the tube. The solution above the interface is pipeted off and the interface itself is then transferred to 2ml of borate. After several hours at 4°C the debris is removed by centrifugation. The supernatant is dialyzed against 500ml borate at 4°C, and then adjusted to a density of 1.40g/ml by addition of CsCl (0.635g CaCl₂/g phage suspension). This material is centrifuged in the SW 39 rotor of a Spinco model L centrifuge at 37,000 rpm, at 5-6°C, for 36 hours. The contents of the tube containing the phage preparation are removed by boring a hole (with a #3 insect pin) in the bottom and collecting five drop fractions into 0.5ml borate.

The fractions are assayed for phage and radioactivity (on a Nuclear Chicago low background counter which counts ³⁵S with an efficiency of 20%). Coincident peaks of radioactivity and infectivity were found in the tenth of the nineteen fractions collected. The tenth fraction was dialyzed twice against 500ml borate at 4°C. The purified virus particles had a specific activity of 3.2×10^{-5} cts/min/plaque former. Since the ratio of ³⁵S:³²S in the growth medium is 1:240 and there are about 1200 sulphur atoms per ~~OX~~ particle (5) the average ³⁵S content per particle is five atoms. About 20% of the total particles were plaque formers as calculated, knowing the sulphur content per virus particle, and comparing the specific activity of the growth medium with that of the plaque formers.

Starvation Synchronization

Cells are grown to log phase in KC broth, at 37°C, pelleted by centrifugation, washed once with starvation buffer (SB), and then resuspended in SB. Starvation is achieved by aeration in SB for 60 minutes at 37°C. Phage, diluted in SB, are added to the starved cells, and adsorption is allowed to proceed for ten to fifteen minutes. The infected, starved cells are centrifuged, the supernatant discarded, and the pellet resuspended in 0.1M-NaCl. To initiate phage development the cells are now diluted (usually about tenfold) into prewarmed tryptone broth (TB). The technique of resuspending the infected cells in 0.1M-NaCl (in which no development should take place) followed by dilution into TB is used to insure accurate timing of the beginning of phage development.

Cyanide Synchronization

Cells are grown to log phase in KC broth at 37°C. The culture is made 0.009M with respect to cyanide by adding an aliquot from a 0.9M-KCN solution. After five minutes at 37 or 40°C phage, diluted in KC broth, are added and allowed to adsorb for ten minutes. Cyanide and unadsorbed phage are removed by filtering the infected cells onto an HA (0.45 μ mean pore size) Millipore filter (Millipore Filter Corp., Bedford, Massachusetts) and washing once with 0.1M-NaCl. The washed filter is placed into the appropriate volume of 0.1M-NaCl and vigorously stirred to aid transfer of the infected cells from the filter to the solution. As in the starvation synchronization the infected cells are diluted into prewarmed TB. In experiments where 30 and 40°C growth curves are run simultaneously the cells in NaCl are divided into two equal portions,

one part is diluted into TB at 30°C, the other into TB at 40°C. Usually about 50% of the infected cells can be recovered from the filter. Attempts to remove cyanide by centrifugation of the infected cells gave erratic results, characterized by extended latent periods and low phage yields.

Growth Curves

Infection is synchronized by one of the methods outlined above. Infective centers are determined by removing 0.1ml from the infection tube, diluting through ice cold TB (in which they may be left for several hours without change in titer) and plating. Unadsorbed phage are determined by titering the filtrate in the case of cyanide synchronization or the supernatant of the centrifuged infected cells in starvation synchronization.

Samples for assay of intracellular phage and SBP are obtained by removing 3ml from the infection tube and immediately freezing in a dry ice acetone bath. When convenient the samples are thawed, placed in an ice water bath and 0.1ml lysozyme (2mg/ml dissolved in 0.25M Tris-Cl, pH 8; Worthington Biochem. Co.) plus 0.2ml 4% EDTA (dissolved in 0.25M Tris-Cl, pH 8) are added. Thirty minutes after adding the lysozyme and EDTA solutions the samples are removed from the ice water bath, lysed by three cycles of freezing and thawing, and then plated for phage and assayed for SBP.

Intracellular phage titers can also be determined by diluting 0.1ml, from the infection tube, into 0.9ml of an ice cold solution composed of 0.7ml H₂O, 0.1ml lysozyme (as above), and 0.1ml

EDTA (as above). After thirty minutes the samples are frozen and thawed three times and plated for phage.

Inactivation of Phage by Serum

A tube containing 0.9ml of anti- ϕ X serum, diluted in TB, is placed in a temperature controlled water bath and allowed five minutes to equilibrate. At time zero 0.1ml of phage, diluted in TB, is added. The phage titer is adjusted to be 2 to 5×10^6 /ml at the instant after it is delivered to the tube. At the appropriate times 0.1ml is removed from the inactivation mixture, and diluted one hundredfold into 10ml of TB at room temperature; an aliquot of this is now plated to determine the number of phage survivors. Phage inactivation follows the equation $P/P_0 = e^{-Kt/D}$ where P_0 is the phage titer at time zero, P is the phage titer at time t minutes, D is the dilution of the serum, and K is the inactivation rate constant in minute^{-1} (39). The K values are easily determined, since at a surviving fraction (P/P_0) of 0.37 the above equation becomes $K = D/t_{0.37}$.

SBP Assay

A standard curve is usually run with each set of unknown samples and is set up as follows: a stock of purified ϕ X⁺ is diluted to a particle concentration of 10^{10} /ml; the diluent is the medium in which the unknowns are contained (most often TB or sucrose solutions). This is followed by nine serial twofold dilutions, which yield ten tubes spanning the range from 10^{10} to 2×10^7 particles/ml. 0.1ml from each of these dilution tubes is pipeted into separate

sterile 10mm x 75mm pyrex tubes (SBP tubes) already containing 0.1ml of anti- ϕX serum that has been diluted 7×10^4 (not counting the twofold dilution upon adding the standard phage) times (about $K=8 \times 10^{-2}$) in TB. A blank, run in duplicate, containing 0.1ml of the diluent in which the phages are diluted, plus 0.1ml TB, and a control tube containing 0.1ml diluent plus 0.1ml serum are also prepared. Each tube is tightly stoppered with a sterile solid rubber (000) stopper, and set in a 48°C incubator for 16 hours (8 hours at 48°C gives essentially the same results as 16 hours, but 8 hours at 37°C is not sufficient for the reaction to reach completion).

The residual neutralizing power of the serum is measured by addition of 0.1ml of tester phage (from a crude stock of $\phi X_{H_A H_B}$), diluted in TB so that its concentration is about 5×10^6 /ml at the instant after it is delivered to the tube. After the tester phages are added the SBP tubes are placed in a 48°C water bath, and after each has incubated exactly two hours 0.1ml of its contents is removed and diluted into 10ml cold TB. Each of these is now titered for surviving tester phage by plating on Bresch plates, with Bresch top agar, and E. coli C_{ab} as the indicator bacteria. Under these conditions the plating efficiency of ϕX^+ is 10^{-6} that of $\phi X_{H_A H_B}$, and hence none of the ϕX^+ still active in the SBP tubes form plaques.

Only 50 to 60% of the tester phages survive two hours at 48°C , in the complete absence of serum, as evidenced by the titers of the blanks. The surviving fractions of the tester phages in the ten tubes composing the standard curve are computed by dividing the titer in each tube by the averaged titers of the blanks. These values are not true surviving fractions; rather they represent the amount of inactivation due to the effects of the residual serum only, and eliminate the contribution of heat inactivation. The survival of testers in the control tube containing serum, but no blocking antigen, is computed

in the same way, and is 5 to 10%. The standard curve is a semilog plot of tester survival versus the number of ϕX^+ particles/ml initially added to each SBP tube (see Figure 2).

Samples possessing unknown amounts of SBP are assayed by mixing 0.1ml of sample with 0.1ml diluted serum and proceeding in precisely the fashion just outlined for the standards. The survival of testers in each of the unknown SBP tubes is equated to ϕX^+ phage equivalents/ml (the unit of SBP) by use of the standard curve. Samples with SBP titers greater than 10^{10} /ml are diluted until they are in the range covered by the standard curve. About 5×10^7 phage equivalents/ml is the lower limit of SBP detectable by the assay.

In some of the experiments 0.5ml of serum (10^5 dilution) and 0.5ml of either standard phage or unknown sample is incubated 16 hours at 48°C in a stoppered sterile 12mm x 100mm tube. In these experiments 0.1ml of tester phage is added to a final concentration of about 5×10^6 /ml.

Sucrose Gradient Centrifugation

5% and 20% (w/v) sterile sucrose solutions, in 0.05M Tris-Cl pH 7.5, are used to prepare linear sucrose gradients. The gradient making apparatus is an all glass U-tube type device with a stopcock between the two arms of the U and another stopcock at the outflow arm; in principle the apparatus is exactly like the one described by Britten and Roberts (40). 25ml gradients are made by starting with 12.5ml of 20% sucrose in the outflow arm and 12.5ml of 5% sucrose in the other arm of the device. The gradients are formed at 4°C in 1 inch x 3 inch nitrocellulose centrifuge tubes. The gradients are allowed to sit at 4°C for 15 to 18 hours before 1ml samples are

layered on top of them. The samples are prepared as described under Growth Curves. However, before layering on the sucrose gradients they are centrifuged at 10,000 rpm for ten minutes to remove a granular white precipitate which often forms after the three cycles of freezing and thawing. About 80% of both the SBP and infective phage are recovered in the supernatant solution.

The gradients are now placed in a SW 25 rotor and centrifuged for 3.5 hours, at 37,000 rpm, at 5 to 6°C, in a Spinco model L. A fractionating device that makes use of a Technicon peristaltic pump, described in detail by Leif and Vinograd (41), is used to collect 23 to 26 fractions of identical volume, at 4°C, after centrifugation. The fractions are stored at 4°C until they are assayed.

RESULTS

PART I - THE SERUM BLOCKING POWER ASSAY

Serum Inactivation of ϕX^+ and $\phi XH_A H_B$

It seems appropriate to begin by describing the serum inactivation of ϕX , since it is upon this reaction that the SBP assay is based. Figure 1 shows serum inactivation curves of ϕX^+ and $\phi XH_A H_B$, the tester phage, at 48°C . These two types of ϕX are inactivated with essentially the same kinetics, and hence are probably serologically identical. The K value of the serum against both phage types, at this temperature, is about 5700 min^{-1} . The small differences between the two curves in their last two points can be attributed to the sampling error in plating. Since only 1 to 3% of the phage have survived when the last two points are taken only 50 to 100 plaques result when they are plated; the plates from which survival at the first four times is determined have 200 to 300 plaques each. The slight upward concavity of the curves has previously been observed in ϕX serum inactivation (10, 42).

The SBP Standard Curve

Two SBP standard curves are shown in Figure 2. The standard phage stocks contained 3% and 20% plaque formers for Curves A and B respectively. Curve A was set up by the 0.1ml standard phage plus 0.1ml serum procedure, Curve B by the 0.5ml standard phage plus 0.5ml serum procedure. The two curves agree fairly well over the range 5×10^7 to 5×10^9 ϕX^+ particles/ml (they differ by no more than a factor of two at any point).

The control tubes containing serum, but no added ϕX^+ blocking antigen, gave tester survivals of 0.1 for Curve A and

0.06 for Curve B. This coincides with the survival obtained with 2 to 5×10^7 added ϕX^+ blocking particles/ml. Essentially complete blockage of the inactivating power of the serum is achieved with 5×10^9 to 10^{10} ϕX^+ particles/ml, which gave a tester survival of 0.8 to 1.0. In practice 5×10^7 ϕX^+ equivalents/ml is considered the lower limit of the assay, and 5×10^9 ϕX^+ equivalents/ml the accurately measurable upper limit.

Most of the experiments to be presented have been done by the 0.1ml plus 0.1ml procedure. In conjunction with the experiments several dozen standard curves, with standard phage stocks ranging from approximately 1 to 25% plaque formers, have been run. These curves are all very similar to Curve A of Figure 2. It seems clear from these results that both the active and inactive phage in a purified ϕX^+ preparation decrease the neutralizing potency of anti- ϕX serum with equal efficiencies. Thus all the standard curves are plotted in terms of total ϕX^+ particles/ml rather than infectious titer/ml.

The unknown samples are unpurified lysates, in TB medium, prepared as described under Growth Curves, or unpurified lysates that have been spun at low speed and then centrifuged through a sucrose gradient. The former samples contain bacterial debris, and the lysozyme and EDTA used in the lysing procedure. The samples from sucrose gradient experiments are all contained in sucrose of various concentrations, ranging linearly from 20% (at the bottom of the centrifuge tube) to 5% (at the top of the tube), depending on the portion of the gradient they come from. Furthermore, the sucrose gradient samples still contain the lysozyme and EDTA (these would be expected only in the topmost fractions of the gradient) and perhaps remnants of bacterial debris.

Naturally it is important to know how these different constituents effect the SBP assay. To this end a number of control standard curves (set up by the 0.1ml plus 0.1ml method) have been run. A standard curve in which the diluent is a lysate of uninfected cells, grown in TB medium, and lysed by the lysozyme-EDTA, freeze and thaw procedure is essentially like Curve A of Figure 2. In fact bacterial debris, in TB medium, does not alter the kinetics of the $\phi X_{H_A H_B}$ serum inactivation shown in Figure 1. In this regard it is probably significant that the TB medium is a poor one for adsorption of ϕX to bacteria. Separate standard curves in which the diluent is either 5%, or 12.5%, or 20% sucrose in Tris buffer are also similar to Curve A of Figure 2. It appears that bacterial debris, lysozyme, EDTA, and sucrose do not profoundly affect the SBP assay. In general then when a series of unknown samples from an unpurified lysate in TB are run, a standard curve in which the diluent is TB is run in parallel; when the unknowns are fractions from a sucrose gradient, a standard curve in which the diluent is 12.5% sucrose in Tris buffer is run in parallel.

Other controls have shown that ϕX single stranded DNA is not measured in the SBP assay.

The SBP of 70S and 5S

Lysates of ϕX infected cells would be expected to have ϕX coat protein in several forms other than complete virus particles. For example it is known that the 70S particles exist; it seems likely that chemical subunits, or subunits like 5S, or more highly aggregated subunits are also present and may possess SBP.

Since purified preparations of 5S and 70S were available it was possible to test them for SBP. Both of these preparations, though extremely pure chemically, still contained about 10^6 infectious ϕ X particles/ml. The 5S was freed of phage by filtration through a VF millipore filter (mean pore size $10\text{m}\mu$). No attempt to remove the residual phage from the 70S preparation was made. However, it is certain that essentially all the material is 70S, because first the UV adsorption spectrum is that of 70S, and second the ratio of infectious ϕ X particles to 70S particles is 1 to 10^5 , which means that the ϕ X present would have to be $10^{-3}\%$ plaque formers (an unlikely low figure) for there to be a 1:1 ratio of total ϕ X particles to 70S particles.

Figure 3 shows standard dilution curves for 5S and 70S which are plotted in terms of ϕ X protein equivalents/ml and thus are directly comparable to the ϕ X standard curve also shown for comparison. It is immediately obvious that 5S, even at a concentration of 10^{11} ϕ X protein equivalents/ml, has no SBP. At all concentrations of 5S examined a tester survival of about 0.06 is obtained, which is identical, in this experiment, to that of the control containing serum but no added ϕ X antigen. The absence of SBP in 5S appears to mean that these subunits do not possess the antigen(s) necessary for ϕ X adsorption. An alternative speculation is that the adsorption antigen(s) although originally present, has somehow been distorted or destroyed by the prolonged treatment at high pH used to produce the 5S subunits.

A somewhat similar situation has been observed in other small spherical viruses. Rappaport *et. al.* (43) reported that subunits of turnip yellow mosaic virus are antigenically distinct from complete virus and top component (empty protein coats). These authors suggested that significant conformational changes resulting from

assembly of subunits into virus particles might account for their results. Scharff et. al. (44) have shown that degradation of poliovirus with guanidine leads to a subunit of the coat protein which is antigenically distinct from either complete virus or empty coats. Furthermore, they found a soluble protein synthesized in poliovirus infected cells that was antigenically similar to the guanidine prepared subunits.

70S particles clearly do have SBP, and it seems as though the assay registers them with roughly one half the efficiency of intact ϕX , over the range 3×10^8 to 3×10^9 particles/ml. The reason for the large amount of scatter in the points of the 70S curve above 3×10^9 equivalents of ϕX protein/ml is unknown.

It will be shown, in Part IV, that subunits of about 15S which are detected in the SBP test appear in ϕX lysates. Since a purified preparation of these subunits was not available it is impossible to estimate their specific SBP relative to whole phage.

Duplicate ϕX standard curves sometimes vary by as much as + a factor of 2 (but rarely more than 2) at any given point, and thus no great significance can be placed on the observed relative efficiencies of measuring whole phage, and 70S particles in the SBP assay. In all the experiments that follow SBP titers are expressed relative to ϕX standard curves, and no efforts are made to correct for possible differences in SBP efficiency of the several forms in which ϕX coat protein is found.

To briefly summarize the findings of this section:

- 1) Viable and nonviable whole ϕ X particles are detected with equal efficiencies in the SBP assay.
- 2) 5S subunits are not measured at all.
- 3) 70S subunits are measured, perhaps with 50% the efficiency of whole ϕ X.
- 4) 15S subunits are measured, but the efficiency is unknown.

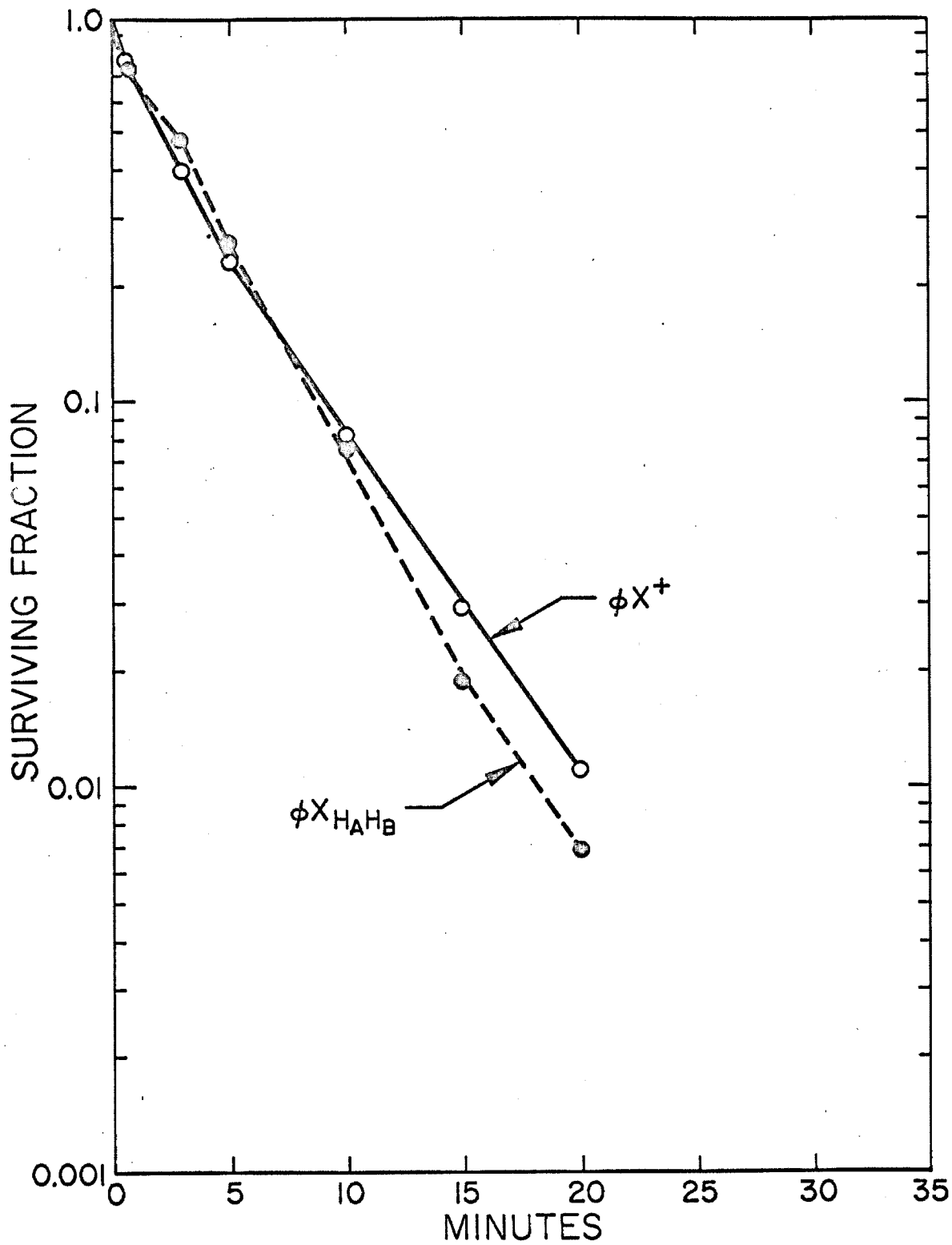


Figure 1

Legend to Figure 1

Serum inactivation of ϕX^+ (0—0) and $\phi X_{H_A H_B}$ (●---●) at 48°C.
The serum is diluted 2×10^4 times in TB. Survivors for both curves,
which are run independently, are assayed on tryptone plates with
E. coli C as the indicator bacteria.

