

BACTERIOPHAGE ØX174: VIRAL GENES AND FUNCTIONS

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ABSTRACT

Mutants. Conditional lethal mutants of bacteriophage $\emptyset X174$ ($\emptyset X$) have been selected. These include nonsense mutants of the am (amber nonsense triplet - UAG) and op (opal nonsense triplet - UGA) types. Missense mutants of the ts (temperature-sensitive) and ss (solvent-sensitive) types have also been isolated. The ss mutants are abnormally sensitive, during plaque formation, to the presence of solvents such as dimethylsulfoxide, ethylene glycol, or urea in the growth medium. Host range mutants are also described. Double and triple mutant strains have been constructed from specific single mutants.

Cistrons. Complementation studies (principally with am and ts mutants) have defined seven complementation groups or cistrons. It is believed that these represent the genes for seven essential $\emptyset X$ -coded proteins. At least six of these cistrons are homologous, by complementation, to cistrons of the related phage S13.

Functions. Studies of abortive infection by conditional lethal mutants under restrictive conditions have been performed in order to elucidate the functions of the mutant cistrons. Properties of phage particles produced by mutants have been examined in order to identify cistrons which code for components of the phage coat. The cistron functions deduced from these studies are: cistron I - lysis of the host. Mutants in this cistron produce progeny at a normal rate, and for an extended time, but are not able to lyse the host cell. cistron II - spike component. This cistron codes for a protein which is a component of the

spikes which project from the 12 vertices of the isometric ϕX capsid. cistron III - spike component. This cistron codes for a second protein component of the phage spike and contains the serum-blocking antigen of the phage particle. cistron IV - phage coat. This cistron codes for a protein of the phage coat which is probably, by elimination, the main structural component of the capsid. cistron V - ?. The function of this cistron product is not known. cistron VI - RF replication. The product of this cistron is necessary for replication of the double-stranded form of ϕX -DNA (RF). cistron VII - spike component. The product of this cistron is believed to be a component of the phage spike which determines the host range of the particle.

These physiological studies suggest that assembly of phage, or defective phage particles is necessary for the synthesis of viral single-stranded DNA. RF replication (which requires cistron VI function and cistron V function are also necessary for single-strand synthesis.

Recombination experiments (involving both two and three factor crosses) indicate that the order of the cistrons is II-III-VII-V-I-IV-VI, possibly joined end to end to form a circular map. It is of interest that cistrons which code for the spike components (II, III, and VII) appear to be contiguous on the map. A polar am mutant in cistron IV is deficient also in cistron VI function. This is interpreted to mean that reading proceeds from left to right on the map as shown (5' to 3' direction of the viral DNA and ϕX mRNA).

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1. GENERAL INTRODUCTION

The existence of a class of minute bacterial viruses has been known since about 1932 (1,2). Beginning in 1959, when Sinsheimer reported his physical characterization of bacteriophage $\emptyset X174$ and its DNA, the study of small bacteriophages has proliferated and is now a major area of molecular biology. Sinsheimer (3,4) showed that each particle of $\emptyset X174$ contains a single molecule of DNA, consisting of a single polynucleotide chain only a few thousand nucleotides in length. The subsequent finding that purified DNA, free of all detectable $\emptyset X174$ coat protein, could infect suitably treated cells of E.coli to produce complete infective virus particles, conclusively demonstrated that this DNA molecule contains all the essential genetic material of the phage (5-8). The small size of the $\emptyset X$ genome made it a particularly appealing object for detailed genetic study. This size set a limit on the number of functions which could be coded by the virus, and therefore made a complete enumeration and characterization of the viral genes seem possible. The small size of $\emptyset X$ DNA, as well as its single stranded nature, also made it a particularly suitable subject for further detailed physical and chemical study. $\emptyset X$ was therefore the material of choice for an experimental approach which combined the power of genetic analysis with that of physics and chemistry in an attempt to understand the process of virus infection in molecular detail.

Such a double-barreled approach to the $\emptyset X$ problem has been carried out in Sinsheimer's group at Cal Tech for almost eight years now. This thesis will be concerned primarily with the genetic aspect of the problem. It will describe how $\emptyset X$ mutants have been used to define and

enumerate ϕ X genes, as well as how physiological experiments making use of these mutants have elucidated many of the viral functions.

This introduction will begin by reviewing the process of infection with ϕ X. The infection will be discussed from a genetic point of view, however the evidence presented will be non-genetic wherever possible. An attempt will be made to estimate the number of genes necessary for the process on the basis of known physiological steps in the infection. Next the literature related to previous genetic studies of ϕ X will be discussed. The introduction will conclude with a brief review of physiological genetics of bacteriophage, particularly the application of conditional lethal mutants.

1.1 The Process of Infection with Bacteriophage $\emptyset X174$

The virus particle. The mature virus particle, completely isolated from the host cell, is the best defined stage in the cyclic process of virus infection, and therefore the easiest place to start.

$\emptyset X$ is an isometric virus with a diameter of 25 to 27 $m\mu$ (9,10) an $S_{20,w}$ of 114 and a molecular weight of 6.2×10^6 Daltons (3). It contains one ring of single-stranded DNA with a molecular weight of 1.6 to 1.7×10^6 Daltons (4). The remaining 4.5×10^6 Daltons presumably compose the protein coat of the virus. The possibility of small amounts of other molecular components has not been excluded.

The Viral DNA. The viral DNA contains about 5500 nucleotides (1800 T, 1350 A, 1000 C, and 1350 G) (calculated from 4). For comparison, a single strand derived from phage T4 would be about 35 times as large, and one derived from phage lambda would be about 10 times the size of $\emptyset X$ DNA (calculated from 13). Assuming a triplet code (14,15) it is apparent that the maximum amount of protein sequence which can be coded by the virus is some 1800 amino acids. This would correspond to 5 to 10 proteins of normal size (180 to 360 amino acids). Several unproven assumptions are involved in such an estimate of the number of $\emptyset X$ genes. Two of the weakest are: 1) We assume that all of the $\emptyset X$ DNA codes for protein. It seems possible that some regions might play some structural role in the assembly of the phage, they might code for some non-messenger RNA, or for some other reason not be used to code for protein. 2) There may well be $\emptyset X$ coded proteins which fall outside the normal range of sizes. Proteins outside the normal size range do exist.

For example, ferredoxin contains 55 amino acids (16) and the A chain of insulin contains 21 amino acids (17), while the peptide chains of β -galactosidase consist of 400-500 amino acids (18).

Two other features besides its small size make $\emptyset X$ DNA genetically interesting. These are the single-stranded nature of the DNA (4) and its ring structure (19,20). Although single-stranded rings of larger phages, for example lambda (21), can be infective, the occurrence of these rings as a normal step in the process of infection might well have observable genetic effects. A special gene (or genes) might be required for the production of such a molecule. Although the $\emptyset X$ ring appears to contain only normal phosphodiester linkages, there appears to be one region in the DNA which is relatively resistant to the action of exonuclease (see 22 for a review). It has been suggested that this is a self-complementary region of the single-strand which forms a double helix and is therefore rendered resistant to the enzyme. This might represent a region with a function other than coding for protein, as mentioned above.

The protein coat. The structure of the protein coat of $\emptyset X$ is of obvious interest with respect to the problem of $\emptyset X$ gene products. Almost all viruses code for the structural proteins of their own coats. Larger bacteriophages appear to devote a large number of genes to the structure and assembly of their coats, presumably for some good evolutionary reason. In phage T4 at least 46 genes appear to be involved in morphogenesis of the coat (23), while in phage lambda 18 genes are implicated (24). On the other hand, there exist viral genomes which

do not code for their own capsids. Rous sarcoma (under some conditions of infection) is an example of such a virus (25,26). In this case the coat is provided by another viral genome, but there appears to be no reason in principle why the host cell itself, or some episomal element associated with the host, could not contribute structural components of a viral coat. At present a genetic approach provides the only reasonable method for determining whether coat proteins are virus coded.

A complex mechanism for the transfer of nucleic acid from phage to bacterial cell appears to be a general phenomenon (with the possible exception of $\emptyset X$ and its close relatives). All large phages show some sort of a tail (see 27 for a review). The two other classes of small phages, the RNA phages and the filamentous single-stranded DNA phages, appear to attach to F-pili of E. coli and quite likely parasitize the transfer mechanism of the host (28,29).

A theoretical approach to the problem of virus structure, was based on a suggestion by Crick and Watson (30), and elaborated by Caspar and Klug (31). This theory considers the most efficient ways in which genetic information can be used to code for a protein coat if the only function of the coat is to enclose space. Persistent application of this theory to $\emptyset X$ has resulted in its continued use as an example of the simplest case of the theory (T=1,12 capsomeres) in spite of gradually accumulating evidence for its structural complexity (see the text by Luria and Darnell, for example (13)).

Electron microscopy of negatively stained particles of $\emptyset X$ has shown that the $\emptyset X$ coat has icosahedral symmetry. The most significant

feature which distinguished ϕX from a sphere is a set of knobs or spikes projecting from the 12 vertices of 5-fold symmetry (9-12).

Various interpretations of the ϕX structure have resulted from electron microscopic studies. Shadowed preparations gave the impression of 12 approximately spherical morphological subunits located at the vertices of an icosahedron (11). Negative staining has revealed a somewhat more complicated picture. Tromans and Horne (12) concluded that the protein coat was composed of 12 morphological subunits but that these appeared to be complex structures, and suggested that the spikes might be involved in a mechanism for phage DNA transfer. Maclean and Hall (9) observed partially disrupted particles of ϕX in the electron microscope. These observations led them to propose a model of ϕX in which only 20% of the protein composes the 12 knobs of the icosahedrally symmetric outer shell. The remaining 80% of the protein was supposed to be directly combined with the phage nucleic acid in the form of a nucleoprotein.

Results to be presented in this thesis demonstrate that the 12 apical spikes do not form a complete shell over the viral surface, but rather are attached to the vertices of a shell which is stable even in their absence. This shell probably consists of more than 12 morphological units. Electron micrographs of the closely related phage ϕR by Bradley (32,33) support this type of model.

Carusci and Sinsheimer (34) characterized a protein preparation derived from the ϕX coat. It appeared to be physically homogeneous with a molecular weight of 25,000 and a chain length of 225 amino acids.

The information required to code this protein would be 675 nucleotides or 1/8 of the $\emptyset X$ DNA. It was suggested that the protein coat of the virus was composed of 180 of these molecules. More recently, electrophoretic evidence of Dann-Markert, Deutch, and Zillig (35) and of Burton (36), and chemical evidence presented by Poljak (37), indicate the presence of two major protein components in the $\emptyset X$ coat. In view of these results it appears likely that the material studied by Carusi and Sinsheimer was a mixture of two proteins of about the same molecular weight, which if coded by the virus could account for $\frac{1}{4}$ of the viral genetic information.

Rolfe and Sinsheimer have shown that the $\emptyset X$ particle is antigenically complex (38). They observed two antigenic sites on intact phage particles, and a third was revealed when the particle was partially disrupted by heating. This third antigen was also present in the 70 S particles usually found in $\emptyset X$ lysates.

It seems reasonable to assume in view of these various lines of evidence that the $\emptyset X$ capsid is a complex structure. It should be possible to associate a gene with each protein in the coat, to determine the structural arrangement of the various protein molecules in the coat, and finally to elucidate the functional significance of the phage structure. Such an analysis has been initiated in collaboration with Marshall Hall Edgell, and has yielded a number of interesting results (see Part 3 of this thesis). In our present working model four $\emptyset X$ genes code for structural proteins of the coat. This coat consists of a shell composed of one protein which encloses the DNA, and 12 structurally com-

plex spikes which are involved in the specific attachment of ϕ X to the host and the subsequent injection of ϕ X DNA. A preliminary report of these findings has been presented (39).

Attachment, eclipse, and injection. Newbold (40) has been able to resolve the initial stages of ϕ X infection into these three steps.

1) First the virus attaches to the sensitive host cell. This attachment is dependent on the ionic conditions of the medium (41). There is considerable host specificity for attachment. ϕ X will attach to E. coli strains C or 15, to Shigella paradyenteriae strain Y6R, and to Salmonella typhosa strain 373, but will not attach to E. coli strains K12 or B (41,5). These facts suggest that both the viral coat protein and host cell gene products in the cell wall are involved in attachment. Treatment of cell wall fragments with lysozyme destroys their ability to attach ϕ X, while treatment with trypsin or phenol does not (42).

Genetic studies have revealed that mutations of both the phage and the host cell can affect attachment (see below). It has been possible to transfer ϕ X sensitivity into E. coli K12 derivatives by bacterial recombination (43), thereby making it possible to apply a variety of useful bacterial mutants to the study of ϕ X. It has also been possible to produce mutants of K12 which are sensitive to the closely related phage S13 (44).

Attachment may be reversible in the sense that a suitable treatment (elution in borate-versene buffer) will remove the phage completely intact and infective (40). At 15°C or below only attachment occurs.

2) After attachment comes the irreversible process of eclipse. When phage attached at 15°C are shifted to 37°C, it rapidly becomes im-

possible to remove them in an infective state. Washing with borate-versene now removes the phage in a non-infective state, and with lowered efficiency (40).

Recently a class of cold-sensitive mutants has been reported, in which eclipse cannot occur at 25°C (45). These mutants occur in a gene which we have shown to code for a structural component of the phage coat, and so support the idea that the ϕ X coat is involved in a real injection process.

3) Next the ϕ X DNA is injected into the host cell. Little is known concerning the mechanism of this process. The infecting phage DNA becomes resistant to elution by the borate-versene procedure as injection proceeds. It appears that protein coats may still be eluted following injection, and that they are converted to 70 S particles, although it is not completely clear that the observed 70 S coats are derived from those phage particles which are capable of initiating infections (40,46). It has not been determined whether the DNA enters the cell as a single-stranded molecule, or whether it perhaps is pulled into the cell by a process involving the synthesis of its complement as suggested by Guthrie (47). It would appear that both the phage coat proteins and some cellular mechanism might be involved in the injection process.

Formation of the parental RF. Within about three minutes after attachment of the infecting phage, its DNA is converted to a double-stranded ring consisting of the original viral (+) strand and a newly synthesized complementary (-) strand (48,49). This double-stranded form of ϕ X-DNA

is called the replicative form (RF) and the molecule containing the infecting viral strand is referred to as the parental RF, as opposed to progeny RF which does not contain infecting material. Some of the parental RF molecules consist of two covalently closed strands (RFI), while in other molecules one strand may contain a "nick" (RFII) (50).

Formation of the parental RF does not seem to require any protein synthesis. It cannot be blocked by chloramphenicol at a concentration of 100 mg/ml (51,52) nor by starvation for an essential amino acid when infection is performed in an auxotrophic host (53). These results suggest that the complementary strand is synthesized by pre-existing enzymes, presumably host enzymes (although they could conceivably be brought into the cell from the phage particle). The formation of parental RFI in the absence of protein synthesis suggests the involvement of both a polymerase and a ligase contributed by the host cell. Under conditions in which protein synthesis is completely blocked (as in high levels of chloramphenicol) the infective process is halted following formation of the parental RF. No progeny RF is formed (51,52), and no later steps in the infection can proceed.