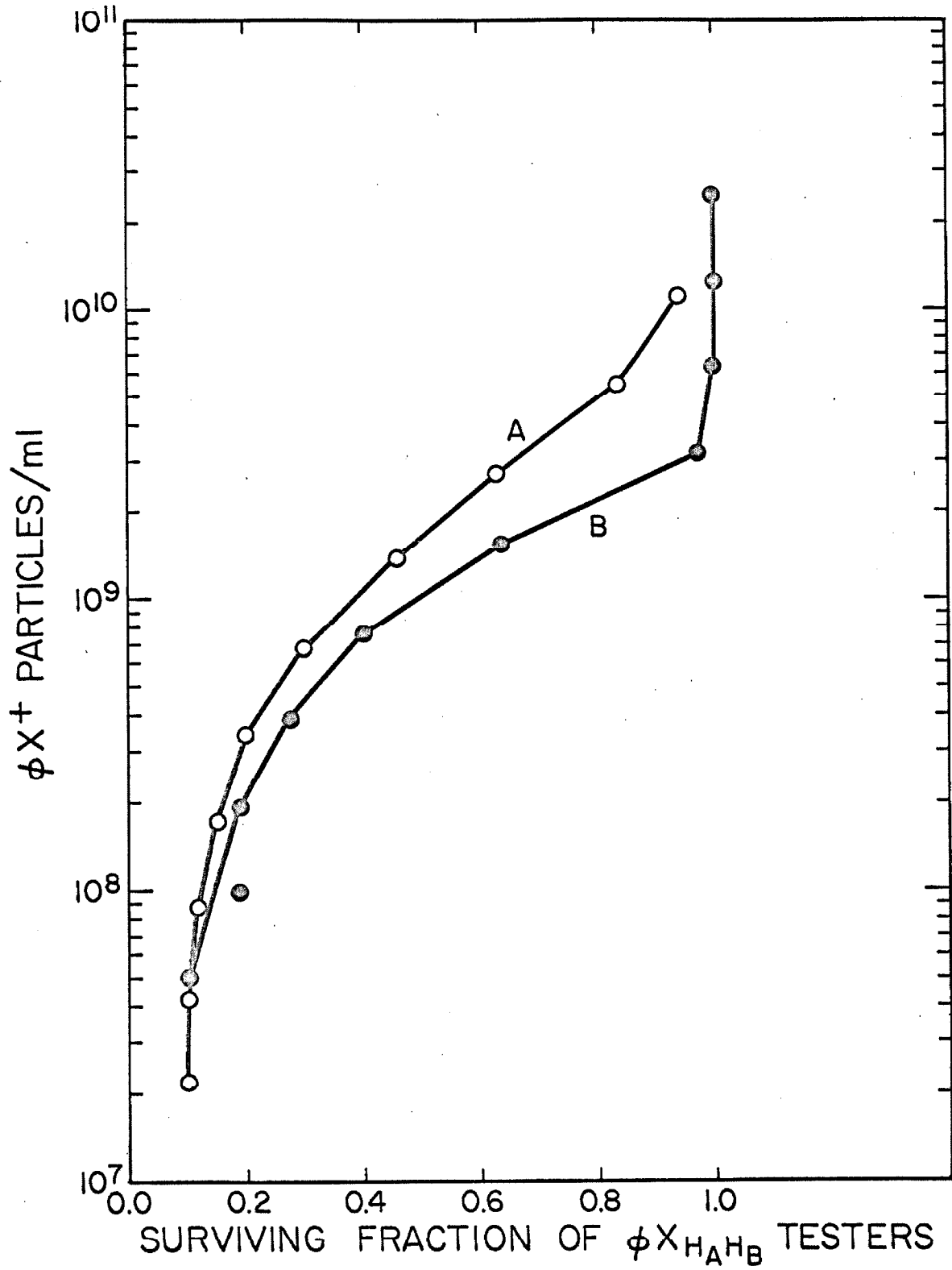


Figure 2

Legend to Figure 2

SBP standard curves. Curve A was run on 6 July 1965 according to the 0.1ml standard phage plus 0.1ml serum procedure. The standard phage stock contained 3% plaque formers. Curve B was run on 26 July 1962 according to the 0.5ml standard phage plus 0.5ml serum procedure. The standard phage contained 20% plaque formers.

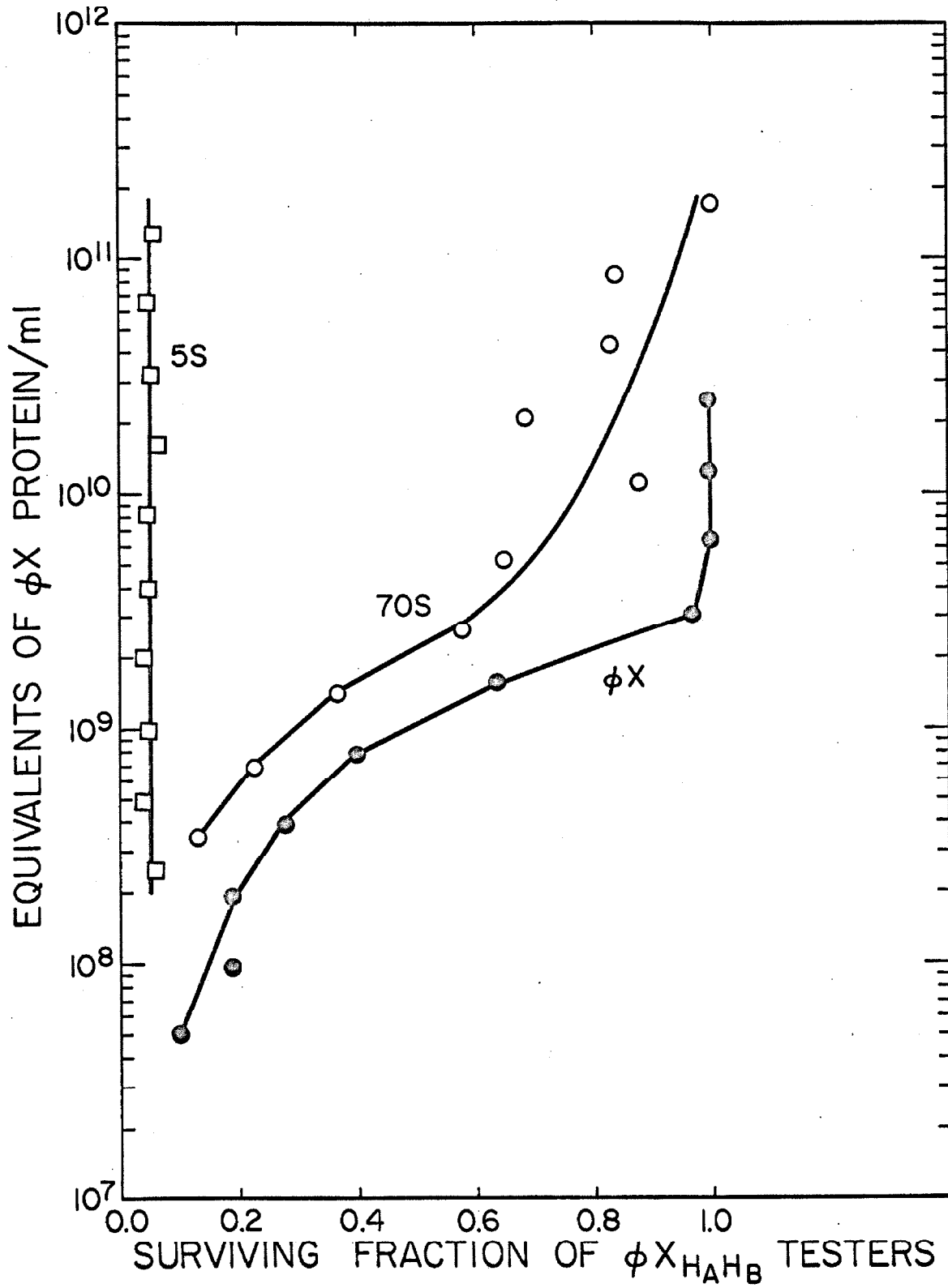


Figure 3

Legend to Figure 3

SBP standard curves for ϕX (●—●), 70S (○—○), and 5S (□—□). These are done by the 0.5ml plus 0.5ml method. The ϕX curve is identical to Curve B of Figure 2. Both the 70S and 5S are from purified preparations of known protein concentrations, and are plotted in terms of ϕX protein equivalents/ml. The conversion factor between protein concentration and ϕX particles is 7.6×10^{-12} protein/ ϕX particle (5).

PART II - SERUM BLOCKING POWER SYNTHESIS IN ϕX^+ INFECTED
CELLS

This section contains results on ϕX^+ growth and SBP development under normal conditions, and also in the presence of several metabolic inhibitors.

Growth Curves at 37°C

Figure 4 shows the time course of infective centers, intracellular phage, and SBP development in *E. coli* C infected by ϕX^+ at 37°C, at a multiplicity of 5. The minimum latent period is 14 min, the end of the eclipse period (the time at which there is an average of one intracellular phage per infected cell) occurs at 6 min, and the average burst size (the average yield of phage per infected bacterium) is 133.

As early as 2 min after development has begun there are 14 phage equivalents of SBP per infected cell. However, it seems unlikely that much, if any, of the ϕX coat protein present at this time is the result of de novo synthesis during the first two minutes of development. The 14 equivalents of SBP per infected cell are probably due to a background of coat protein present in the infecting phage stock. The reasoning behind this statement is as follows: if it is assumed that the SBP of adsorbed phage is the same as that of free phage then the expected amount of background coat protein at time zero should be equal to the sum of the active plus inactive input phage particles that adsorb to the bacteria. Adsorption of the plaque formers is always in excess of 90%, under the conditions of infection used. However, a variable fraction (0.22 and 0.55 in two independent

measurements) of the non-plaque formers adsorb, as will be demonstrated in Part IV, where experiments that utilize a purified ^{35}S labeled stock of ϕX^+ that is 20% active are presented. In the experiment of Figure 4 the multiplicity of infection is 5 (this is based on plaque forming ability only) and the phage stock is 20% active. The expected background of phage coats due to the plaque formers is 9×10^7 /ml. The contribution from inactive particles is harder to assess, since the fraction that adsorb is variable. But, if it is assumed that 50% of the inactive particles adsorb, then the background of phage coats they give is $(3.6 \times 10^8$ /ml) (0.5) or 1.8×10^8 /ml (in a 20% active stock there are 4 non-plaque formers for each plaque former, which gives rise to the factor 3.6×10^8 /ml above). Thus, the total predicted background would be $(9 \times 10^7$ /ml) + $(1.8 \times 10^8$ /ml) or 2.7×10^8 /ml, which is agreeably close to the observed SBP titer of 2.5×10^8 /ml at 2 min. The idea that the 2 min SBP is attributable to background material seems reasonable. Neither Figure 4 nor any of the SBP curves that follow are corrected for background.

Another argument against the 2 min SBP being the result of de novo synthesis is that the first RF is not completed until 3 minutes (18), and no ϕX specific protein synthesis should take place before RF is present (22).

At 5 min after development has begun a threefold increment in SBP has occurred and there are 44 phage equivalents of coat protein per infected cell. Between 5 and 8 min there is a fourfold increase in SBP, and at 8 min there are 160 phage equivalents of coat protein per infected cell. The SBP and intracellular phage titers both continue to increase up until about 25 min, at which time all the infected cells have lysed. At the end of the growth cycle there are 2000 phage equivalents of SBP per infected cell. Unfortunately these

data are not detailed enough to permit an accurate determination of the time at which infected cells first begin making ϕX coat protein. A rough estimate, based on these data, is 5 min.

A striking feature of Figure 4 is that the SBP titers exceed the intracellular phage titers at all times. At 2 min the ratio of SBP/intracellular phage is 250. At 5 min the same ratio is 90, at 8 min it is 30, at 10 min it is 17, and at all times from 13 to 55 min it is approximately 13. The large ratio at 2 min is in a sense an artifact; it is due to the combination of significant amounts of background SBP, and the intracellular phage being at their furthest stage of eclipse. The ratios of SBP/intracellular phage during the first 10 min of development depend, to a degree, on the extent of eclipse achieved by the intracellular phage, and the quantities of background protein added with the infecting phage stock. The maximum eclipse in the intracellular phage curve of Figure 4 is a factor of 18 (at 2 min) below the infective centers baseline. The maximum eclipse observed in other experiments is often a factor of 100, or more, below the infective centers baseline. The end of the eclipse period, however, invariably occurs at 6 to 8 min, at 37°C . It has been reported (20) that the eclipse period for ϕX ends at 10 min, at 37°C ; the reason for the small difference between this value and the one obtained in the present work is not clear.

Despite variations, in different experiments, in the maximum eclipse and amounts of background coat protein present, the pattern just described is qualitatively quite reproducible; that is at early times a high ratio of SBP/intracellular phage is seen. This is followed by a gradual decline until at about 14 min a ratio of 13 is reached. For the remainder of the growth cycle the ratio SBP/intracellular phage persists at about 13.

It is not surprising that SBP is always in excess of intracellular phage. For, intracellular phage is a measure only of plaque forming ability, while SBP measures whole phage, be they plaque formers or not, and 70S particles, and 15S subunits. One obvious meaning of the above results is that at all times after infection the ϕ X SBP in the form of complete plaque forming viruses is never more than about 7% of the total amount of SBP present. The higher ratios of SBP/intracellular phage at earlier times, as compared to later times, means that a greater proportion of the coat protein is in a form other than infectious virus.

Figure 5 contains the same SBP and intracellular phage data as Figure 4, but in 5 they are normalized to the number of infective centers at time zero and plotted on linear scales. Over the time span 12.5 to 19 min both curves are straight lines; hence, SBP and intracellular phage are being synthesized at constant rates. SBP synthesis, during this interval, occurs at a rate of 108 phage equivalents/min, the rate at which intracellular phage are made is 13 phage/min.

Growth curves qualitatively similar to those of Figure 4 have been obtained at low (0.1) multiplicity of infection. However, these curves are complicated by the fact that they do not level off at late times; instead all the titers keep on rising, at a rate somewhat slower than the rapid rise observed in the first cycle of growth. This increase in titer at the end of one cycle of growth is due, no doubt, to second cycles of infection, since in a low multiplicity experiment most of the bacteria are initially uninfected. It is interesting to note that the second cycles of infection take place in spite of the cells being in the TB medium, which is a poor medium for ϕ X adsorption because it lacks calcium. Apparently the procedure

(see legend to Figure 4) of growing cells in KC broth, which contains calcium, starving and infecting in the starvation buffer, which also contains calcium, followed by washing and resuspending in TB medium, the medium in which development and lysis takes place, is not sufficient to prevent reabsorption.

Growth Curves at 30 and 40°C

Figure 6 contains growth curves of ϕX^+ at 30 and 40°C. These are shown so that a comparison between the growth curves of ϕX^+ and several ts mutants (growth curves of these will be presented in Part III) can be made later on. At 30°C the minimum latent period is about 32 min, the end of the eclipse period is 18 min, and the average burst size is roughly 100. At 40°C the minimum latent period is about 15 min, the end of the eclipse period is 12 min, and the average burst size is 100. The maximum eclipse for both intracellular phage curves is more than a factor of 500 below the infective centers baseline.

During the first 8 min of development the 30°C SBP curve remains constant at about 5×10^8 phage equivalents/ml. Between 8 and 60 min the SBP titer gradually rises, and eventually reaches 10^{10} /ml. The final yield of SBP per infected cell is about 1700. The SBP/intracellular phage ratio at 2 min is greater than 5×10^4 , and at 60 min it is 17.

The SBP titer at 40°C remains constant, at 3×10^8 /ml, for the first 8 min of development. From 8 to 60 min the titer rises and attains a maximum of 1.7×10^{10} /ml. The final yield of SBP per infected cell is about 3000. The SBP/intracellular phage ratio at 2 min is 3×10^4 , and at 60 min it is about 30.

The infection in this experiment was done with a ϕX^+ stock that contained only 4% plaque formers. Thus a large background of coat protein, due to the non-plaque formers was to be expected. A calculation like the one described for the 37°C growth yields 2.3×10^8 phage equivalents/ml as the predicted titer at time zero. This is reasonably close (considering the ambiguity as to the number of inactive particles that adsorb) to the observed values between 2 and 8 min for both the 30 and 40°C curves. The reason for the small difference between the two curves over this time interval is not understood. The background of ϕX coat protein is, in fact, so high in this experiment that the kinetics of SBP synthesis during the first 8 min of growth are completely obscured. At later times the SBP titers do, however, rise more than ten times above the background level.

Growth Curves at 37°C in the Presence of Chloramphenicol

Chloramphenicol (CAP) at concentrations as low as 10 µg/ml is a potent inhibitor of microbial protein biosynthesis (45). There is some evidence that it acts by interfering with the attachment of messenger RNA to ribosomes (46). It was decided to test the effects of CAP on ϕX coat protein synthesis. In a sense this experiment is a control to determine that the SBP assay truly is a measure of protein synthesis. Of course the anticipated result is that SBP synthesis will be completely inhibited. A SBP standard curve in the presence of 30 µg/ml of CAP was identical to the standard curves in the absence of CAP.

Figure 7 shows that CAP at a concentration of 30 µg/ml effectively inhibits the production of progeny virus as well as SBP. The SBP titer remains constant at the background level, for this experiment,

of 8.5×10^7 /ml until at least 40 min after development is initiated. Infective centers and intracellular phage also remain constant at 8.5×10^6 /ml and 2.8×10^5 /ml respectively, at all times examined. The observed intracellular phage titer may well be due to residual unadsorbed phage that have not been removed by the procedure of spinning down the infected cells and discarding the supernatant. The measured unadsorbed phage titer (on the supernatant in the above procedure) was 1.5×10^6 /ml. The intracellular phage titer (2.8×10^5 /ml) is 20% that of the unadsorbed phage. If it is assumed that only 80% of the unadsorbed phage have been removed (which is reasonable) then the intracellular phage titer can be completely accounted for by the small number of residual unadsorbed phage. That the titer of infective centers remains constant must mean that the deleterious effect of CAP is overcome upon dilution and plating out. Or, put another way, a bacterium infected by ϕX in the presence of CAP becomes capable of producing infectious phage (since it yields a plaque) upon removing it from the CAP containing medium and plating it in the absence of CAP.

The average burst size, minimum latent period, and end of the eclipse period observed on the control curves, in the absence of CAP, in Figure 7, all agree very well with the values obtained from Figure 4. The SBP curve starts out normally, but at 10 min abruptly levels off for some unknown reason. Despite the atypical halt in net synthesis of SBP at 10 min there has already occurred a 20 fold increase in the total SBP per infected cell, as opposed to no increase in the presence of CAP.

Since RF is synthesized in the presence of CAP, this experiment also demonstrates that the RF molecule lacks SBP. One last point in regard to Figure 7 is that the infective centers titer, even

in the control curve, is only about one half the expected number. It is possible that some losses are incurred in the process of sedimenting and resuspending the starved, infected cells. This, however, seems unlikely because uninfected cells can be completely recovered by sedimenting and resuspending; and low multiplicity experiments usually give the expected number of infective centers at time zero. It seems that in some experiments at high multiplicities of infection many of the potential infective centers are somehow lost.