The observation that viral mutants may be blocked at this same stage (Part 3 of this thesis) suggests that synthesis of some phage-coded protein is necessary at this point in the infection. So the next step must involve the synthesis of a ϕX specific messenger.

Synthesis of ϕX mRNA. Hayashi, Hayashi, and Spiegelman (54) detected the presence of ϕX specific messenger RNA in infected cells by a hybridization technique. This pulse labeled mRNA would hybridize with

denatured ϕ X RF (which contains a mixture of (+) and (-) strands) but not with purified viral (+) strands, indicating the ϕ X mRNA is transcribed specifically from the complementary (-) strand. It was not possible to determine whether this specificity was absolute. At late times of infection it could be seen that less than 5% as much of the pulse labeled material would hybridize with (+) strands as with a mixture of (+) and (-) strands. At early times (5 to 6.5 min) much less ϕ X-specific mRNA was found, so that background problems became more severe and it was only possible to conclude that at least 80% of the radioactivity hybridizable with ϕ X DNA was complementary to sequences in the (-) and not the (+) strand. It should be noted that the conditions of infection employed in this work were quite different than those used in this laboratory, and it seems possible that the infection could proceed during the adsorption period so that the "early" pulse labelling experiment did not actually represent a very early stage of the infection.

Hayashi and Hayashi (55) have found a RF-mRNA complex in the ϕ X-infected cell. This complex consists of small (3-3.5 S) pieces of pulse labeled RNA hydrogen bonded to RF. Again these pieces of RNA hybridize specifically with the (-) strand. The material was isolated from cells infected for 2 hours in the presence of 50 μ g/ml of chloramphenicol, so it is not clear whether the material is characteristic of an early or a late phase of normal infection.

Sedat (56) has made use of the technique of chromatography on BNC (benzoylated-naphthoylated-DEAE-cellulose) (57) to isolate mRNA from ϕ X-infected cells. The mRNA labeled during a pulse after 7 min of infection

hybridized specifically with (-) strands. Sedimentation of this material in a sucrose gradient revealed that although it was somewhat heterogeneous in size, a considerable amount sedimented slightly faster than $\emptyset X$ (+) strands. A similar pattern of mRNA synthesis was observed by Hayashi when $\emptyset X$ RF was transcribed in vitro (58).

The available evidence indicates that $\emptyset X$ mRNA is synthesized using the (-) strand of the RF as a template. The molecules are heterogeneous in size following extraction and purification, but some of the material is of a size comparable to the complete viral genome. No evidence for the formation of mRNA complementary to the (+) strand exists, although in view of the existence of early and late mRNA in other phage systems (59) the possibility of such molecules should not be rejected until careful experiments at early times of infection have been performed. The best working model is that the whole $\emptyset X$ genome is transcribed into a single polycistronic mRNA molecule, 5500 nucleotides in length, which is then translated into the viral proteins. Such a model requires that $\emptyset X$ mRNA (or at least the first molecule synthesized) is made using a pre-existing polymerase, presumably a host enzyme (since no phage enzyme could be made before the synthesis of $\emptyset X$ mRNA).

The existence of polar mutants, to be described in this thesis, provides genetic evidence for the existence of such a polycistronic message, and provides evidence for the direction of transcription relative to the order of the genes as determined by recombination.

The bacterial site. Evidence of various sorts exists that the parental RF molecule must become associated with a special site in the host

cell in order to initiate a successful infection. Denhardt and Sinsheimer (60) showed that the parental (+) strand plays some essential role in the infection. If this strand is inactivated by ^{32}P decay, even late in the infection when many copies exist within the infected cell, production of progeny phage may be halted. Yarus has made measurements of the "participation number" of $\emptyset X$ in the formation of progeny (making use of genetically distinguishable $\emptyset X$ strains) which indicate that only a limited number of infecting phage particles may contribute their genetic information to the formation of progeny (61). This is especially pronounced in starved cells, in which case the majority of cells can only produce progeny of a single parental genotype. Exponentially growing cells can produce up to four genotypes in single bursts, although not as frequently as would be expected on the basis of an infinite "participation number". Stone (52) studied the replication of $\emptyset X$ RF following shifts from $^{15}N^2H$ to $^{14}N^1H$ medium as a function of time following infection, and concluded that only parental RF is involved in replication. Knippers (62) found that the parental RF is preferentially associated with a "membrane fraction" when infected cells are artificially lysed and sedimented at low speed, while progeny RF appears in the supernatant fraction. Parental RF attaches to this "membrane fraction" even in the presence of 100 $\mu g/ml$ of chloramphenicol.

These results suggest that the parental RF molecule is attached to a specific site on the bacterial membrane, that the number of such sites is limited (only one in starved cells), and that such attachment is necessary for subsequent RF replication and synthesis of progeny

phage. The structure of this site is presumably determined by genes of the host cell. Apparently no new protein synthesis is required for attachment.

Genetic complementation studies in starved cells, to be presented in this thesis (Part 2), indicate that attachment to the bacterial site is necessary for the performance of several phage functions and hence may be required for transcription. Site attachment may therefore occur prior to the synthesis of the first mRNA molecule or even during the synthesis of the parental RF. The *rep*⁻ mutant of the host, described by Denhardt, Dressler and Hathaway (63), may represent an alteration in the bacterial site. This mutant allows the synthesis of the parental RF molecule, but does not permit RF replication, or the performance of later ϕX functions (e.g. lysis of the host).

Replication of the RF. Replication of the parental RF occurs in a semi-conservative manner (48,52). The parental (+) strand remains associated with the site on the membrane (62) but exchanges the complementary (-) strand during replication. Pulse label incorporated into replicating RF first appears in parental RFII (either the (+) or the (-) strand may be open) associated with the membrane fraction (62). This label can be chased into the cytoplasm of the cell where it initially appears as RFII, but is subsequently converted to RFI (62,64). At 12-15 min following infection 15-20 RF molecules are present in the infected cell. This is mainly RFI.

Evidence will be presented in this thesis that ϕX gene products are necessary for RF replication, since certain ϕX mutants are blocked

in this process. Since RF replication can proceed in the presence of 30 μ g/ml of chloramphenicol (48) the $\emptyset X$ proteins involved must be synthesized in the presence of this drug. Suppression of host protein synthesis by chloramphenicol has allowed the identification and partial purification of one $\emptyset X$ gene product necessary for RF replication (65). The function of this protein has not been further characterized. There exist bacterial mutants, temperature-sensitive in the synthesis of bacterial DNA, which are unable to replicate $\emptyset X$ RF at the restrictive temperature (66). This result demonstrates that host functions as well as phage functions are involved in $\emptyset X$ RF replication. Quite a few proteins could be involved in the process: 1) some DNA polymerase, 2) a ligase, 3) a nuclease (for opening a strand of RFI to permit replication), 4) proteins involved in the structure of, and attachment to, the bacterial site.

Synthesis of the serum blocking power (SBP) antigen. During the RF replication phase of the infection (from about 3 to about 12-15 minutes after infection at 37 $^{\circ}$ C), synthesis of $\emptyset X$ coat protein begins. Krane (46) developed a serum blocking assay for the $\emptyset X$ coat antigen which reacts with anti- $\emptyset X$ serum resulting in inactivation of the plaque forming ability of the phage. This assay has made it possible to selectively measure the SBP antigen without purification in extracts of infected cells. Newly synthesized SBP antigen begins to appear by 5 minutes after infection at 37 $^{\circ}$ C and continues until the time of lysis.

Experiments with mutants of $\emptyset X$ (Part 3 of this thesis) indicate that the SBP antigen is present on just one of the structural proteins of the phage coat.

The early synthesis of a phage coat protein is consistent with the picture of ϕX mRNA synthesis presented above, in which a single mRNA molecule the size of the ϕX genome is synthesized. This result shows further that cistrons of the mRNA may be translated into protein starting early in the infection, even though the protein may not be required until some later stage.

Superinfection exclusion. The use of ϕX mutants has revealed the phenomenon of superinfection exclusion in ϕX infection (Part 3 of this thesis). If E. coli C is infected with one strain of ϕX and is then superinfected several minutes later with a distinguishable mutant strain, only the first strain is found among the yield of progeny phage. By the use of a non-lysing mutant of ϕX as the primary infecting phage it has been possible to exclude a trivial explanation, namely that the primary infection induces cell lysis before the secondary infection can produce mature progeny. The excluded phage DNA is not found in RF when the intracellular DNA is analyzed following superinfection, but rather appears to remain an infective, therefore presumably intact, single strand.

This exclusion begins to be established during the early (RF replication) phase of the infection. Exclusion of the superinfecting phage from the performance of the lysis function could be detected by 5 minutes following infection at 37°C and was essentially complete by 10 minutes.

Superinfection exclusion is not established if the infection is carried out in the presence of chloramphenicol (30 or 100 μ g/ml), indi-

cating that protein synthesis is a prerequisite for the exclusion. It is not yet known whether specific ϕ X gene products are directly responsible for the phenomenon of superinfection exclusion.

Switchover to the late phase of infection. Several events occur approximately simultaneously at about 10-15 minutes after infection. Synthesis of host DNA is halted (67) as is net RF synthesis (67,68), and mature progeny phage (containing single-stranded DNA) begin to appear. It is not known whether this switch from double to single-stranded DNA synthesis may represent some specific regulatory process. Alternatively the switchover could be a kinetic phenomenon, controlled by the levels of various phage gene products produced concurrently.

Shutoff of host DNA synthesis. Synthesis of bacterial DNA ceases 12-14 minutes after infection with ϕ X (67). This can be most easily demonstrated during infection with certain mutants of ϕ X (blocked in lysis and in synthesis of single-stranded DNA) which will be described in this thesis, but the effect can be observed in wild-type infection as well. If chloramphenicol is present during the infection then host DNA synthesis is not shut off. However, chloramphenicol added after the shutoff will not cause re-initiation of host DNA synthesis. This indicates that the synthesis of some protein is required for an irreversible shutoff to occur. This could conceivably be either a phage or a host coded protein. Evidence acquired by the use of ϕ X mutants demonstrates that this shutoff can occur even when phage maturation is blocked and no single-stranded DNA is made.

Cessation of net RF synthesis. There is essentially no increase in the number of RF molecules in the normal infected cell after 15 minutes of infection (67,68). This shutoff is blocked by the addition of chloramphenicol. Even if chloramphenicol (at a concentration of 30 μ g/ml) is added late in the infection (after shutoff) RF synthesis is re-initiated (68), suggesting that whatever proteins are required for the suppression of RF synthesis must be continuously supplied. Experiments with $\emptyset X$ mutants show that this cessation of RF synthesis is independent of phage maturation and single-stranded DNA synthesis (67).

Single-strand synthesis and phage maturation. At about 10 minutes after infection (at 37°C) mature progeny phage begin to appear in the infected cell (69). Single-stranded DNA is apparently incorporated into phage particles almost immediately when it is synthesized, as no free single strands are observed (48).

At this time the rate of $\emptyset X$ DNA synthesis increases dramatically, with essentially all the synthesis being (+) strands. Pulse labelling with tritiated thymidine reveals that newly synthesized $\emptyset X$ DNA first appears in RFII, but then is chased into progeny phage or with lower probability into RFI. This synthesis occurs mostly in the cytoplasmic RF, but also in the membrane bound fraction. Pulse label in cytoplasmic RFII appears mostly in open (+) strands. The pool of RFI present in the cell is mostly converted to RFII which then can participate in single-strand synthesis (64).

Experiments with ϕ X mutants defective in coat protein synthesis (Part 3 of this thesis) indicate that assembly of the ϕ X coat is necessary for single-strand synthesis to occur. This result leads to a model in which the formation of single strands and the assembly of progeny phage form an integrated process.

Several proteins appear to be involved in the late phase of ϕ X infection. These probably include: 1) A DNA polymerase which synthesizes viral (+) strands. 2) A ligase which joins the newly synthesized (+) strand to form a ring. 3) A nuclease which converts RFI to RFII which can then participate in single-strand synthesis. (These three enzymes could either be the same ones involved in the replication of RF or they could be new ones.) 4) The coat proteins of the phage.

Phage continue to be matured within the infected cell at a constant rate (usually 20-30 phage per cell-minute) until lysis of the host (68,69).

Lysis. In a normal ϕ X infection, the host cell lyses about 20 minutes after infection (at 37° C) and releases a burst of mature progeny phage (70). There is considerable spread in lysis times among the cells. An appreciable number of cells begin to lyse by 13-14 minutes after infection, and 90% of the phage have been released by about 35 minutes. Phage from a single infected cell are released in a burst, within 30 seconds or less. Microscopy of lysed cells indicates that lysis is a result of breaks in the cell wall rather than any general dissolution of the wall structure (71). Lysis can be inhibited by the addition of sucrose (20% w/v) (71) or spermine (10^{-2} M) (72) to the infected culture.

Markert and Zillig (71) used metabolic inhibitors to study ϕX induced host cell lysis. They showed that synthesis of both DNA and protein following infection was essential for normal lysis, while formation of mature progeny phage or even mature progeny DNA was unnecessary. Most interestingly, they found that protein synthesis was required at a time later than the onset of coat protein production in order for lysis to occur. It still remained to be determined whether this synthesis was phage or host specific. Thirteen ϕX temperature sensitive (ts) mutants studied by Markert and Zillig all lysed normally at the restrictive temperature. Since no mutants showing defective lysis were found, the question of phage control of lysis remained open.

Some unsuccessful attempts to detect a lytic enzyme in the mature phage particle and in infected cells have been reported (42,71,73).

Denhardt and Sinsheimer (69) have described a mutant of ϕX with a longer mean latent period than wild-type. This mutant (ϕX_p) also shows reduced lysis when grown in concentrated culture, suggesting some degree of phage genetic control over the lytic process. However, the rather complicated behavior of this mutant, in particular the concentration dependence of its effect, makes it difficult to study.

This thesis includes a description of the selection and characterization of mutants of ϕX which are unable to lyse the host cell. The existence and properties of these mutants demonstrate the involvement of a ϕX -coded protein in the lytic process.

Summary. Table 1 is an attempt to summarize present knowledge and hypotheses concerning the gene products involved in the various steps

of ϕ X infection. The observable functions which take place in the infected cell are listed in approximate chronological order. The infection is divided into initial (approximately 0-3 min at 37^0 C), early (3-12 min), and late (12-20 min) stages. The requirement of protein synthesis in order for a step to proceed is indicated by a "+" while a "-" indicates that the step in question can occur in the absence of protein synthesis. Gene products of the ϕ X genome, and of the host cell, which have been suggested in this introduction are listed beside the related function.

It is quite possible that functions may occur in the ϕ X infected cell which are not observable by present experimental techniques. However, the number of viral proteins implicated by this survey of the physiology of ϕ X infection is at least consistent with the earlier estimate (5-10) based on the information content of ϕ X DNA.