Growth Curves at 37°C in the Presence of 5-fluorouracil Deoxyriboside

5-fluorouracil deoxyriboside (FUDR) is an inhibitor of DNA synthesis which is known to induce thymineless death in *E. coli* (47). In the bacteria FUDR is converted to 5-fluorouracil deoxyribotide (FUDRP); this nucleotide irreversibly inactivates the enzyme thymidylate synthetase (47). Normally this enzyme mediates the conversion of deoxyuridylic acid to thymidylic acid, by methylation of the 5 position of the pyrimidine ring with formaldehyde activated by tetrahydrofolic acid. This inhibition is exclusively a property of FUDRP, neither 5-fluorouracil (FU), nor 5-fluorouracil riboside (FUR), nor phosphorylated derivatives of FUR induce a thymine deficiency; they must first be converted to FUDRP before they exert any effect on DNA metabolism. However, it is known that FU can be extensively incorporated into the RNA of *E. coli* (48), and result in modification of proteins, and even reverse the phenotype of certain T4 phage mutants (49). It is also a fact that when FUDR is given to bacteria a small fraction of the material that gets into the cells is degraded to FU (47). Thus, for FUDR to act as a specific inhibitor

of DNA synthesis it is best used in combination with small amounts of uracil to avert the possible effects on RNA metabolism.

The interest in FUDR in relation to ϕ X growth was to determine whether ϕ X DNA alone, in the absence of RF, could produce SBP. An infection done in the presence of FUDR will not permit even the first RF molecule to be made. Figure 8 shows such an experiment. It is clear that no SBP is made. For as long as 100 min the titer remains constant at the background level of 10^8 phage equivalents/ml. Furthermore, about 90% of the infective centers present at time zero have been killed by 60 min. Thus FUDR, in contrast to the results seen with CAP, is irreversibly killing most of the infective centers. Since the intracellular phage decline faster and further than the infective centers, unadsorbed phage can account for only a small fraction of the infective centers. The more extensive decline in intracellular phage is probably due to residual unadsorbed phage adsorbing to cells.

It is tempting to conclude from this experiment that single stranded ϕ X DNA is not the template for functional ϕ X messenger RNA synthesis. This agrees with the result of Hayashi et. al. (22) which strongly implied that the DNA strand complementary to ϕ X DNA, in the RF, is the template for messenger RNA synthesis. It must be admitted, though, that the result presented here is, for two reasons, somewhat tentative. First, the effect of FUDR on the SBP assay itself is not known. This could be easily checked by running a standard curve in the presence of FUDR. Second, the TPG plus FUDR medium used was not supplemented with uracil; which leaves open the possibility that messenger RNA really was made, but was not functional, or produced a ϕ X coat protein so altered that it was not detected in the SBP assay.

Judging from the work of Cohen et al. (47) about 50% of the FUDR administered gets into cells, and of this only 10% is converted to FU and various ribose derivatives of FU. In the present experiments this might mean that the bacteria have roughly 1 μ g/ml of FU and its derivatives within them. The concentrations of FU used to affect RNA metabolism in *E. coli* are in the range 10 μ g/ml to 50 μ g/ml (48, 49), the actual quantities that enter the bacteria are no doubt lower. From these considerations it seems unlikely that the complete inhibition of SBP synthesis is due entirely to messenger RNA altered by FU incorporation.

Of interest here is the observation of Ebisuzaki (50) that FUDR does not prevent the synthesis of SBP in T4-infected cells. But, since the DNA of this phage is double stranded the result in no way conflicts with the data on ϕ X.

Growth Curves at 37^oC in the Presence of Phleomycin

Phleomycin is an antibiotic of unknown structure that is an inhibitor of DNA synthesis. Although the mechanism by which it acts is not clear it is known that it binds to adenine-thymine rich regions of DNA, in vitro (51). Cells infected by ϕ X in the presence of phleomycin are able to convert the parental DNA into a partially complete RF, but allow no further replication of DNA (52).

Figure 9 shows that cells containing the partially completed RF do not synthesize SBP. The points scatter about 4×10^8 phage equivalents/ml for the first 30 to 40 min, but by 50 min the titer has dropped, for some unknown reason, to 5×10^7 /ml. Unfortunately, no standard curve in the presence of phleomycin was run.

At -12 min, even before infection or addition of cyanide, phleomycin has already killed over 90% of the colony formers. However, in the experiments cited above (52) it appears that the cells which no longer form colonies still produce the incomplete RF upon infection. Any infective centers that are formed are rapidly killed to the level of residual unadsorbed phage.

These results are in a way complementary to the FUDR experiments and make it very probable that at least one complete RF must be made in order for SBP synthesis to occur.

The results of this section can be summarized as:

- 1) Much more SBP than is incorporated into infective virus is made during the course of infection. The ratios of SBP/intracellular phage at the end of the growth cycles at 30 and 37°C are both about 15. At 40°C the final SBP/intracellular phage value is about 30.
- 2) At 30 and 37°C about 2000 phage equivalents of SBP/infected cell are made. At 40°C about 3000 phage equivalents of SBP/infected cell are made.
- 3) CAP, FUDR, and phleomycin when present at the time of infection all produce essentially complete inhibition of ϕ X SBP synthesis.

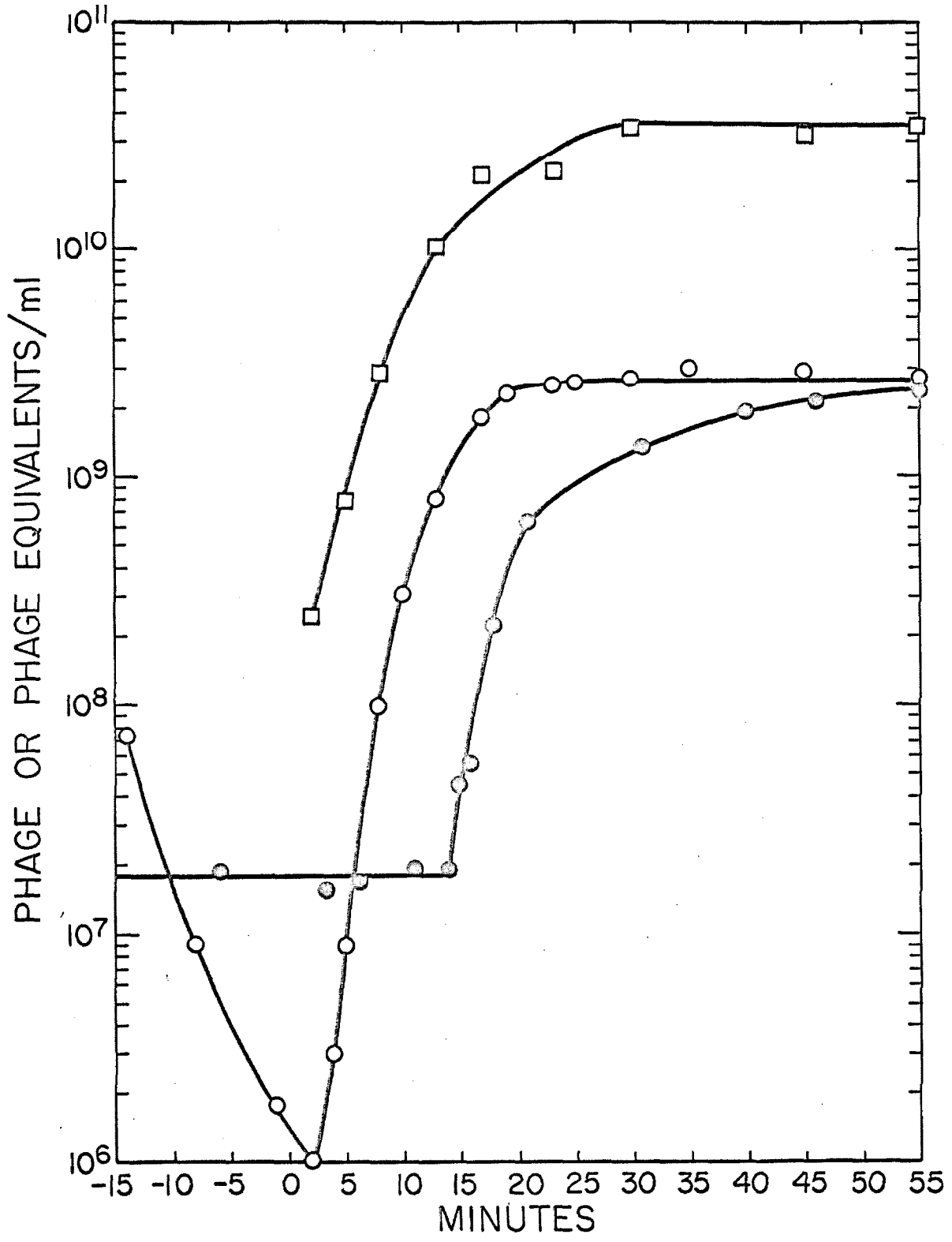


Figure 4

Legend to Figure 4

Growth curves of ϕX^+ at 37°C. Infective centers (●—●), intracellular phage (○—○), SBP (□—□). *E. coli* C was grown to 2×10^8 /ml in KC broth, starved for 60 min then infected at a multiplicity of 5. After allowing 15 min for adsorption 10 ml of the infected cells were spun out of the starvation buffer and the pellet resuspended in 10 ml of 0.1M-NaCl. (Unadsorbed phage was measured on the supernatant. In this experiment 98% of the input phage adsorbed). Phage development was initiated by diluting the infected starved cells tenfold into TB prewarmed at 37°C. The titers shown in the time span -15 to 0 min were corrected (multiplied by 10^{-1}) for the difference in concentration of infected cells before and after time zero. The samples were prepared and assayed as described under Growth Curves. In this and all subsequent experiments, on the time course of SBP development, the infective center titers are adjusted to be approximately 10^7 /ml at time zero. This is because the lower limit of the SBP assay is 5×10^7 phage equivalents/ml, and working at higher levels of infective centers would only aggravate the problem of background coat protein added with the infecting phage stock (see text referring to this figure).

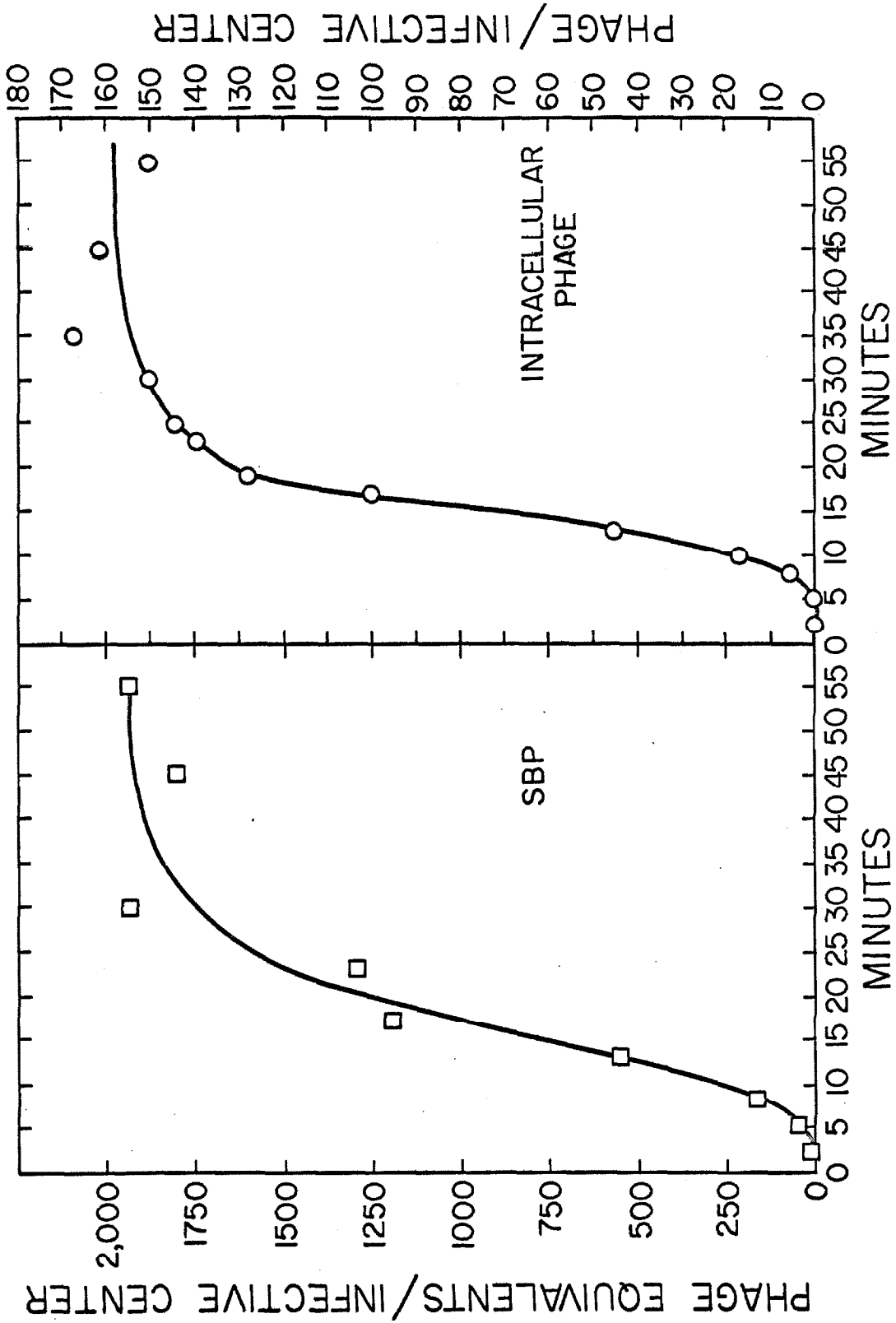


Figure 5

Legend to Figure 5

The data of Figure 4 normalized to the number of infective centers obtained at time zero (1.8×10^7 /ml) and plotted on linear scales.

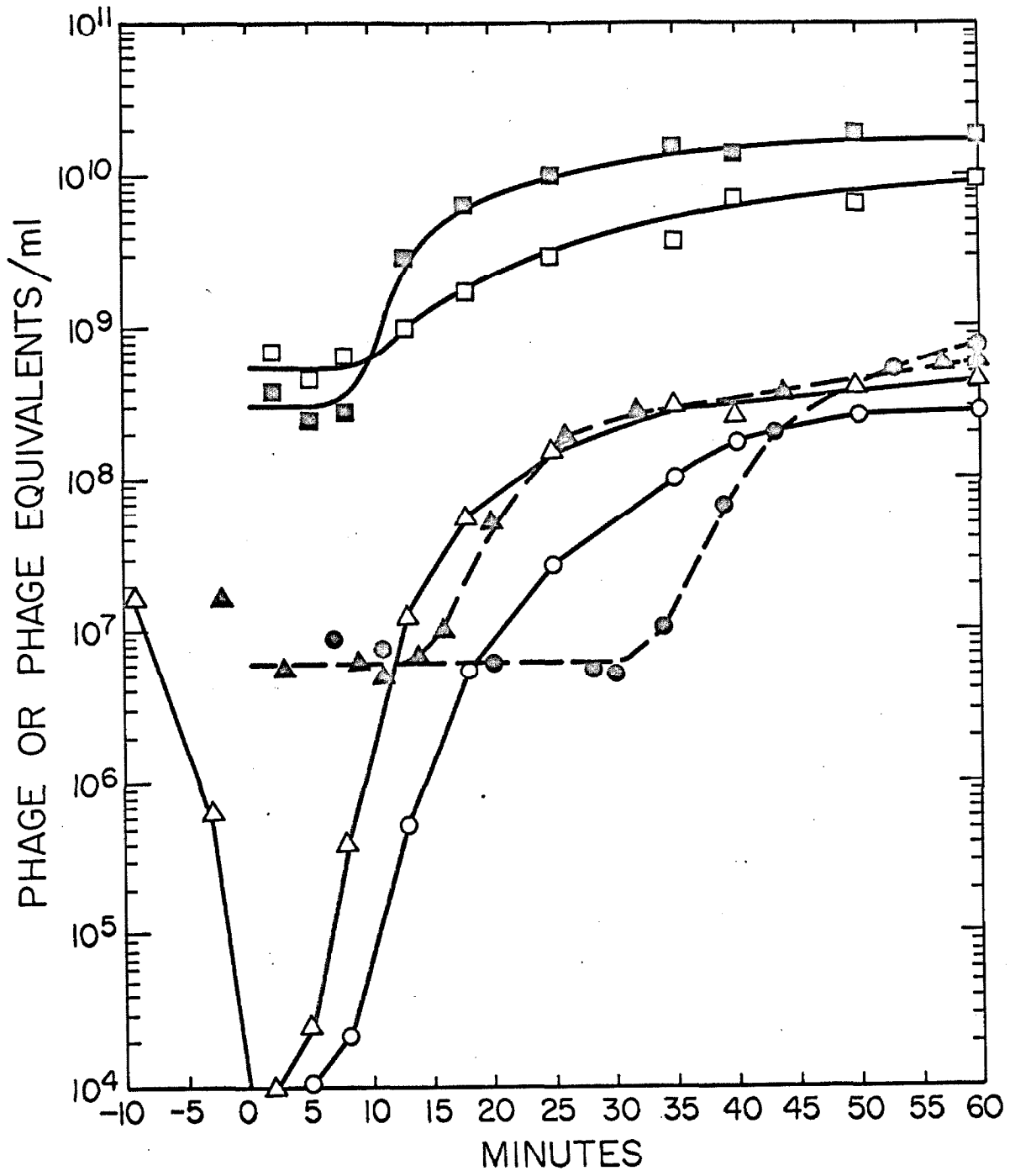


Figure 6

Legend to Figure 6

Growth curves of ϕX^+ at 30°C , infective centers (---●---), intracellular phage (—○—). SBP (—□—); and at 40°C , infective centers (----▲----), intracellular phage (—△—), SBP (—■—). *E. coli* C was grown to 3×10^8 /ml, in KC broth, at 37°C . At -15 min the culture was shifted to 40°C and cyanide was added. At -10 min enough ϕX^+ to give a multiplicity of 3 was added. At time zero 9ml of the infected culture was filtered onto an HA Millipore filter. (Unadsorbed phage was measured on the filtrate. In this experiment 99% of the input phage adsorbed). The filter was washed with 9ml 0.1M-NaCl, and then transferred to 9ml 0.1M-NaCl. After vigorous stirring 4ml of the washed infected cells were diluted 10 fold into TB prewarmed at 30°C ; another aliquot of 4ml was diluted 10 fold into TB prewarmed at 40°C . Titers between -10 and 0 min were corrected as in Figure 4. The samples were prepared and assayed as described under Growth Curves. The infective centers point at -2 min was 1.6×10^7 , and the infective centers baseline after time zero was 6×10^6 ; thus only 38% of the infective centers were recovered from the filter.