1.2 Previous Genetic Studies of Bacteriophage $\emptyset X$ 174

This section will review the limited literature related to $\emptyset X$ genetics which was available prior to the work presented in this thesis. More recent work related to the molecular effects of various mutagens on $\emptyset X$ and related phages will also be discussed. Genetic work concerned with the number and functions of the viral genes in other small phages, particularly the closely related S13, will be discussed later in comparison with the results to be presented.

$\emptyset X$ mutants. In 1958 Zahler (41) reported the isolation of a number of mutants of $\emptyset X$, and of phage S13 (which he found to be serologically related to $\emptyset X$). These included 1) mutations affecting the salt requirements for plaque formation on several host strains, 2) host range mutants able to form plaques on bacterial mutants resistant to wild-type phage, and 3) plaque morphology mutants. These were all spontaneous mutants.

Tessman (74) produced host range (h) mutants of $\emptyset X$ by in vitro treatment of $\emptyset X$ with nitrous acid. These mutants were able to form clear plaques on a mixture of E. coli C and a mutant of C which was resistant to wild-type $\emptyset X$. Nitrous acid inactivated $\emptyset X$ in a strictly exponential fashion, while the fraction of h mutants among the surviving phage increased linearly with time (as expected). Data were also presented which indicated that $\emptyset X$ mutants induced by such in vitro nitrous acid treatment arise in pure clones whereas similar treatment of bacteriophage T4 results in clones containing a mixture of the mutant and the wild-type. These data were interpreted in terms of the evidence that $\emptyset X$ contains

only a single-strand of DNA while T4 DNA has the usual double-stranded structure. (It should be noted, however, that in this experiment $\emptyset X$ was inactivated to a very low survival level. Thus if $\emptyset X$ were double-stranded we would expect any strand containing an h mutant to be paired with a strand containing lethal hits. The h⁺ allele from this strand would then have to be rescued by recombination to yield a mixed burst. Since recombination only occurs infrequently in $\emptyset X$ as compared with T4 (see below), mixed bursts might not arise.)

Various plaque morphology and host range mutants were also isolated by Epstein (75).

In addition to the extended host range mutants mentioned above, limited host range and inverted host range systems have been reported (76) which allow the selective detection of both forward and reverse mutations.

Mutagenesis of single-stranded DNA. Tessman and co-workers (76, 78) have reported that they can identify a number of specific host range mutations in phage S13 by phenotypic characterization (using a variety of sensitive hosts). They have made use of these mutations to analyze the action of a number of mutagenic agents on single-stranded DNA. The results indicate that: 1) Hydroxylamine (in vitro) specifically induces C \rightarrow T, 2) Ethyl methansulfonate (in vitro) induces all transitions, 3) Nitrous acid (in vitro) induces all transitions, 4) 5-Bromo-deoxyuridine (in vivo) preferentially induces T \rightarrow C and A \rightarrow G, but in one case induced G \rightarrow A, 5) 2-Aminopurine (in vivo) induced C \rightarrow T and G \rightarrow A, and in some cases T \rightarrow C and A \rightarrow G, 6) Ultraviolet irradiation

(of both phage and host) produced C \rightarrow T and possibly non-transition mutants. More recently it has been reported that in vivo mutagenesis by hydroxylamine can induce all four transitions (79).

Recombination. In 1959 Tessman and Tessman (80) reported their discovery of genetic recombination in phage S13. Following ultraviolet irradiation of the parental phage they were able to detect recombinant genotypes at frequencies of approximately 0.1% in crosses between several pairs of mutants. Plaque morphology and host range mutants were used in this study.

Recombination in phage \emptyset X was first observed by Pfeiffer (81), using a selective system for the detection of recombinant types. A three step extended host range mutant, $H_a H_b H_c$ (a triple mutant), was constructed. This \emptyset X mutant could form plaques on a triple mutant of E. coli C, C_{abc}, which was resistant to infection of wild type \emptyset X by virtue of three mutational steps. A variety of revertants from $H_a H_b H_c$ which were unable to form plaques on C_{abc} were then isolated. Many pairs of these revertants could form recombinants of the genotype $H_a H_b H_c$ which could be selectively detected by virtue of their ability to infect C_{abc}. The frequency of such recombinants among progeny from mixed infections varied depending on the pair of mutants used from 1×10^{-5} to 5×10^{-4} . The revertants could be classified into three groups which showed relatively low recombination frequency in intra-group crosses, but showed higher frequencies in intergroup crosses. It was not possible to order the three clusters of mutants on the basis of the data obtained, presumably because of a large amount of negative

interference which was indicated by the results of several crosses. Since all the mutations involved in this study affect host range, it is presumed that structural proteins of the phage coat are altered. It is not known whether all three loci affect the same protein. Aach (82) showed that the H_b mutation alters the electrophoretic mobility of $\emptyset X$ particles, a fact which has been helpful in the analysis of $\emptyset X$ coat proteins (see Part 3 of this thesis).

The discovery that recombination between $\emptyset X$ and S13 can be detected, by means of a suitable selective host range system, has strengthened the idea that these two phages are very closely related (83).

1.3 Physiological Genetics of Bacteriophage - Conditional Lethal Mutants

In order to enumerate and characterize the genetic functions performed by $\emptyset X$ DNA it is necessary to have some method for selecting mutations in various phage genes. Edgar, Epstein, and their collaborators have shown that certain types of conditional lethal mutations are nearly ideal for this purpose, and have made extensive use of such mutations in their analysis of phage T4 (84-86). A conditional lethal mutant is a strain which can be propagated under one set of growth conditions, but which cannot propagate under some other experimentally defined condition due to the mutation. Conditional lethal mutations include such diverse phenomena as mutation to nutritional requirement in fungi or bacteria (the mutant can grow in the presence but not in the absence of an added nutrient) and rII mutations in phage T4 (rII mutants reproduce fairly normally except in cells lysogenic for phage lambda). The importance of the technique described by Edgar and Epstein lies in the use of types of conditional lethals which are not selected to affect any specific phage function, but rather result in alterations of a general nature in protein structure or synthesis. These mutations may arise in most, if not all, phage genes to produce conditionally functional genes. The important properties of several types of such general conditional lethal mutations will be discussed. These include mutations which result in a block to virus replication under certain environmental conditions, such as extremes of the temperature range acceptable to the wild-type virus. Other conditional lethal mutations result in a re-

quirement (for virus reproduction) for certain bacterial suppressor genes in the infected cell.

In discussing conditional lethal mutations and their applications it is usual to refer to the experimental conditions during infection as:

1) "permissive" - the condition under which virus replication proceeds, and the mutant gene functions fairly normally, or 2) "non-permissive" or "restrictive" - these terms are used interchangeably to denote the experimental condition under which the mutant gene function is blocked, resulting in a block to the normal infective process of the virus.

The non-permissive or restrictive growth conditions must be permissive for the wild-type virus.

Conditional lethal mutants are used chiefly for two purposes. The first is for the enumeration and genetic labeling of the various genes of a virus. Complementation experiments allow the classification of mutants into functionally related groups called "cistrons". Generally, if bacterial cells are mixedly infected with two conditional lethal mutants of a bacteriophage under restrictive conditions, and if the mutations affect different functions or cistrons, then a burst of progeny bacteriophage will be produced "by complementation". In this process each mutant is able to supply the function which is defective in the other. If the mutations alter the same cistron, usually no phage will be produced by complementation (exceptions will be discussed below). These cistrons, defined by complementation experiments, appear to be equivalent to the structural genes for single polypeptide chains. Classification of a large number of mutants into cistrons has allowed the

identification of 81 genes (cistrons) in phage T4 (103). Mutations in these genes have been useful in many purely genetic experiments, such as detailed mapping of the T4 chromosome (86).