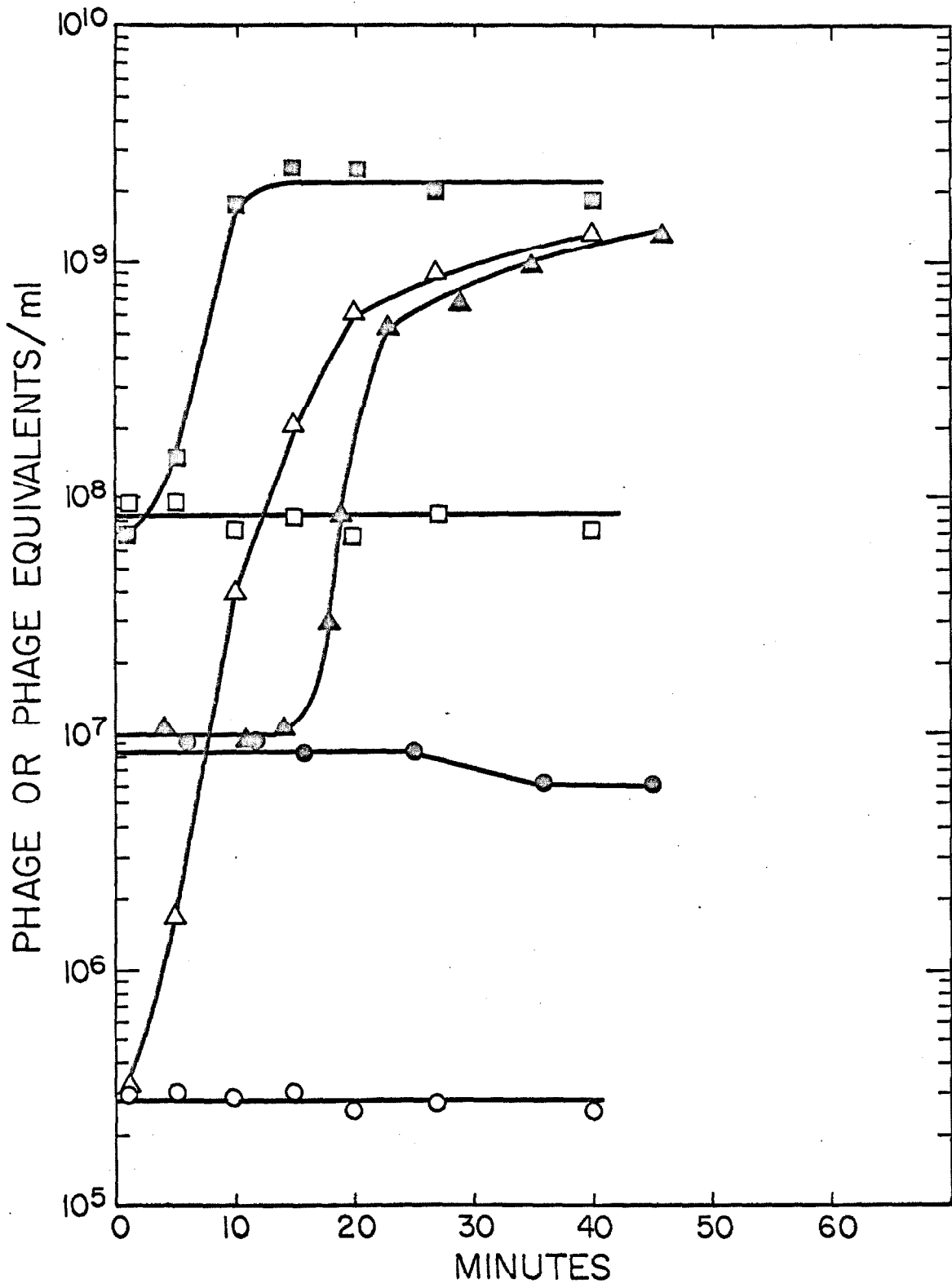


Figure 7

Legend to Figure 7

Growth curves of ϕX^+ at 37°C in the presence of chloramphenicol; infective centers (—●—), intracellular phage (—○—), SBP (—□—). Control curves in the absence of chloramphenicol are also shown; infective centers (—▲—), intracellular phage (—△—), SBP (—■—). The protocol was precisely the same as for Figure 4, with the following exceptions. A different phage stock, one that was 10% active, was used. The multiplicity was 4. After resuspending the starved, infected cells in 0.1M-NaCl the culture was divided into two parts; one of these was diluted into prewarmed TB containing 30 μ g chloramphenicol/ml, the other part into prewarmed TB without chloramphenicol. 99% of the input phage adsorbed.

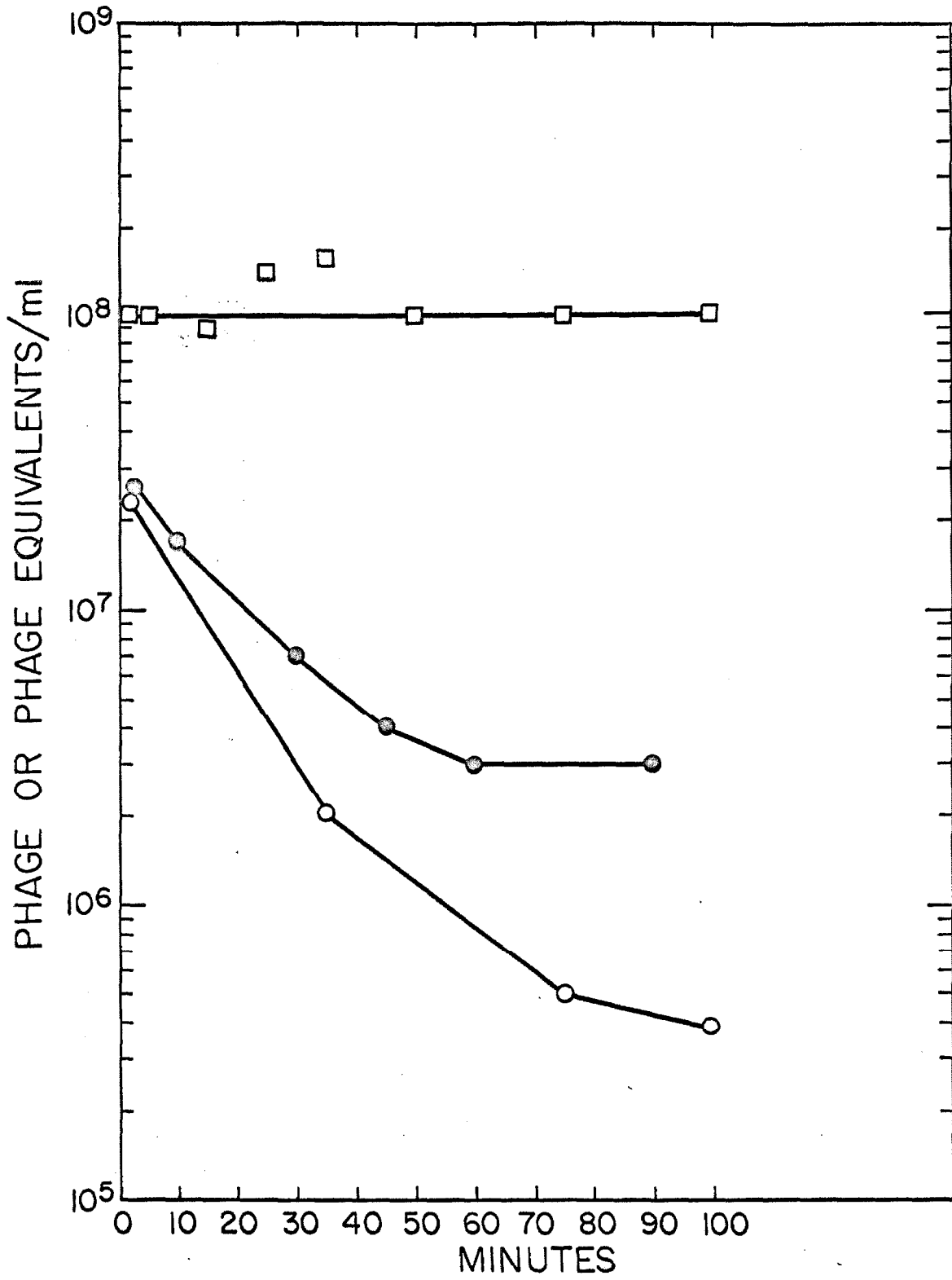


Figure 8

Legend to Figure 8

Growth curves of ϕX^+ at 37°C in the presence of 5-fluorouracil deoxyriboside (FUDR); infective centers ($\text{---}\bullet\text{---}$), intracellular phage ($\text{---}0\text{---}$), SBP ($\text{---}\square\text{---}$). *E. coli* CT^- was grown to $10^8/\text{ml}$ in the TPG medium supplemented with $50\mu\text{g}/\text{ml}$ of thymine. The cells were spun down and washed two times with TPG medium supplemented with $20\mu\text{g}/\text{ml}$ of FUDR. The washed cells were restored to their original volume in the TPG plus FUDR medium. After 15 min of aeration the cells were infected with ϕX (from a 15% active stock) at a multiplicity of 0.1. This corresponds to time zero. Unadsorbed phage were not removed in this experiment.

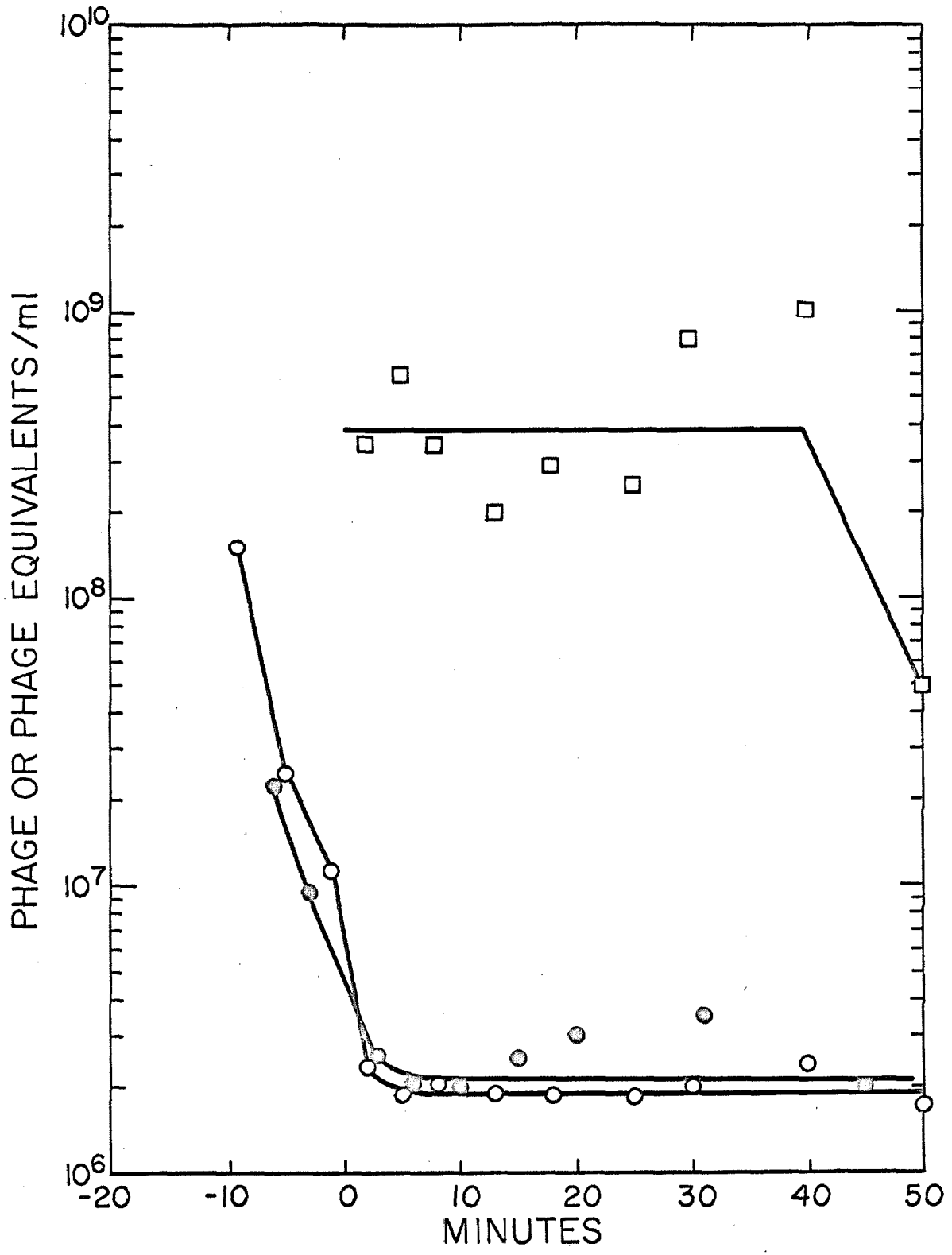


Figure 9

Legend to Figure 9

Growth curves of ϕX^+ at 37°C in the presence of phleomycin; infective centers (—●—), intracellular phage (—○—), SBP (—□—). *E. coli* C was grown to 10^8 /ml, in KC broth, and at -20 min enough phleomycin (from a 3mg/ml stock solution) to give a final concentration of $30\mu\text{g}/\text{ml}$ was added to 10ml of the culture. At -15 min cyanide was added, and at -10 min the culture was infected with ϕX (from a 10% active stock) at a multiplicity of 2. After allowing 10 min for adsorption the culture was filtered onto an HA Millipore filter, and then washed with 10ml of 0.1M-NaCl. The filter was resuspended in 2ml of 0.1M-NaCl (effecting a fivefold concentration of the infected cells), and after vigorous stirring the entire 2ml was diluted into 8ml of TB (restoring the original cell concentration) prewarmed at 37°C and containing $30\mu\text{g}/\text{ml}$ of phleomycin. 97% of the phage adsorbed. At -12 min the titer of colony forming cells was 7×10^6 /ml.

PART III - SERUM BLOCKING POWER SYNTHESIS IN CELLS
INFECTED BY ϕ X ts MUTANTS

Investigations on coat protein synthesis by ϕ X ts mutants were undertaken for two reasons. First, the mutants can be used as an important tool for defining the physiological functions dictated by the ϕ X genome. The combined results of studies on coat protein synthesis and genetic complementation tests should make it possible to identify the cistron(s) responsible for making the coat protein. Many of the ts mutants have already been studied, under nonpermissive conditions, with respect to their ability to induce cell lysis (53), and synthesize infective DNA (35); the heat stability of some of the mutant particles has also been investigated (53).

The second motive for studying coat protein synthesis by the mutants is related to the observation, recorded in Part II, that the ratio SBP/intracellular phage during the course of a wild type infection starts out at a high value (always in excess of 100) and then drops to a much lower value (15 to 30) by the end of the latent period. As explained before background coat protein is, in part, responsible for the high values of SBP/intracellular phage at very early times. During the interval 5 to 8 min (in Figure 4, for example) the SBP titer rises above the background level; the ratio SBP/intracellular phage however, is still significantly higher than at later times.

One hypothesis that might explain these results is that SBP is initially in the form of subunits which are precursors to the coat protein of mature phage. During the initial 5 to 8 min of development the rate of synthesis of these subunits would be much faster than the process of phage maturation. As the growth cycle continues the rate

of phage maturation increases (perhaps because other necessary components, like phage DNA, become available), material from the pool of precursor subunits is incorporated into whole phage, and the ratio of SBP/intracellular phage declines. It seemed reasonable that some of the ts mutants, which did make SBP under nonpermissive conditions, might synthesize much or even all of their SBP in the form of the hypothetical subunit. A lysate derived from such a mutant would probably be the best place to identify, isolate, and characterize this presumptive subunit.

Three complementation groups, based on ts mutants, have been identified in ϕX (53). The mutants studied in detail in this thesis are ts 4, 9, γ , and 79. The mutants γ and 79 are in the same complementation group, ts 9 is in a second group, and ts 4 in the third of the ts complementation groups.

Figure 10 shows that the three ts mutants 4, 79, and γ are all inactivated by anti- ϕX^+ serum with essentially the same kinetics as the tester phage, $\phi XH_A H_B$. In the first set of curves the serum has a K of 5000 min^{-1} against both $\phi XH_A H_B$ and ts 79. For the initial 3 min of inactivation both these curves are identical, but for the remainder of the experiment ts 79 is inactivated at a slightly slower rate than $\phi XH_A H_B$. In the second set of curves the serum has a K of 5400 min^{-1} against $\phi XH_A H_B$, ts γ , and ts 4. The small difference between the K of the serum measured against $\phi XH_A H_B$ in the two sets of curves is not significant; and shows that the serum inactivation of ϕX proceeds at the same rate in TB and KC broth.

Since these ts mutant particles are inactivated by anti- ϕX^+ serum it seems almost certain that they will produce protein measurable by the SBP assay, when grown at 30°C . Whether they make SBP at 40°C is the principal concern of Figures 11 to 14.

These figures show detailed growth curves of the ts mutants at 30 and 40°C. Table I summarizes and compares the results of these growth curves with those for wild type.

At 40°C all of the mutants, with the exception of ts 79, do make SBP. However, the final yield of SBP per infected cell (at 60 min after development is initiated) produced by the mutants is in all cases lower than the wild type yield. For ts 9 and ts γ (both infections were done with crude stocks) the yield is only 7 and 6% respectively, that of wild type. Infection with a crude stock of ts 4 gave 12% the yield of ϕX^+ , but infection with a purified stock produced 58% the SBP yield of ϕX^+ . Why crude and purified stocks should give different results in these experiments is not clear. The yield of SBP made by ts 79 is at most 0.3% that given by ϕX^+ . It is unlikely that ts 79 makes any de novo SBP at 40°C, since the titer remains essentially constant at 5×10^7 /ml (the lower limit of the SBP assay) throughout the growth cycle (see Figure 14).

At 30°C all the mutants make SBP, but, even here, in sub-normal amounts when compared to wild type. The mutants 9, γ , and 79 synthesize 17, 18, and 40% respectively, the SBP of ϕX^+ . The disparity between infections performed with crude versus purified stocks of ts 4 seen at 40°C is again in evidence at 30°C. The SBP yield with the crude versus purified stock was 21 versus 85% that of ϕX^+ . This difference is especially confusing here since the burst size resulting from infection by the crude stock was three times higher than the one with the purified stock.

The data recorded in Table I under the headings burst size, minimum latent period, and end of eclipse period require no special comments. The ratios of SBP/intracellular phage (at 60 min after development is initiated), at 40°C, for the mutants are, as expected,

all quite high. This is simply a reflection of the fact that the mutants are making no infectious intracellular phage at 40°C.

At 30°C the ratios of SBP/intracellular phage are all, with the exception of the ts 4 infection performed with the purified stock, lower than the corresponding values for wild type. This result is the consequence of the mutants giving essentially normal yields of phage, but subnormal yields of SBP. One implication of this is that the SBP the mutants do make at 30°C is more efficiently incorporated into mature phage than is the case for wild type.

In a joint effort with Clyde Hutchison III many other ts mutants have been examined for their ability to make SBP at 40°C. These results are recorded in Table II. Unfortunately, all of these experiments were done with crude stocks (in some cases the stocks were probably also low in % plaque formers), hence background for some of the mutants is so high that a decision as to whether SBP is made is not possible.

A brief summary of the results of this section follows:

- 1) At 40°C ts γ , and 9 make SBP, but in subnormal amounts when compared to wild type at this temperature. At 40°C ts 4 makes SBP, and the yield is closer to that of wild type than is the case for the other mutants. The mutant ts 79 does not make any SBP at 40°C.
- 2) At 30°C all of the mutants make SBP, but again in subnormal amounts when compared to wild type at this temperature. However, an infection performed with a purified ts 4 stock gave an essentially normal yield of SBP.

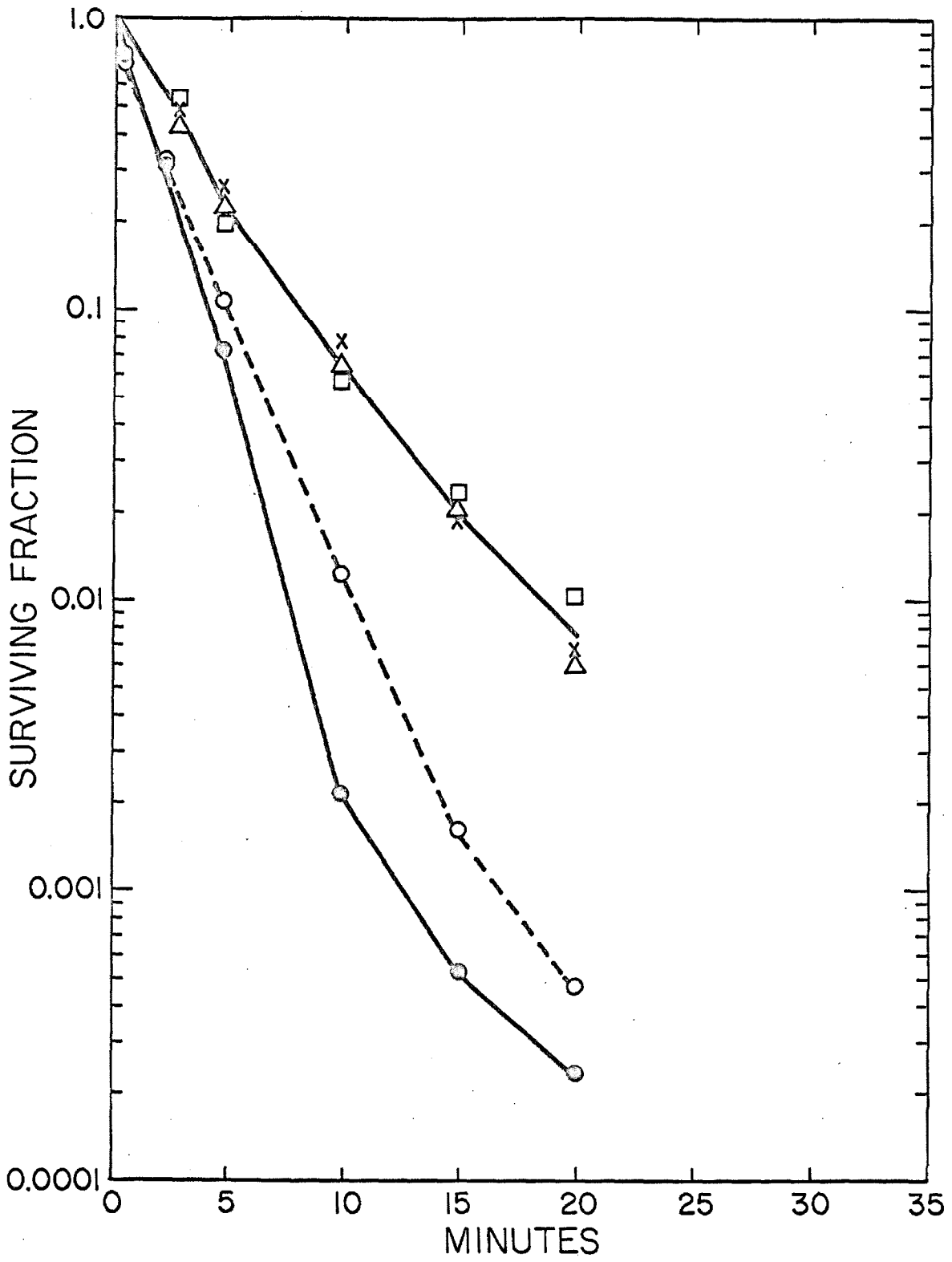


Figure 10

Legend to Figure 10

Serum inactivation at 48°C of several ts mutants and $\phi\text{XH}_{\text{A}}\text{H}_{\text{B}}$. The figure represents two separate sets of experiments. The first set consists of $\phi\text{XH}_{\text{A}}\text{H}_{\text{B}}$ (—●—), and ts 79 (--0--) run at a serum dilution of 10^4 in KC broth. Both phage types were inactivated simultaneously in the same tube. Survivors were plated on a mixed indicator of one part E. coli C to three parts E. coli C_{ab}, and plates incubated 5 hr at 30°C . Under these conditions $\phi\text{XH}_{\text{A}}\text{H}_{\text{B}}$ gives clear plaques, while ts 79 gives turbid plaques. The second set of curves was run at a serum dilution of 2×10^4 in TB and contains $\phi\text{XH}_{\text{A}}\text{H}_{\text{B}}$ (-x-), ts γ (-□-), and ts 4 (—△—). These were all run in separate tubes, and each was plated on E. coli C, and incubated 5 hr at 30°C . The stocks of ts γ and ts 4 were purified as described under Methods, the other phage types were from crude stocks.

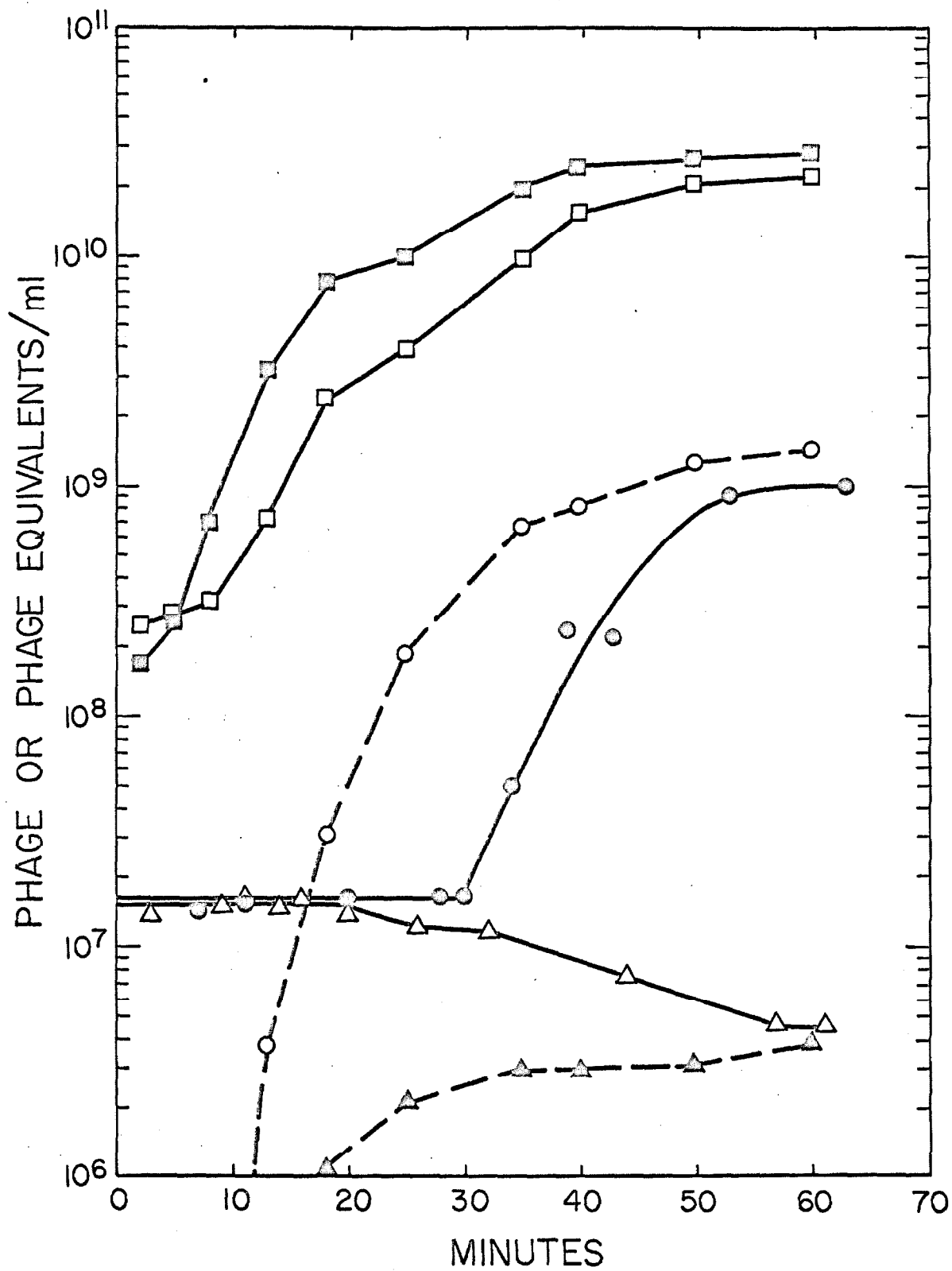


Figure 11

Legend to Figure 11

Growth curves of ts 4 at 30°C, infective centers (—●—), intracellular phage (--0--), SBP (—□—); and at 40°C, infective centers (—△—), intracellular phage (--▲--), SBP (—■—). *E. coli* C was grown to 10⁸/ml in KC broth at 37°C. The culture was shifted to 40°C and at -15 min cyanide was added. At -10 min enough phage, from a purified stock, was added to give a multiplicity of 2. After filtration and resuspension in NaCl (effecting a threefold concentration in this experiment) one half of the infected cells were diluted tenfold into TB at 30°C, the other half was likewise diluted into TB at 40°C. 99% of the phage adsorbed.

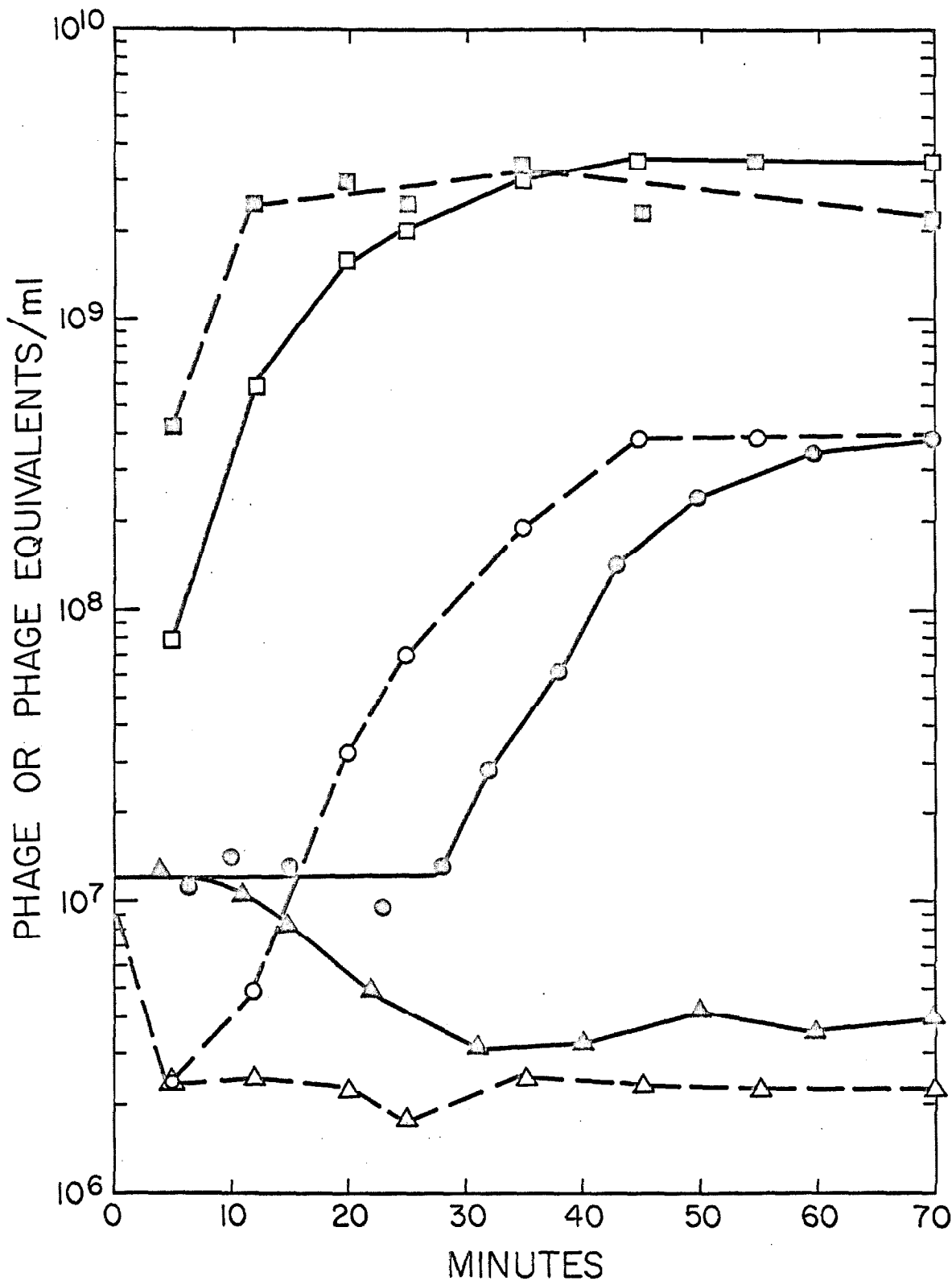


Figure 12

Legend to Figure 12

Growth curves of ts 9 at 30°C, infective centers (—●—), intracellular phage (--0--), SBP (—□—); and 40°C infective centers (—▲—), intracellular phage (--△--), SBP (--■--). *E. coli* C was grown to 10⁸/ml in KC broth at 37°C. After 60 min of starvation the culture was diluted fivefold, in SB, shifted to 40°C and infected (phage were from a crude stock) at a multiplicity of 3. The starved infected cells were spun down and resuspended in NaCl (effecting a fivefold concentration). One half of the culture was diluted fivefold into TB at 30°C, the other half was likewise diluted into TB at 40°C. 94% of the phage adsorbed.

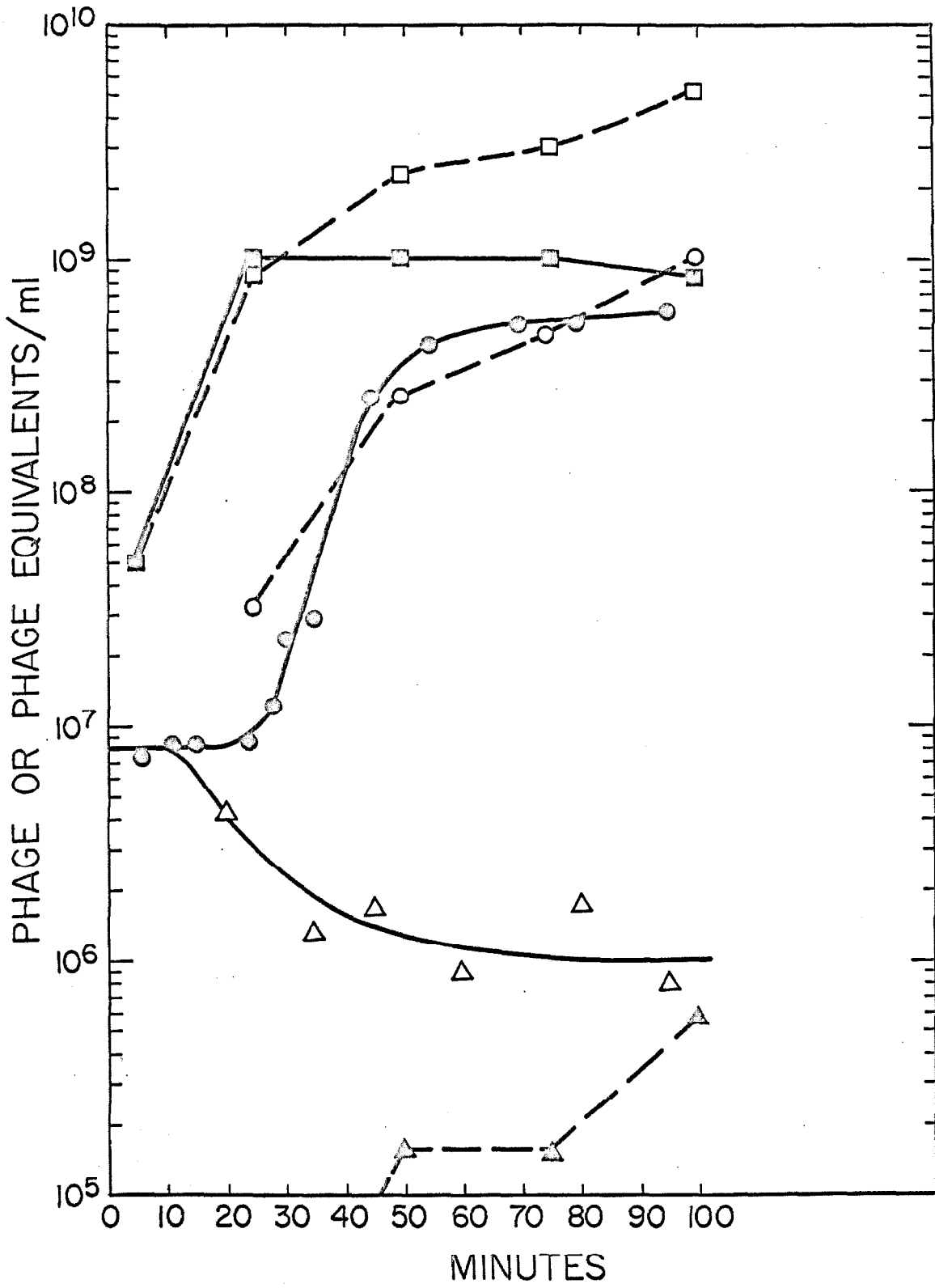


Figure 13

Legend to Figure 13

Growth curves of $ts\ \gamma$ at 30°C , infective centers (—●—), intracellular phage (--0--), SBP (--□--); and 37°C infective centers (—△—), intracellular phage (--▲--), SBP (—■—). *E. coli* C was grown to 2×10^8 /ml in KC broth at 37°C . After 60 min of starvation the culture was infected at a multiplicity of 0.1 (phage were from a crude stock). The starved, infected cells were spun down and re-suspended in NaCl (effecting a fivefold concentration). One half of the culture was diluted tenfold into TB at 30°C , the other half was diluted likewise into TB at 37°C . 93% of the phage adsorbed. This mutant was isolated with 37°C as the nonpermissive temperature.

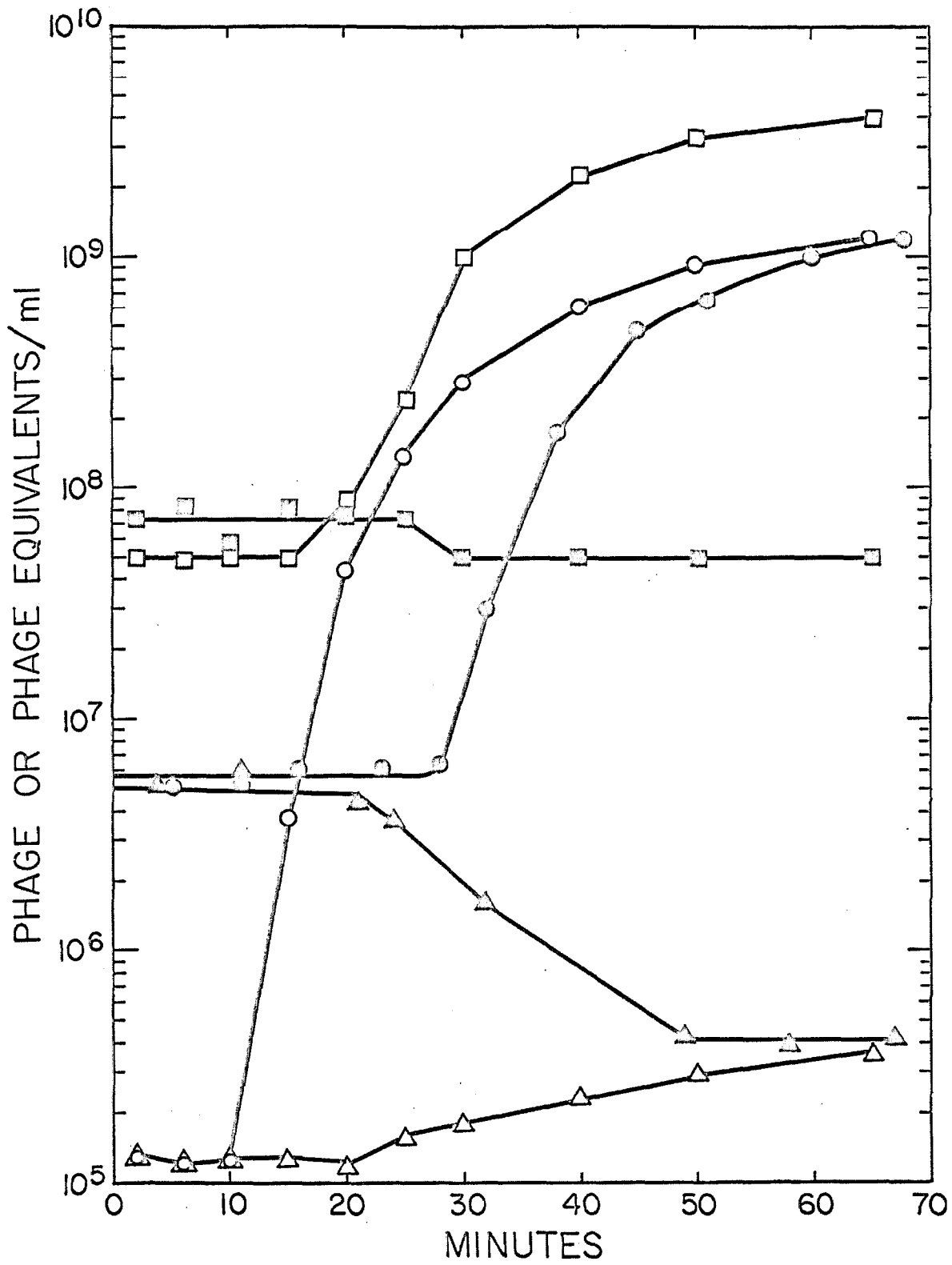


Figure 14

Legend to Figure 14

Growth curves of ts 79 at 30°C, infective centers (—●—), intracellular phage (—○—), SBP (—□—); and 40°C, infective centers (—▲—), intracellular phage (—△—), SBP (—■—). *E. coli* C was grown to 10⁸/ml in KC broth at 37°C. The culture was shifted to 40°C and at -15 min cyanide was added. At -10 min enough phage, from a crude stock, to give a multiplicity of 2 was added. After filtration and resuspension in NaCl one half of the infected cells was diluted tenfold into TB at 30°C, while the other half was diluted tenfold into TB at 40°C. 98% of the phage adsorbed.