The other principal use of conditional lethal mutants has been in the detailed study of the roles played by various phage genes in the normal infective process. When a conditional lethal mutant infects under restrictive conditions, the abortive infection is blocked by the malfunctioning of the mutant gene. A variety of techniques have been used to identify the particular step in the infective process blocked by mutation in various phage genes (84,85). Many of the steps controlled by genes of phage T4 have been investigated at a molecular level in experiments which capitalize on the existence of the conditional lethal mutants.

Temperature sensitive mutants. Mutation may alter the temperature range over which an organism can reproduce. Mutants of Neurospora which grow at 25° C but not at 35° C were reported as early as 1950 by Horowitz (87,88). Such mutations occurred approximately at random among genes controlling known biochemical reactions as well as in genes which could not be identified by the standard techniques of biochemical genetics. Edgar and Lielausis (86) isolated temperature-sensitive (ts) mutants of phage T4D which could form plaques at 25° C but not at 42° C . Wild type T4D forms plaques with no change in "efficiency of plating" over this temperature range. Such mutations occur in a large number of genes in T4. Reproduction of the mutants is blocked at various steps during intracellular growth under the restrictive conditions (42° C) (84-86).

Analysis of the components present following abortive infection with such mutants has yielded a great deal of useful information with regard to the functions of the various mutant genes.

It is believed that ts mutations cause amino acid substitutions in the mutant protein, resulting in a molecule which cannot function at the restrictive temperature. Two types of ts mutants can exist: 1) the formation of the mutant protein is temperature sensitive, whereas the mutant protein formed at low temperature can function at high temperature, 2) the mutant protein is inactivated at high temperature even when formed at low temperature (89). It appears that most ts mutations in structural components of the phage particle are of type 1, being temperature sensitive only during the formation of the phage particle, and resulting (at 25°C) in the formation of phage particles of nearly normal thermal stability (86). Mutations in proteins with enzymatic function may be of either type 1 or 2. Both types of mutations have been observed in the structural gene for deoxycytidylate hydroxymethylase (90), for example.

Complementation between ts mutants altered in the same structural gene occurs frequently (85). Presumably this is directly related to the fact that ts mutations result in missense, allowing the formation of an altered protein subunit which may be able to form a functional multimer when combined with subunits bearing some different, and complementary, alteration.

Temperature sensitive mutations in a number of genetic systems have now been reported. These include several bacteriophages (91-93), animal

viruses (94-96), and bacteria (97).

Amber mutants. Amber (am) mutants were originally defined by Epstein, et al (84) as mutant strains of phage T4D which could form plaques on E. coli strain CR63, but not on strains B or S/6. Like ts mutations, am mutations may occur in a large number of T4D genes. Again the mutations result in blocks at various stages of intracellular development, and their general significance in the analysis of T4 infection is similar to that of ts mutations. However, the biochemical basis of the am phenotype is quite distinct from that of the ts phenotype.

It has been shown that am mutations result in premature termination of the mutant protein chain during infection of a restrictive host cell (98). In the permissive host the am mutation results in the production of a protein of normal size which contains a single amino acid replacement in the position immediately following the site of premature termination in the restrictive host (99). (Some of the prematurely terminated peptide is also made in the permissive host.) This is interpreted to mean that the am mutant contains a triplet which is nonsense (resulting in chain termination) in the restrictive host but is missense (resulting in an altered but functional protein) in the permissive host.

Mutational studies indicate that am mutations result in the codon UAG at the altered site in mRNA (100).

Bacterial strains are permissive for am mutants by virtue of suppressor mutations which they carry. A number of different am suppressors have been characterized (see 100 for a review). These differ in the particular amino acid which is inserted at the point specified by the am

codon (UAG). It has been shown that an am suppressor results in the production of an altered sRNA molecule which directs the incorporation of an amino acid in response to the am codon (101).

Intragenic complementation is not observed with am mutants (85). Thus two am mutants can complement only if they cause termination of different peptides, and an am mutant and a ts mutant which alter the structure of a single protein chain cannot complement. This is presumably a direct result of the fact that the mutation produces nonsense in the restrictive host, resulting in a prematurely terminated peptide which is not functionally related to the complete protein. This property makes am mutants more useful than ts mutants in defining phage genes.

Amber mutations have been reported in several bacteriophages as well as in bacteria (see 100 for a review). It appears that the mechanism of action of super-suppressors in yeast may be similar to that of am suppressors (102).

Ochre mutants. Ochre (oc) mutants are suppressible nonsense mutants similar in nature to am mutants, characterized by the mutant codon UAA. They are distinguishable from am mutants by the fact that they are not suppressed by the same bacterial mutations. These mutants are generally less useful than am mutants in studies of the physiology of phage infection, since all known oc suppressors are quite weak (100).

Opal mutants. Opal (op) mutants (103) are analogous to am and oc mutants but are characterized by the mutant codon UGA. Recently a strong suppressor specific for this triplet has been reported (104). Such

mutants should be quite useful in the same way that am mutants are, and might be expected to provide additional sites for conditional lethal mutations in a phage genome.

Other conditional lethal mutants. A number of other general types of conditional lethal mutants could conceivably be exploited. For example, Campbell has described pH-sensitive mutations in phage lambda (91). Mutations producing sensitivity to various conditions which affect the stability of proteins (such as the presence of denaturing agents in the growth medium) can be easily imagined.

This thesis describes the selection and characterization of ts, am, and op mutants of $\emptyset X$. Mutants sensitive to the presence of urea and ethylene glycol during formation of plaques, have also been found. Conditional lethal mutants have provided useful materials for the physiological and biochemical analysis of a number of steps in the infective process.

Possible limitations on the use of conditional lethals to completely enumerate the genes of an organism will be discussed in Part 4 of this thesis.

TABLE 1
GENE PRODUCTS REQUIRED FOR ϕ X INFECTION

STAGE	FUNCTION	ϕ X-CODED PROTEINS	HOST PROTEINS	IS PROTEIN SYNTHESIS REQUIRED?
INITIAL (0-3 min)	Attachment	ϕ X coat protein(s)	cell wall determinants (receptor)	NO
	Eclipse	ϕ X coat protein(s)	cell wall determinants, DNA polymerase?	NO
	Injection	ϕ X coat protein(s)	cell wall determinants, DNA polymerase?	NO
	Parental RF synthesis	none	DNA polymerase, ligase	NO
	Attachment to bacterial site	?	determinants of membrane structure	NO

EARLY (3-12 min)	ϕ X mRNA synthesis	probably none	RNA polymerase	?
	RF replication	ϕ X protein is required: polymerase? ligase? nuclease?	YES	
	SBP synthesis	ϕ X-SBP protein	(protein synthetic machinery)	YES
	Superinfection exclusion	?-some ϕ X protein may be required	?	YES

STAGE	FUNCTION	ϕX -CODED PROTEINS	HOST PROTEINS	IS PROTEIN SYNTHESIS REQUIRED?
LATE (12-20 min)	Halt to net RF synthesis	?	?	YES
	Shutoff of host DNA	?	?	YES
	SS synthesis	polymerase? ligase? ϕX coat proteins	polymerase? ligase? nuclease?	YES
	phage matur- ation	coat proteins, other factors?	?	YES
	block to cell division	?	?	?
	lysis of the host	ϕX protein is required	cell wall determin- ants, repair?	YES

2. MUTANTS OF OX: SELECTION AND GENETIC CHARACTERIZATION

2.1 MATERIALS AND METHODS

2.1.1 Media

KC broth contains 10g Bacto Tryptone (Difco), 5g KCl and 0.5 ml 1M CaCl_2 per liter of distilled water.

Bottom agar contains 10g Tryptone, 2.5 g NaCl, 2.5g KCl, 10g agar and 1 ml 1M CaCl_2 per liter of distilled water. Ten ml of bottom agar are used per plate.

Top agar contains 10g Tryptone, 5g NaCl and 8g agar per liter of distilled water.

2.1.2 Bacterial strains

All the strains are E. coli except as noted.

C is the usual host for $\emptyset X$ used in this laboratory, BTCC No. 122.

CR is a recombinant derived from a cross between the E. coli strains CR34 and C416. It was described by Denhardt & Sinsheimer (49). It is $\emptyset X^S$ and carries an am suppressor.

C_G is a spontaneous variant of C. It is $\emptyset X^S$, differs from C morphologically. These cells are predominantly rod shaped, whereas C cells are usually small doublets.

C₁ is a spontaneous mutant from C_G which is resistant to $\emptyset X$ wild-type, but sensitive to certain $\emptyset X$ host range mutants.

C_{ab} is a spontaneous double mutant of C which is resistant to $\emptyset X$ wild-type but sensitive to certain $\emptyset X$ host range mutants. It was isolated by Pfeiffer (81) who kindly supplied the strain.

CR/ΦX is a spontaneous mutant from CR which is resistant to ΦX wild-type but sensitive to certain ΦX host range mutants.

HF 4711 is a C-K12 hybrid strain derived by Paul Howard-Flanders who kindly provided this strain. It is Hfr-J2, ΦX^S, ade⁻, met⁻, arg⁻, str^r.

HF 4714 was derived from the cross HF 4711 x AB 2487 and kindly provided by Paul Howard-Flanders. It is ΦX^S, and carries an am suppressor.

CAJ 70, is lac⁻ (deletion), su⁺_{UAG}, str^S, ΦX^r, (A), F-lac₂ (UGA). This strain was isolated in Brenner's laboratory and kindly supplied by R.S. Edgar.

S26, S26 RIE, S26 RID, S26 R4C, and U11 RID are a set of strains obtained from A. Garen. They translate the mRNA triplet UAG as, respectively, nonsense, serine, glutamine, tyrosine, and lysine. The strains are believed to be isogenic except for these differences in the suppression of the am triplet. All the strains are ΦX^r.

Shigella parady-enteriae strain Y6R was obtained from J. Huppert.

C600 is the permissive host for assay of sus mutants of phage λ .

K37 and K38 are permissive and nonpermissive hosts for su mutants of phages f1 and f2. They were kindly supplied by R.C. Valentine.

WWU, U6-44, Ub-41, U6-25, and 34-S-3 are a set of strains obtained from F. Funk. They are ΦX^S, and believed to be isogenic except that they translate UAG as, respectively, nonsense, serine, glutamine, tyrosine, and (probably) lysine (see 108).

C-22 is a ΦX^S recombinant from a cross between strains C and K12. It was obtained from J. Bertani.

2.1.3 Bacteriophage strains.

$\emptyset X$ wt is wild-type bacteriophage $\emptyset X174$ characterized by Sinsheimer (3,4).

λ sus mutants were isolated by Campbell (91) and are suppressed by the same bacterial suppressors as T4 am mutants. J. Weigle kindly supplied stocks of several of these strains.

su mutants of phages f1 and f2 were isolated in N. Zinder's laboratory and kindly provided by R.C. Valentine. These are also am mutants as judged by their specificity of suppression.

T4D wild-type and op mutants were kindly supplied by R.S. Edgar.

su mutants of phage S13 were kindly supplied by E.S. Tessman.

am mutants of $\emptyset X$ have been isolated in the laboratory of M. Hayashi, who generously provided representatives of each of his complementation groups. C.E. Dowell kindly supplied the $\emptyset X$ mutant ts 41D.

2.1.4 Biological assays

Plating bacteria are grown in KC broth with aeration to a concentration of about 5×10^8 per ml, then stored in the cold until used.

Plaque assays are performed by the agar layer technique. We use 2.5 ml of top agar and 0.3 ml of plating bacteria per plate.

Dilutions of phage or infected cells for plating are made through KC broth.

Free phage are measured after killing infected cells by shaking with chloroform. This procedure inactivates plaque formation by $\emptyset X$ -infected cells even at late times when intracellular phage are present. In some

experiments unadsorbed phage are removed by incubating the infected cells for 5 min with anti- ϕ X serum with a K of 2 min^{-1} . Cells are then diluted at least 10^4 fold away from the serum before assay.

Intracellular phage are measured by lysing the infected cells with lysozyme and EDTA. To 1 ml of infected cells in KC broth at 0°C is added 0.1 ml of lysozyme solution followed by 0.1 ml EDTA (solutions as in Guthrie & Sinsheimer, (105)). After incubation for 30 min at 0°C the spheroplasts are disrupted by freezing and thawing 3 times in a dry-ice acetone bath before plating.

Infective DNA is assayed by the spheroplast procedure of Guthrie & Sinsheimer (105), modified by incubating the infected protoplasts at 30°C for 2 hours.

DNA is prepared by phenol extraction. Bovine serum albumin is added to the phage sample to give a concentration of 1 mg/ml. An equal volume of phenol, equilibrated with 0.05 M sodium tetraborate, is added and the mixture heated to 70°C until the solution forms a single phase, then cooled until the solution becomes cloudy. The solution is alternately heated and cooled for 3 min. The phases are then separated by centrifugation at room temperature. The aqueous phase is recovered and phenol is removed by extraction with ether. Nitrogen is then bubbled through the solution to remove the ether. ϕ X DNA samples are kept frozen when not in use.

Irreversible attachment measurement. Cells were grown to a turbidity of $A_{650 \text{ m}\mu} = 0.20$ in KC broth (about 1×10^8 cells/ml). KCN was then added to a concentration of 0.003 M. Phage were diluted to a con-

centration of 1×10^7 plaque-forming units/ml in KC broth. 0.1 ml of phage was added to tubes containing 0.9 ml of cells. The tubes were incubated 20 min at 30°C and then chilled to 0°C . Each tube was then diluted tenfold into KC broth saturated with chloroform and shaken at room temperature. Surviving phage were then assayed. The chloroform treatment kills infected cells, so that they do not form plaques. Therefore the phage measured after chloroform treatment should represent those that have not made an irreversible attachment during the 20-min adsorption period. This fraction was calculated by comparison with a control in which phage were subjected to the same procedure except that cells were absent.

2.1.5 Mutagenesis

Nitrous acid mutagenesis was used extensively to produce a broad spectrum of mutants, since it can induce all transitions (76). One volume of 1 M sodium acetate, one volume of 1 M acetic acid, and two volumes of 1 M sodium nitrite are mixed to give the inactivation medium. Phage (either in 0.05 M sodium tetraborate or in KC broth) are diluted 3-10 fold into the inactivation medium at room temperature ($20-25^{\circ}\text{C}$). The reaction may be halted by dilution and by raising the pH. Samples for the selection of mutants are usually diluted 10-fold from the inactivation mixture into 1.0 M Tris buffer, pH 8 at 0°C . These samples are frozen if not to be used immediately. Under these conditions plaque forming ability is inactivated exponentially, at the rate of a factor of 10 every 10 min, for at least 50 min (surviving fraction of 10^{-5}).

5-Bromodeoxyuridine substituted stocks of $\emptyset X$ mutants ts \checkmark , ts4,
ts9, and ts41 were grown and made available by M. Yarus.