TABLE I
Summary of $\phi\chi^+$ and ts Mutant Growth Curves

Phage Type	Average Burst Size			Minimum Latent Period (in Minutes)		End of Eclipse Period (in Minutes)		*SBP per Infected Cell, at 60 Minutes			SBP per Intra-cellular Phage, at 60 Minutes				
	30°C	37°C	40°C	30°C	37°C	40°C	30°C	37°C	40°C	30°C	37°C	40°C			
Wild Type (purified)	100	133	100	31to34	14	15	18	6to8	12	1700 (100)	2000 (100)	3000 (100)	17	13	30
ts 4 (purified)	63	-	0.3	31to33	-	-	17	-	-	1440 (85)	-	1750 (58)	16	-	7x10 ³
† ts 4 (crude)	185	-	0.1	32to35	-	-	17	-	-	350 (21)	-	350 (12)	2	-	600
ts 9 (crude)	33	-	0.3	29to31	-	-	16	-	-	290 (17)	-	210 (7)	9	-	800
ts Y (crude)	73	0.1	-	29	-	-	?	-	-	310 (18)	125 (6)	-	7	5x10 ³	-
ts 79 (crude)	210	-	0.1	29to31	-	-	16	-	-	700 (40)	-	≤ 9 (≤ 0.3)	4	-	≤ 120

* Numbers in parenthesis are the percent yield of SBP relative to wild type at the corresponding temperature.

† The data for this experiment is not presented in any of the figures. All the other data is derived from Figures 4, 6, 11, 12, 13, and 14.

TABLE II

SBP Synthesis by ϕ X ts Mutants at 40°C

Mutant Number	SBP/ml at 0 min	SBP/ml at 50 min	Is SBP Made ?
wild type	5×10^8	1.0×10^{10}	+
4	5×10^7	7×10^8	+
10	5×10^8	4×10^9	+
17	4×10^8	5×10^9	+
19	5×10^8	3×10^9	+
28	1×10^9	5×10^9	?
34	3×10^9	5×10^9	?
41	2×10^8	5×10^9	+
53	8×10^7	3×10^9	+
70	4×10^9	10^{10}	?
72	4×10^8	5×10^9	+
74	2×10^8	5×10^9	+
76	5×10^8	4×10^9	+
78	5×10^8	5×10^9	+
79	4×10^7	9×10^7	-
81	5×10^8	3×10^9	+
84	5×10^8	4×10^9	+
89	2×10^9	4×10^9	?
91	1×10^8	2×10^9	+
95	3×10^9	5×10^9	?
96	5×10^7	5×10^9	+
99	7×10^8	2×10^9	?
101	2×10^9	4×10^9	?
102	2×10^8	4×10^9	+
106	2×10^9	5×10^9	?

Legend to Table II

E. coli C was grown to 7.4×10^7 /ml in KC broth at 37°C , and then starved for 60 min. The starved cells were shifted to 40°C and infected at a multiplicity of about 6.5 (crude stocks were used in all cases). After allowing 15 min for adsorption development was initiated by adding an equal volume of KC broth, prewarmed at 40°C , directly to the starved infected cells (this is time zero in the experiment). A 1ml sample was taken immediately after adding the phage (0 min sample), and another 1ml sample was taken at 50 min. A standard curve was run in which the diluent consisted of 50% KC broth and 50% starvation buffer; it was similar to the standard curves for ϕX presented in Part I.

It was, more or less arbitrarily, decided to assign a (+) to any mutant that made a greater than fivefold increase, and a (-) to any mutant that made less than a fivefold increase of SBP between the two times sampled. If the increase was less than fivefold, but the titer at 0 min was very high (10^9 or greater), due to background, it was not possible to determine whether or not a significant amount of SBP synthesis occurred; these mutants are designated (?). It is not clear why the mutants 4, 53, 79, and 96 gave SBP titers at 0 min significantly lower than the expected minimum (5×10^8 /ml).

The results of Hutchison's complementation tests on these mutants show that γ , 10, 41, 53, (74), 79, 81, (84), (101), and 106 are all in the same complementation group. The mutants 9, 70, and (95) are all in a second group distinct from the first. The mutants 4, 28, and 34 are in yet a third group. Mutants listed in parenthesis have only been tentatively assigned. Those mutants in the table not mentioned in any of the complementation groups have not yet been run in complementation tests.

PART IV - THE PHYSICAL STATE OF SBP SYNTHESIZED, AT 40°C,
BY ϕ X ts MUTANTS AND ϕ X⁺

Although it was demonstrated in Part III that many ts mutants make SBP at 40°C these results give no information as to the physical state of this SBP. It might be imagined that a given ts mutant might, at 40°C, make its SBP in one of several different forms; for example, protein subunits or aggregates of protein subunits, or perhaps complete or nearly complete virus particles. The experimental approach to this problem has been to analyze the SBP of lysates of cells infected, at 40°C, with a particular type of ts mutant or wild type by the technique of sucrose gradient velocity centrifugation.

The S values listed in the results below, especially those for slow sedimenting SBP, are really gross approximations. In fact, due to the inaccuracies in the S values it is possible that the different forms of slow sedimenting SBP (12S for ts γ , 18S for ts 9, 7S to 13S for wild type, perhaps even 21S in the case of ts 4) observed all represent the very same type of subunit. In this section (Part IV) the calculated S values will be used; however, to simplify the Discussion that follows the slow sedimenting SBP will be referred to as "15S" (the average of the observed values).

ts γ

Figure 15 shows the patterns that result after sucrose gradient centrifugation of lysates derived from cells infected by a crude stock of ts γ . The 1 min gradient contains a significant peak in the region of 84S. This material must be background SBP present in the infecting phage stock. But the exact origin of this SBP is

unclear. It probably arises from parental phage coats (both active and inactive). It will be shown, in Figure 19, that this same peak is observed in the course of a wild type infection. Later in the infection the parental coats are found in a broad band centered roughly at 70S. Since the experiment in Figure 15 was performed with a crude stock of ts γ , it is conceivable that some of the 1 min SBP (particularly in the slower sedimenting part of the band) is 70S or 70S like material present in the stock.

The 15 min gradient was titered on two separate occasions (these were duplicate titerings of the same gradient), 4 weeks apart. The most predominant feature of both titerings is the large peak of SBP at 12S which comprises about 50% of the total SBP in the gradient. In the first titering smaller peaks at 78, 60, and 48S also appear. It is assumed that the material under the 78S peak is largely the background SBP seen in the 1 min gradient, but that the other peaks, especially the 12S, are composed of de novo SBP.

The second titering of the 15 min gradient still shows the large peak at 12S; but the peaks in the region of 78S and 60S have declined, and the 48S peak has disappeared completely. It appears that the SBP in the fastest peaks, particularly at 48S, is unstable to prolonged storage at 4°C, in sucrose. Fluctuations in the SBP assay itself could account for much of the variation seen at 78S and 60S.

It is tempting to speculate that the SBP at 12S represents a subunit of ϕ X coat protein such as was alluded to in Part III. This point will be elaborated on in the Discussion.

Several other sucrose gradient experiments have been done on lysates (prepared between 50 and 75 min after development was initiated) derived from cells infected, at 40°C, by ts γ . The patterns of SBP in these gradients were all qualitatively similar to

the 15 min pattern shown in Figure 15. That is, most of the SBP was in a slow sedimenting peak at about 15S; smaller peaks in the region of 70S were also found; which may, however, have been, at least in part, background material.

ts 9

Essentially all of the SBP in the 1 min ts 9 pattern shown in Figure 16 is found in a broad band in the middle of the gradient, with a peak at 78S. This material is exactly analogous to the background SBP (84S) found in the ts γ gradient at 1 min which was discussed above. There is also a small peak at 18S.

In this experiment the 15 min gradient was also titered two times, 7 weeks apart. The first of these titerings showed a peak at 78S coincident with but smaller than the peak seen at 1 min, another peak at 54S and two adjacent peaks at the top of the gradient. The second titering revealed a situation somewhat similar to the ts γ case. The peak in the region of 78S was diminished, the peak at 54S had vanished, and one large peak appeared near the top at 18S, in place of the two smaller peaks in the first titering. Thus the 54S SBP, like the 48S material in the ts γ gradient, seems to be genuinely unstable. Two alternatives which might explain this instability seem possible. One is that these particles aggregate on prolonged standing, the second is that they are degraded to protein which no longer possess SBP. The situation at the top of the ts 9 gradient is quite confusing; however, the differences might, in part, be explainable by variations in the SBP assay. It is clear, though, that appreciable amounts of SBP stay near the top of the gradient. This slow sedimenting SBP may be subunits of ts 9 coat protein.

The experiment shown in Figure 16 is the only sucrose gradient that has been run on ts 9.

ts 4

Some preliminary sucrose gradients run on lysates prepared from cells infected by crude stocks of ts 4 showed that at all times after infection SBP was located mainly at about 70S. This made for a complex situation since both the background and de novo SBP appeared in essentially the same region of the gradient. It was hoped that if the infection was performed with a purified stock of ts 4 some of the background SBP might be eliminated (certainly most of the 70S particles will be eliminated, and furthermore purified stocks, especially when prepared by the phase separation system described under Methods, usually have a larger fraction of their particles as plaque formers than do nonpurified stocks).

Figure 17 shows the sucrose gradient pattern of SBP obtained from lysates of cells, made at 5 and 25 min, infected by purified ts 4. Although the input sample for the 5 min gradient had 3×10^8 phage equivalents of SBP each of the 23 fractions contained at most 5×10^7 phage equivalents of SBP (the background value in the SBP assay). Either the small amount of input SBP has become so spread out during centrifugation that no region of the gradient measures above the background level of the assay, or else the input SBP has somehow been inactivated.

About 50% of the SBP in the 25 min gradient sediments as a broad band centered at 71S. Two smaller but significant peaks occur at 43S and 21S. A very small amount of SBP also appears at about 107S. Even though the 5 min gradient appears to contain no SBP,

there might be some SBP in the 25 min gradient that is background, derived from the purified infecting stock. However, it is clear that most of the material under the 71S peak is de novo, because the total amount of SBP under this peak is about three times greater than the entire SBP content of the 5 min input sample.

Thus, about 50% of the SBP produced by ts 4 at 40°C is in the form of particles that sediment at 71S. As will be pointed out in the Discussion section these particles are probably not equivalent to the 70S particles formed during a normal infection.

wild type

The results of a sucrose gradient centrifugation on wild type lysates are shown in Figure 18. The infection was performed at 40°C with a purified ³⁵S labeled stock, that contained 20% plaque formers. Although a 6 min gradient (not shown) was run all the fractions contained only the background titer (5×10^7) of SBP, despite the fact that the input sample possessed 5×10^8 phage equivalents of SBP. This is exactly the situation observed with the 5 min gradient of ts 4. The reasons that might account for this were discussed under the ts 4 section.

It must be noted here that the ϕ X growth cycle took place at an abnormally slow rate in this experiment. At 13 min there was only 0.5 intracellular phage/infective center, whereas the corresponding normal value at this time is about 2. At 25 min there were about 50 intracellular phage/infective center, which is about normal. Thus, it is not clear how to regard the 13 min gradient, but perhaps it is best thought of as really representing a time earlier in the latent period than 13 min. Essentially all of the SBP in the 13 min gradient remains near or at the top of the centrifuge tube.

The 25 min gradient has three large SBP containing areas. The fastest of these sediments at 114S, and corresponds to whole phage. None of the mutants showed a peak at 114S. However, just such a peak was expected in a wild type infection, since infective progeny phage are made. The infective phage titer at the 114S peak was 4×10^8 /ml; since the SBP titer was 1.35×10^9 /ml it may be estimated that the phage contained 30% plaque formers. The largest area of SBP lies under the peak at 74S. Significant amounts of SBP remained near or at the top of the gradient (13S to 7S). In addition small quantities of SBP appeared at the very bottom of the gradient, and also at 34S. The three principal regions of SBP in the 25 min gradient can be interpreted as whole phage (114S), 70S particles (74S), and some sort of subunit of the ϕ X protein coat (13S to 7S).

With the ^{35}S labeled stock it was possible to directly measure the fraction of non-plaque forming particles that adsorb to host cells. It was known that only 20% of the particles were plaque formers, and that essentially all of these adsorb to host cells (by infectivity measurements). Thus, 80% of the input counts in an infection, with this stock, reside in the non-plaque formers, but 100% of the un-adsorbed counts (measured either on the HA Millipore filtrate, or supernatant of spun down infected cells - depending on the protocol used) would be attributable to the non-plaque formers. In two independent determinations the fraction of counts in inactive phage that remained adsorbed to host cells was 0.22 (supernatant of spun down infected cells) and 0.55 (filtrate from HA Millipore filtration). The difference between these two might, in part, be due to some inactive particles which are not really adsorbed to host cells sticking to the filter.

Figure 19 shows the pattern of parental ^{35}S radioactivity obtained from the same sucrose gradients that were described under

Figure 18. At 6 min a small fraction of the counts are still in the form of 114S particles, but the majority of the radioactivity sediments in a peak centered at about 87S. At 25 min even less of the parental coat protein sediments at 114S, and essentially all of the counts are found in a broad peak in the region of 67S. At both times a tiny fraction of the counts remained at the top of the gradients. It should be noted that the amount of SBP directly attributable to parental SBP is almost negligible (10% at most) when compared to the measured SBP in the region of 70S in the 25 min gradient. These results warrant the following conclusions: parental phage (both active and inactive) are converted to 70S particles during the course of infection, there is no transfer of parental coat protein to progeny virus, and very little, if any, of the parental coat is converted to slow sedimenting subunits.

A brief summary of this last section follows:

- 1) At 40°C the main SBP component produced by ts γ sediments at about 12S. A 48S component that has SBP, but which seems to be unstable is also made. SBP also appears at 78S and 60S in the sucrose gradient; however, it is probable that the 78S material is background SBP present in the infecting stock.
- 2) At 40°C the main SBP component synthesized by ts 9 sediments in the vicinity of 18S. 78S and 54S SBP is also found in the sucrose gradient; the former is very likely background material, while the later is unstable.
- 3) At 40°C about 50% of the SBP made by ts 4 sediments at 71S. Smaller peaks of SBP were observed at 107S, 43S, and 21S.

- 4) At 40°C the principal SBP containing components synthesized by wild type sediment at 114S (complete virus particles), 74S (no doubt the typical 70S particles produced by ϕX), and near the top of the gradient (13S to 7S). Furthermore, during the course of infection practically all of the parental wild type coat protein is converted to 70S particles.

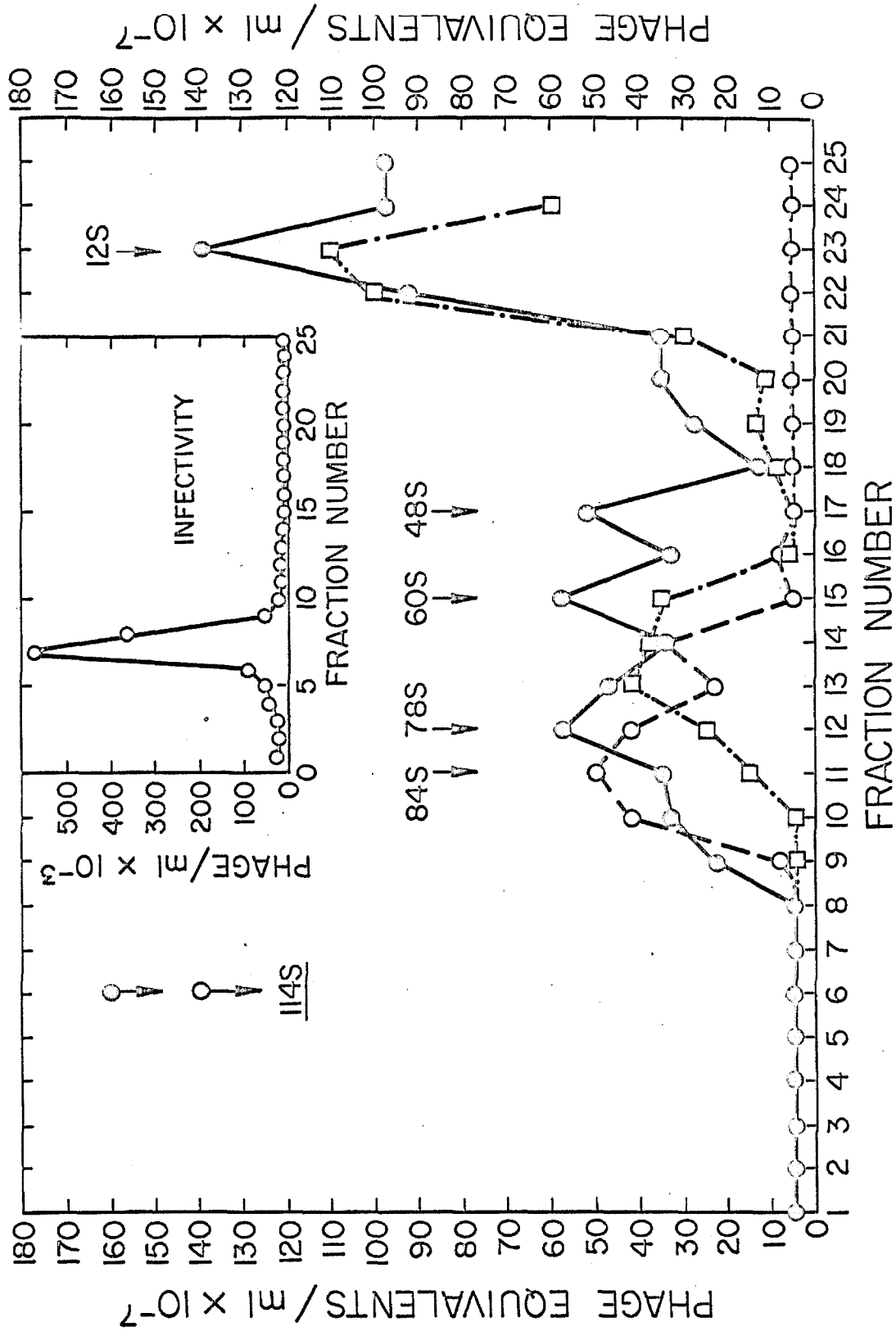


Figure 15

Legend to Figure 15

Sucrose gradient velocity centrifugation of lysates derived from *E. coli* C infected, at 40°C, by ts γ . Lysates were made 1 min (---0---, titered 5 November 1964) and 15 min (—●—, titered 31 August 1964; --□--., titered 28 September 1964) after development was initiated. The inset graph shows, in detail, the pattern of phage infectivity obtained for the 15 min gradient. Peaks of infectivity for both gradients are indicated by the arrows ↓ (15 min) and ↓ (1 min). In this and the sucrose gradient experiments that follow the infectivity is due to the small number of intracellular phage found (see growth curves in Part III). In all cases the infectivity sediments as one fairly sharp peak, and is used as a sedimentation marker. It is known that wild type ~~α~~ sediments at 114S (3) and is assumed that the ts mutant particles do likewise.

The approximate S values of pertinent peaks of SBP are indicated by arrows (↓). These values are calculated, as outlined by Martin & Ames (54), according to the following equation $114S/\text{distance from meniscus of phage peak} = S \text{ of unknown peak}/\text{distance from meniscus of unknown peak}$. It must be emphasized that in these experiments the listed S values are only rough approximations.

This experiment was synchronized by cyanide and the infection was performed with an unpurified virus stock at a multiplicity of 2. The titer of infective centers at the time development was initiated was approximately 10^8 /ml. At 1 min and 15 min 10ml aliquots were removed; the infected cells were spun out and each sample resuspended in 3ml of 0.033 M Tris-Cl, pH 8.0. These were brought to room temperature and 0.1ml of lysozyme (2mg/ml, in H₂O) was added;

after 2 min 0.15ml of 4% EDTA (in H₂O) was added, and the suspensions were shaken for 3 min. The samples were now lysed by three cycles of freezing and thawing. The lysates were spun at low speeds, to remove bacterial debris, layered onto sucrose gradients, centrifuged and fractioned as described under Methods. In this and subsequent sucrose gradient experiments between 50 and 100% of both the phage infectivity and SBP of the input samples are recovered in the fractions of the gradient, unless otherwise stated.

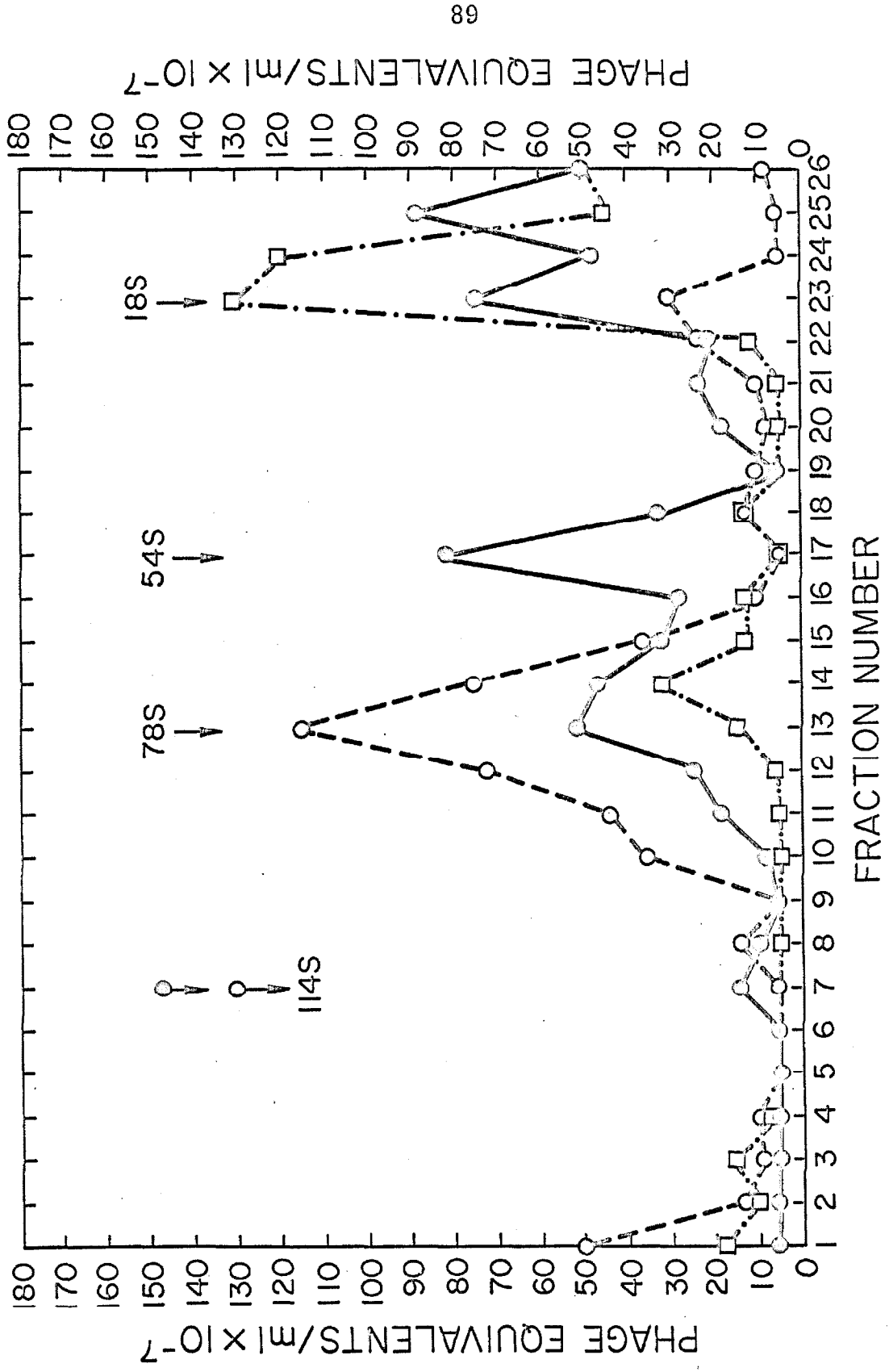


Figure 16

PHAGE EQUIVALENTS/ml $\times 10^{-7}$

PHAGE EQUIVALENTS/ml $\times 10^{-7}$

FRACTION NUMBER

Legend to Figure 16

Sucrose gradient velocity centrifugation of lysates derived from *E. coli* C infected, at 40°C, by a crude stock of ts 9. Lysates were made at 1 min (---0---, titered 15 November 1964) and 15 min (—●—, titered 31 August 1964; --□---, titered 21 October 1964) after development was initiated. Phage infectivity peaks are indicated by ↓ (1 min) and ↓ (15 min). The experiment was performed in precisely the same fashion as described in the legend to Figure 15, except that the multiplicity used here was only 0.5.

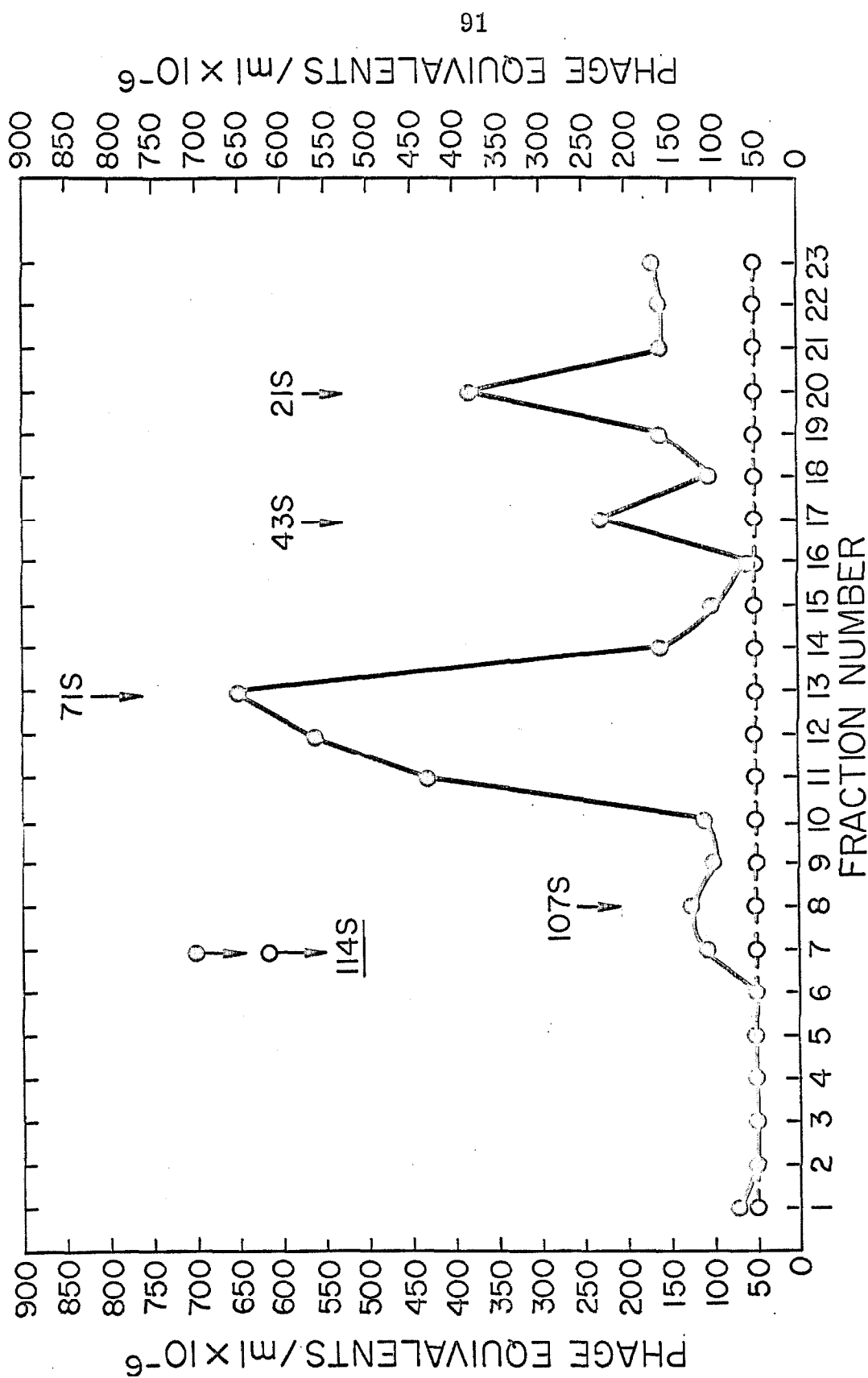


Figure 17

Legend to Figure 17

Sucrose gradient velocity centrifugation of lysates derived from *E. coli* C infected, at 40°C, by a purified stock of ts 4. Lysates were prepared at 5 min (---0---) and 25 min (—●—) after development was initiated (both were titered at the same time). Phage infectivity peaks are indicated by ↓ (5 min) and ↓ (25 min). The samples originated from the experiment described in Figure 11. Sucrose gradient centrifugation was carried out as described under Methods.

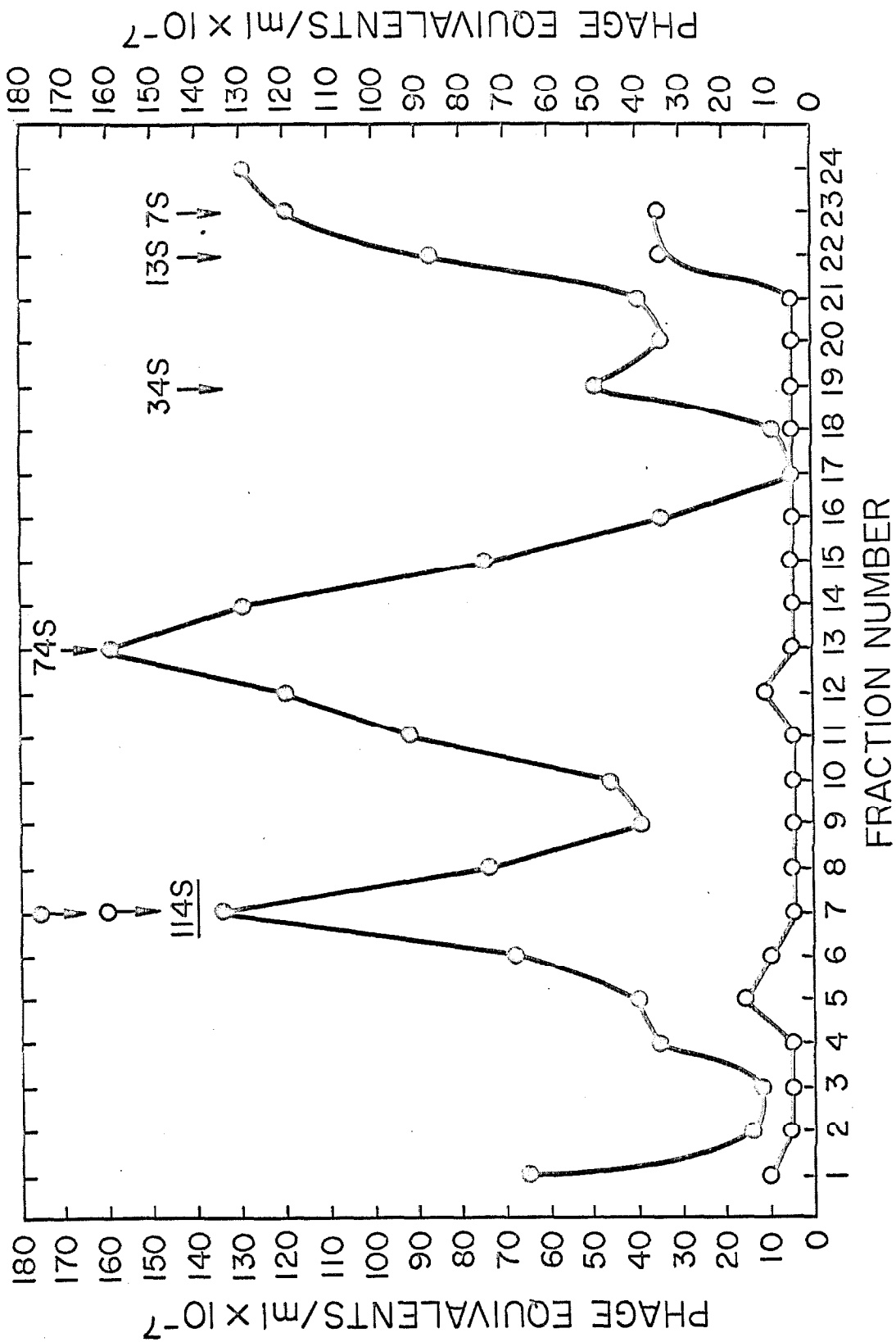


Figure 18

Legend to Figure 18

Sucrose gradient velocity centrifugation of lysates derived from *E. coli* C infected, at 40°C, by ϕX^+ . Lysates were prepared at 13 min (—○—) and 25 min (—●—) after development was initiated (both were titered at the same time). The phage infectivity peaks are indicated by \downarrow (13 min) and \downarrow (25 min). This experiment was performed with a purified ^{35}S labeled stock of ϕX^+ that was 20% active. Cyanide synchronization was used, the titer of infective centers at the time development was initiated was about 10^8 /ml, and the multiplicity was 2. The protocol followed was like the one outlined under cyanide synchronization of the Methods section. Sucrose gradient centrifugation and fractionation were carried out as described under Methods.

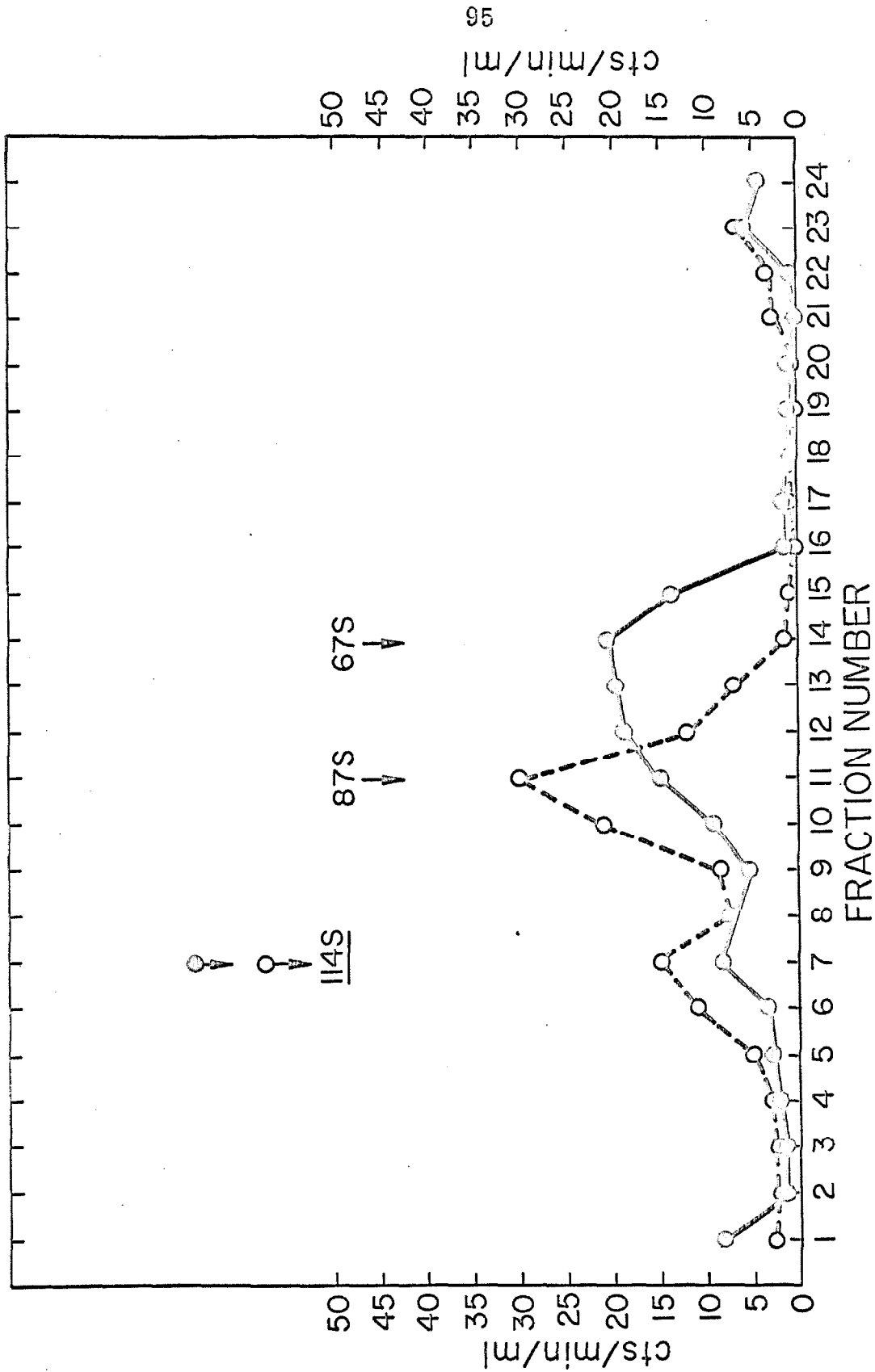


Figure 19

Legend to Figure 19

Radioactivity patterns (^{35}S) for the experiment described under Figure 18. A 6 min lysate (----0----), and 25 min lysate (—●—) are shown. Phage infectivity peaks are represented by ⤴ and ⤵ for the 6 and 25 min gradients respectively. The pattern for the 13 min gradient (the SBP of which was presented in Figure 18) is not shown here because it was heavily contaminated with radioactivity from some unknown source; as evidenced by a tenfold excess of total counts in the fractions of the gradient over the input sample. Radioactivity was determined by applying 0.25ml samples to aluminum planchets, drying, and counting on a Nuclear Chicago low background counter. The counts plotted are the measured values less background; no corrections have been made for possible absorption effects due to the sucrose. 19% and 21% of the counts in the input samples (which in this experiment were contained in TB medium) were recovered in the 6 and 25 min gradients respectively.

APPENDIX TO PART IV

The DNA of mature ϕ X is a single stranded ring containing one E. coli phosphodiesterase resistant discontinuity (15, 16). This discontinuity can be thought of as joining together the two ends of a noncircular molecule, thereby converting it to the circular form. Since only circular ϕ X DNA molecules are infective the discontinuity will probably prove to be an important structural feature. One approach to determining the nature of the discontinuity is to make "intelligent guesses" as to what it might be; specific assays can then be designed to test whether the "intelligent guesses" are correct.

One "intelligent guess" that has been put to the test is that the discontinuity contains sulphur (an element not normally found in DNA). A sulphur containing amino acid or polypeptide could be envisioned. Bendich & Rosenkranz (55) have proposed that amino acids might occasionally be interspersed between the repeating phosphate linkages that make up the backbone of DNA. But, the specific type of linkage in which the sulphur might be contained need not be specified at this point. Since a stock of ^{35}S labeled ϕ X was available (see Figure 19 and Methods) it was an easy task to extract and purify the DNA from the virus particles and ascertain whether it contained any radioactivity.

There are 1200 sulphur atoms in the coat protein of each ϕ X virus particle (5). If the ϕ X DNA molecule contains one sulphur atom, then 1/1200 of the radioactivity in a purified ^{35}S labeled ϕ X stock would reside in the DNA. In the experiment performed here a sample of ^{35}S ϕ X, that contained 10^5 cts/min, was mixed with some D^{15}N ϕ X DNA and extracted with hot phenol. The DNA was then centrifuged to equilibrium in a CsCl density gradient. The

contents of the centrifuge tube were fractioned and assayed for infectivity (by the protoplast system) and radioactivity. If ϕ X DNA contains one sulphur per DNA molecule, then according to the argument outlined above a peak of radioactivity containing about 85 cts/min would be expected coincident with the peak of infectivity due to the ^{14}N DNA (assuming 100% of the DNA is recovered after phenol extraction, centrifugation, and fractionation). The D^{15}N DNA was added as a control for measuring the amount of residual ^{35}S protein that might become associated with DNA after phenol extraction.

The results are shown in Figure 20. Two clearly resolved peaks of infectivity, corresponding to D^{15}N ϕ X DNA and ^{14}N ϕ X DNA (originating from the ^{35}S ϕ X particles) are observed. It can be estimated (see legend to Figure 20) that about 10% of the DNA that was in the original sample was recovered from the gradient. Thus, rather than a peak of radioactivity containing 85 cts/min a smaller peak, one with about 9 cts/min, might be anticipated. None of the fractions under the ^{14}N ϕ X DNA peak of infectivity, nor any other fraction in the gradient had more than 2 cts/min (this is background on the counter). From this I conclude that there is less than one (that is, no) sulphur atom in the DNA of ϕ X.

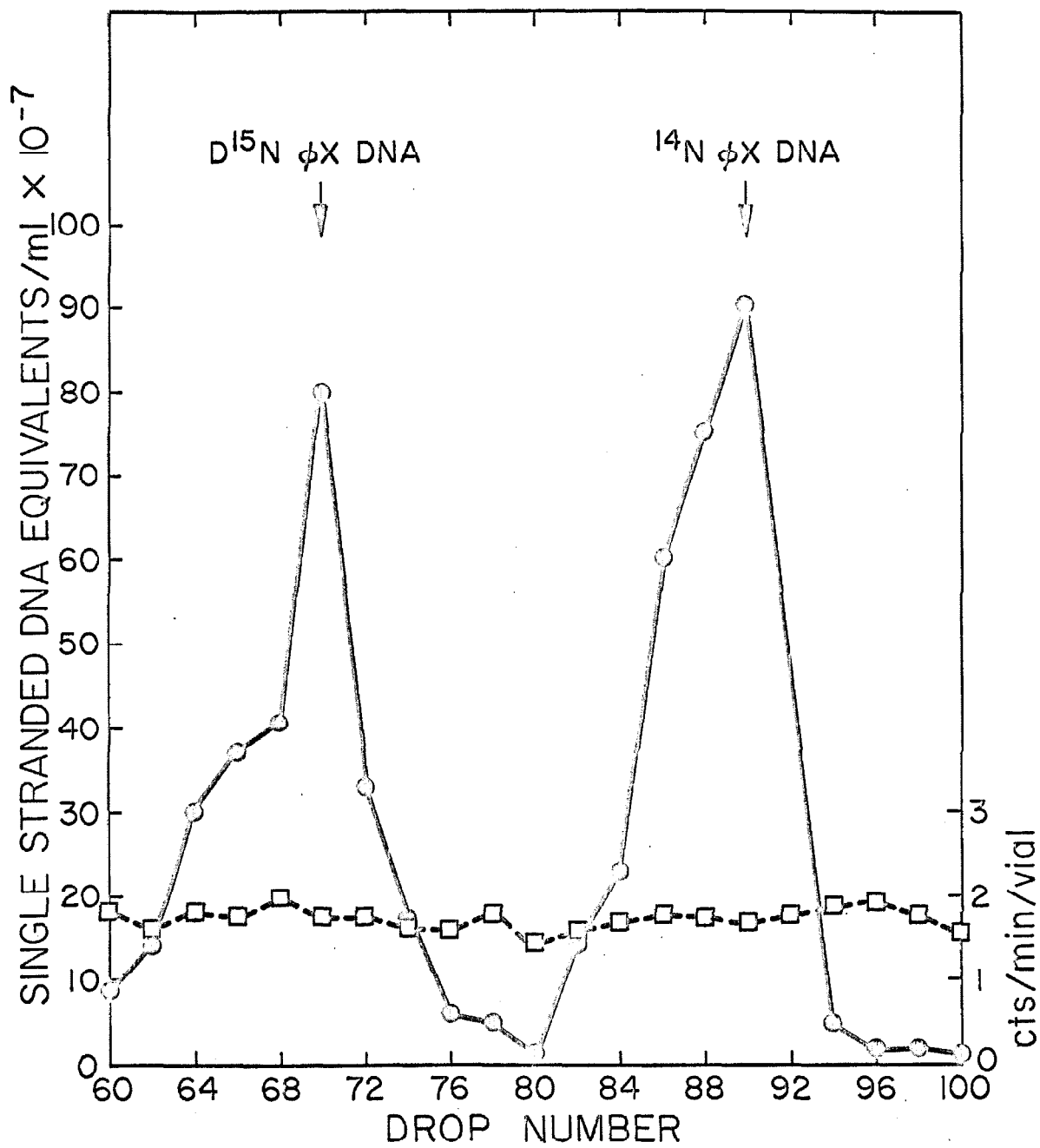


Figure 20

Legend to Figure 20

A 0.3ml aliquot of ^{35}S ϕX (in 0.05M borate) contained 10^5 cts/min, 2×10^9 infective phages, and 10^{10} total phage particles. This was combined with 0.3ml ^{15}N ϕX DNA (in 0.05M borate, containing no radioactive label - a gift from M. Yarus), plus 0.1ml 30% bovine serum albumin (BSA), plus 0.4ml 0.05M borate. The DNA of this mixture was extracted four times by the hot phenol procedure of Guthrie & Sinsheimer (21). Residual phenol was removed from the last aqueous phase by five extractions with ether at 4°C ; residual ether was removed by bubbling nitrogen through the solution for several minutes at room temperature. The volume of the extracted DNA was brought up to 4ml by addition of 0.05M borate. The solution was adjusted to a mean density of 1.670g/ml by addition of CsCl. The solution was centrifuged in the SW-39 rotor of a Spinco model E centrifuge for 48 hr at 37,000 rpm, at 7°C . Alternate drops were collected directly onto planchets and into vials containing 0.9ml of $5\mu\text{g/ml}$ EDTA, both in 0.05M Tris-Cl, pH 7.5. A small aliquot from each vial was taken for assay of infective DNA, performed according to the method of Guthrie & Sinsheimer (21). A standard curve covering the range 10^{10} to 10^1 DNA molecules/ml was also run.

A total of 160 drops were collected, but the results of only drops 60 through 100 are plotted. The bioassay of the other vials yielded zero on the scale plotted here. The peak of infectivity under ^{14}N DNA contains about 10^9 single stranded equivalents. If all of the 10^{10} ϕX particles that were extracted contained potentially infective DNA molecules then only 10% of the DNA was recovered. It has been shown that most of the DNA in nonplaque formers is

infective to protoplasts, implying that nonplaque formers are defective either in adsorption or penetration (52).

The planchets were dried and counted in a low background counter. No planchet contained more than the background value of 2 cts/min. However, afterthought made it seem likely that due to quenching effects of CsCl a small, but significant, peak of activity might be obscured. To eliminate this objection the contents of the vials (after bioassay) under the infectivity peaks were assayed for radioactivity as follows: 100 μ g of calf thymus DNA was added to each vial (to serve as carrier), the DNA was then precipitated by addition of 0.2ml of 10% trichloroacetic acid (TCA) to each vial. The vials were allowed to remain at 4^oC for 30 min, the precipitates were then filtered onto Schleicher & Schuell membrane filters and washed with about 5ml of 5% cold TCA. The filters were dried, glued to planchets and counted - these are the counts plotted (they are not corrected for background).

DISCUSSION

DISCUSSION

This section will focus on first, a discussion of SBP synthesis by ϕX^+ , and second, a discussion of the ts mutants with reference to their functional defects at 40°C.

Wild Type

The growth curves presented in Figure 4 showed that cells infected by ϕX^+ , at 37°C, begin to synthesize SBP at about 5 min, under conditions such that the eclipse ends at 6 to 8 min. It is clear, from these curves, that the SBP titer exceeds the titer of infectious phage at all times. However, as pointed out in Part II the SBP titers measured during the first few minutes of development are erroneously high, due to background SBP added with the infecting phage stock. As the growth cycle progresses the ratio of SBP/intracellular phage declines, and from the end of the minimum latent period (14 min) to the end of the growth cycle (25 min) this value remains constant at about 13. This indicates that only a small fraction of the SBP synthesized is incorporated into plaque forming phage particles.

The excess SBP (that which is not incorporated into mature plaque forming virus) is found in several different forms, as revealed in the sucrose gradient centrifugation of a ϕX^+ lysate, (grown at 40°C) prepared at 25 min, shown in Figure 18. The major component is a broad peak at about 74S containing 40% of the total SBP; this consists of the 70S particles formed during the course of the infection. Roughly 20% of the total SBP appeared at 114S (it was estimated, in Part IV, that about two-thirds of the SBP at 114S was due to non-plaque formers). 13% of the SBP was in "15S" sub-

units. Small amounts of SBP comprising 4 and 3% of the total occurred at the very bottom of the gradient and at about 34S respectively, and also contribute to the SBP not incorporated into plaque formers.

Eigner et. al. (24) demonstrated about an eightfold excess of 70S particles over infectious ϕ X particles at the end of the growth cycle. This is in reasonable agreement with the results shown in Figure 18 here. The experiment of Figure 19 demonstrates that parental phage coats are converted to 70S particles, but also that most of the 70S observed at the end of the growth cycle is made of protein synthesized after infection. Thus, one way in which 70S can arise is by a degradation of complete virus (both plaque formers and non-plaque formers) that involves loss of most of the DNA. It has been suggested that the bulk of the 70S formed during ϕ X infection may be the result of newly synthesized virus undergoing abortive infection by adsorption to bacterial debris or to unlysed cells (5).

It seems likely that the "15S" SBP observed in the 25 min gradient of Figure 18 is composed of surplus subunits that have not been incorporated into phage. Dr. L. Greenlee (56) has demonstrated that "15S" subunits are indeed precursors to both mature phage and 70S particles. He has developed an immune precipitation assay similar to the one described by Reuckert and Zillig (8). Greenlee delivered a pulse of ^{35}S to ϕ X infected cells, at the end of the pulse lysed the cells and centrifuged the lysate through a sucrose gradient. He observed ^{35}S labeled material precipitable by anti- ϕ X serum at 114S, 70S and "15S". If at the end of the ^{35}S pulse a chase of unlabeled sulphate was given, and the cells then allowed to incubate for several minutes before lysing and centrifugation, a decrease in the amount of

serum precipitable ^{35}S at "15S" was observed, which was balanced by an equivalent increase in the total precipitable ^{35}S at 114S plus 70S.

That the "15S" subunits are precursors to whole phage supports the hypothesis (outlined in Part III) that the decline in the ratio SBP/intracellular phage observed during the early part of the growth cycle, is due to incorporation of SBP from a pool of precursor subunits into infectious phage. However, in the absence of measurements on the kinetics of synthesis of the "15S" subunits the hypothesis cannot be convincingly proved.

It is possible that the "15S" subunits are the morphological subunits of the ϕX coat protein. It was estimated that a ϕX morphological subunit might have a M. W. of 3.8×10^5 (see Introduction). There are several globular proteins whose molecular weights and S values are well characterized (57). For example, catalase (M. W. = 2.5×10^5 , $S_{20,w} = 11.3$) and urease (M. W. = 4.8×10^5 , $S_{20,w} = 18.6$). Using these known values a graph of $S_{20,w}$ versus M. W. has been plotted. A sedimentation coefficient of 15 on this graph corresponds to a M. W. of 3.4×10^5 , which is temptingly close to the predicted value for a ϕX morphological subunit. Of course, with the limited information available this argument is purely speculative. Further experiments aimed at purification of the "15S" subunits and an accurate determination of their sedimentation coefficient seem very worthwhile.

It will be recalled that the chemically prepared 5S subunits do not have SBP. A further speculation that may now be made is that in order to possess SBP the adsorption antigen of ϕX must aggregate at least to the state of the "15S" subunit.

DeMars (37) showed that lysates prepared at the end of the T2 growth cycle contain about a twofold excess of SBP over infectious phage. In the case of the T-even phages the SBP assay is a measure only of tail fiber proteins. However, these phages make other structural components such as empty heads in excess of the amount incorporated into mature phage (31, 58). Many examples can be cited from among the small spherical plant and animal viruses in which excess protein in the form of empty particles, devoid of nucleic acid, is synthesized in addition to complete virus (59, 60). Thus, ϕX is certainly not unique in that it produces excess structural protein.

Before concluding this section on ϕX^+ it is worth mentioning again the results presented in Part II which showed that CAP, FUDR, and phleomycin when present at the time of infection each produce essentially complete inhibition of SBP synthesis. This suggests that at least one complete RF must be made in order for SBP synthesis to occur.

ts Mutants

The results presented in Parts III and IV taken together with other work on ts and amber (am) mutants of ϕX allow one to attempt a definition of the defects afflicting some of these mutants when grown under nonpermissive conditions. The am mutants are conditional lethals in that they grow normally in ϕX sensitive strains of E. coli carrying a suppressor of the am mutation (permissive host), but not in strains lacking the suppressor (nonpermissive host). Most importantly studies of this type on large numbers of mutants can provide the basis for understanding the functional organization of the ϕX genome.

Most ts mutants are thought to be the result of base substitutions (34). Thus, the gene in which the base change has occurred will eventually produce a protein with a primary amino acid sequence different from the equivalent protein made by wild type. This should be true both at 30 and 40°C. While the wild type protein can function equally well at either temperature, the mutant protein functions successfully only at the low temperature. The amino acid substitution has rendered the mutant protein sensitive to temperature. One possibility is that alterations in the tertiary structure of the mutant protein occur at the high temperature.

It would appear then that the changes in the SBP protein made by ts 79 at 40°C are so radical that all ability to complex with neutralizing antibodies is lost. If a certain degree of aggregation of the chemical subunits of the coat protein is necessary before SBP can be expressed, then one might theorize that the change in ts 79 at 40°C makes this type of aggregation impossible.

Since ts 79 makes no detectable SBP at 40°C it seems very likely that the complementation group in which it is located represents the structural gene for ~~α~~X SBP. Hutchison (62) has tested seventeen different am mutants for their ability to synthesize SBP in the non-permissive host. Of these only two, am 9 and am 23 failed to make any detectable SBP; both of these mutants are in the same complementation group as ts 79. The other fifteen mutants tested were all in complementation groups different from the one under consideration.

The mutant ts γ is in the same complementation group as ts 79, am 3 and am 23 and does make SBP. The protein which represents the ts γ SBP protein probably has an amino acid sequence different from the SBP protein made by wild type. But the sequence change in ts γ apparently does not as radically alter the three dimensional

configuration of the protein at 40°C, since it still possess SBP, and can form "15S" subunits.

Dowell and Sinsheimer (35) have found that ts γ while it makes a small amount of infectious RF at 40°C does not make any infectious single-stranded DNA. Furthermore, Lindqvist (63) has shown that not even noninfectious single strands are produced. Thus, while ts γ seems to be mutated in the SBP gene it also makes no single stranded DNA at 40°C. Perhaps the following line of reasoning can reconcile these two pieces of data. Free single stranded ϕ X DNA is never found, not even in wild type lysates. Single stranded DNA is found only within phage particles, from which it can be extracted with phenol. It is true that ts γ makes SBP in the form of "15S" subunits; however, suppose these subunits are defective in the sense that they cannot complex with single stranded DNA to form phage particles. If normal single stranded DNA were produced under such circumstances it might be converted to RF (which in this case would have to be non-infectious) or perhaps be degraded by cellular nucleases.

Although particles of ts 79 and ts γ synthesized at 30°C are as stable to heat inactivation as wild type, other mutants in the same complementation group as these are significantly more sensitive than wild type (62). This is consistent with the idea that this complementation group represents a coat protein gene, since only a structural component of the virus could effect the stability of assembled particles.

Finally, as regards the mutants of this complementation group it should be noted that even at 40°C they begin to lyse host cells at about 15 min (62).

The mutant ts 9 is in a complementation group distinct from the ts 79, ts γ group. The following facts are known regarding the

growth of ts 9 at 40°C. SBP is made and it appears to be largely in the form of "15S" subunits. RF, but not single stranded DNA, is synthesized. Host cells begin to lyse at about 15 min after infection. The sensitivity to heat inactivation of ts 9 particles is like wild type, but particles of some other mutants in this same complementation group are more sensitive to heat inactivation than wild type. Mutants that produce particles (when grown at 30°C) more sensitive to heat inactivation than wild type are found only in the ts 9 complementation group and the ts 79, ts γ group.

That some mutants in the ts 9 group are more sensitive than wild type to heat inactivation might be construed to mean that a protein in addition to the SBP protein is found in the coat of ϕ X. Mutants located in the gene for this protein would produce it in altered form at 40°C, and this might preclude assembly of complete virus. The rationale for the absence of single stranded DNA would then be identical to the one outlined for the ts 79, ts γ complementation group.

A completely different hypothesis concerning the gene function of the ts 9 complementation group is that it specifies a ϕ X specific DNA polymerase. (It was pointed out in the Introduction that ϕ X probably produces single stranded DNA off RF using a polymerase specified by the ϕ X genome). A mutation in such a gene would easily lead to defects in the production of single stranded DNA.

At this time our knowledge of the mutants in this complementation group is so scarce that both of the above ideas might prove to be wrong.

The mutant ts 4 is in the third of the ts complementation groups. The following facts are known regarding its growth at 40°C. It makes SBP principally in the form of 71S particles. It makes both infectious RF and infectious single stranded DNA. It does not lyse its host cell.

The last statement might prompt one to immediately suggest that ts 4 is located in the gene concerned with synthesizing the ϕX lytic protein. However, reasoning from the discussion below, this probably is not true.

In addition to the three complementation groups discussed so far (these contain both ts and am mutants) Hutchison (62) has discovered two other complementation groups (making a total of five). Only am mutants have been found in the two new groups. One of these new groups contains the mutant am 3, which has the remarkable property that in the nonpermissive host it makes complete virus particles, but fails to lyse the host cells. In fact if the nonpermissive host is artificially lysed about 2 hr after infection by am 3, several thousand am 3 particles per infected cell (these particles are infective on the permissive host) are liberated. Clearly am 3 is defective only in the lysis function and the gene in which it is located almost surely specifies the ϕX lytic protein. Other am mutants in the same complementation group also fail to lyse their nonpermissive hosts.

It is, however, true that ts 4 also is defective in the lysis function. In addition, although ts 4 does synthesize particles at 40°C these certainly do not resemble normal ϕX particles, for they sediment at about 71S. Furthermore, it seems unlikely that these 71S particles are akin to the 70S particles produced in ϕX infections under normal conditions. The 71S particles contain much of the SBP made at 40°C by ts 4, and it is reasonable to postulate that they also contain the infectious single stranded DNA found by Dowell and Sinsheimer (35). Were this true these particles could be envisioned as nearly completed virus, but with the DNA slightly extruded.

Heating ϕX^+ at 70°C for 30 min causes extrusion of its DNA and results in a particle that sediments at 47S, due to the increased

frictional coefficient relative to 114S particles (4, 64). The DNA of the ts 4 71S particles would have to be less extruded than is the case for the heated wild type particles.

One approach toward testing this hypothesis would be to assay the fractions of a sucrose gradient, like the 25 min one in Figure 17, for infectious DNA (after phenol extraction). In fact this experiment has recently been performed in collaboration with Hutchison, and indeed a peak of infectious DNA appeared at 67S. The fractions from the gradient were also assayed before phenol extraction, and in this case no infectivity (DNA) was observed at 67S. It is not exactly clear why the DNA infectivity sedimented slightly more slowly than the bulk of the SBP. However, these results seem to provide some tentative support for the model of the ts 4 71S particle outlined above.

However, it is still not obvious from the available data on ts 4 what function to ascribe to the gene in which the mutation is located.

The ϕ X genome contains at least five separate genes as defined by complementation tests. The production of the SBP protein and the lytic protein account for two of these genes. A gene for ϕ X DNA polymerase very likely accounts for a third. Some possibilities for the functions specified by the remaining two genes are a coat protein in addition to the SBP protein, an enzyme concerned with ring closure or special properties of the discontinuity in the DNA, an enzyme that mediates assembly of the ϕ X components into mature virus, and enzymes whose functions remain to be guessed at.

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