2.1.6 Preparation of genetically pure mutant phage stocks.

A reference stock of the mutant is plated out under permissive conditions to give less than 50 plaques per plate. A single, young plaque is picked with a piece of sterile glass tubing and blown into 30 ml of sensitive cells (at a concentration of 1×10^8 per ml) growing with aeration in KC broth. All lysates are made at 30°C . The ts mutants are grown on C and the am mutants on CR or on HF 4714. The culture usually lyses in 2-3 hours. In KC broth at the cell concentration employed, most of the infective phage released by lysis readsorbs to cellular debris. Therefore the debris is spun down at $10-15 \times 10^3$ rpm, and the supernatant is discarded. The debris is resuspended in 5 ml 0.05M sodium tetraborate in order to elute the adsorbed phage. After shaking for a few hours in the cold, the debris is recentrifuged. The supernatant is the "stock." Such stocks typically have titers of $2-5 \times 10^{10}$ plaque forming particles per ml. A small fraction of the phage, usually between 10^{-5} and 10^{-7} , are revertants which will form plaques under restrictive conditions. This procedure of stock preparation is advantageous because the phage are very stable in the final borate suspension, and because the stock is relatively free of nutrients and so can be used to infect starved cells (Denhardt & Sinsheimer, 69).

2.1.7 Synchronization of infection

In some experiments synchronization of infection has been achieved by starvation-block or cyanide-block as described by Denhardt & Sinsheimer (69), except that phage adsorption was allowed to proceed for 10 to 15 minutes.

2.1.8 Complementation test

To test the ability of two mutants to reproduce through complementation, C is mixedly infected with a multiplicity of 3-5 of each of the two mutants. The infection is performed at a cell concentration of 2×10^7 per ml, in KC broth containing KCN. After adsorption of the phage, KCN is removed by dilution and infection is allowed to proceed at 40-42°C if complementation of ts mutants is to be measured, and at 30°C or 40°C in experiments involving only am or op mutants. In each experiment the progeny yield of the mixed infection is compared to the yields obtained in selfings of the two parents, and to a wt infection performed in an identical manner. In tests involving only am or op mutants, the infected C cells are sometimes plated on a permissive host (CR or HF 4714) immediately after dilution from KCN. The same controls are performed as in the progeny yield measurements.

For convenience in performing a large number of tests simultaneously, the cyanide-block procedure has been modified. The following procedures have been used, and give similar results.

Procedure 1. Phage are diluted to 2×10^8 per ml in KC broth containing .003 M KCN. To 0.8 ml (0.4 ml of each mutant) of phage-KCN is added 0.2 ml of C freshly grown to a concentration of 1×10^8 per ml

in KC broth at 37°*C*. This adsorption mixture is then incubated for 35 min at 30°*C*. A sample is removed for the assay of unadsorbed phage and anti- ϕ X serum is added to remove residual free phage. Five minutes later the infected cells are diluted 10⁴-fold away from KCN and serum into prewarmed KC broth at the appropriate temperature. If infective centers are to be assayed the samples are immediately diluted and plated. Progeny phage are assayed after an incubation period sufficient to permit lysis of the infected cells (usually 60 min), by plating under conditions which are permissive for the mutants being tested.

Procedure 2. Phage are diluted to 2×10^8 per ml in KC broth containing 0.005 M KCN. C is grown to a concentration of 1×10^8 per ml in KC broth at 37°*C*. In some experiments the cells are treated with KCN before adsorption of phage. KCN is added to a concentration of 0.005 M and the cells are aerated for 5 min at 37°*C*. 0.2 ml of C (either freshly grown or KCN treated) is added to 1.0 ml of phage-KCN mixture (0.5 ml of each mutant) in an ice bath. Tubes containing these adsorption mixtures are then transferred to a waterbath at 30, 37 or 40°*C* for 10-20 min (to allow adsorption to proceed). Tubes are then returned to the ice bath, a sample is removed for assay of unadsorbed phage, and anti- ϕ X serum is added. The tubes are then incubated for 5-10 min at 30, 37, or 40°*C*, after which they are returned to the ice bath. If infective centers are to be assayed the samples are diluted and plated immediately. Samples for measurement of progeny burst size are diluted 10⁴-fold into KC broth, prewarmed to the appropriate temperature, and incubated till lysis (usually for 60 min).

These procedures have been used in routine complementation testing for the purpose of assigning mutants to cistrons. Altered procedures used in certain special purpose complementation experiments will be described with the experimental results.

It should be noted that phage produced in either of these complementation procedures will be assayed only if the lysis function is successfully performed. In these modified cyanide-block procedures little if any phage synthesis occurs during the adsorption period (even if performed at permissive temperature), and wt phage yields after dilution are normal.

2.1.9 Spot test for phage sensitivity

In order to qualitatively determine whether a bacterial strain is sensitive to a phage, or a particular phage mutant, spot tests were used. The bacteria to be tested were grown as described for plating bacteria. A stock of the phage was diluted in KC broth, generally to a concentration of 10^5 to 10^6 plaque forming particles per ml. The test was performed in one of two ways: a) An agar plate was overlaid in the usual manner with top agar containing, as plating bacteria, the strain to be tested. Small drops of the phage strains to be tested were applied to the surface of such a plate after the agar had hardened. A number of phage strains could be tested on a single plate. A clear spot following incubation indicated sensitivity, while the absence of clearing indicated insensitivity. b) A drop of the phage to be tested

was placed in a depression of a Disposo-Tray (Cat. No. 14-6300, Chemical Rubber Co., Cleveland, Ohio). The bacteria were mixed with melted agar at the usual concentration for plating, and 1 ml portions of this mixture were added to each depression and allowed to solidify. Again clear agar following incubation indicated sensitivity, while phage-resistant strains developed a turbid lawn.

2.1.10 Recombination experiments

Mixed infections were initiated as described for complementation tests (procedure 2) in the presence of 0.003 or 0.005 M KCN. Except as noted in the text the infections were always performed under conditions which were permissive for all mutants being crossed (30°C infection of C for experiments which only involved crosses between ts mutants, and 30°C infection of CR or HF4714 for crosses involving am mutants). Unadsorbed phage were generally not removed since they make no detectable contribution to the phage yields. Following adsorption growth was initiated by diluting 100-fold into KC broth. Phage yields were normal following this dilution from KCN. It is realized that residual KCN may have observable effects on recombination. To keep these effects constant, growth of cross lysates was performed at the same dilution from the adsorption mixture in all mapping experiments. (Because of the low recombination frequencies observed with $\emptyset X$ a much greater dilution was not feasible.) The diluted growth tubes were incubated 1 hr before assay.

Control selfings of each mutant used were always performed with every experiment, in order to determine the level of background due to reversion of the mutants.

Total progeny yields were determined by plating under permissive conditions for the mutants involved (in the general case of an experiment involving both ts and am mutants the progeny were plated on CR(su⁺) or HF4714(su⁺) at 30°C). The wt recombinants were selectively scored by plating on C at 40 to 41°C.

Reconstruction experiments showed that possible problems such as interference of large numbers of mutant phage with a minority of wt particles, or recombination on the plate, did not interfere with the selective assay of recombinants down to a level of 10^{-6} recombinants per progeny phage.

2.1.11. Recombination spot test

A spot test has been used to determine qualitatively whether or not wt recombinants may arise from crossing two mutants. The phage to be tested are diluted to a concentration of 5×10^7 plaque formers per ml in KC broth. One drop (about 0.05 ml) of each parent is added to a depression of a Disposo-Tray. Selfings of each mutant are also performed, using 2 drops of the diluted phage. 1 ml of melted top agar containing the usual concentration of C plating bacteria (approximately 20 ml of plating bacteria per 150 ml bottle of top agar) is added to each depression. As soon as the agar has hardened, the Disposo-Tray is placed in a 40°C incubator for 3 to 4 hours. The production of plaques

under these conditions indicates the formation of wt recombinants in crosses between $\emptyset X$ conditional lethal mutants, providing plaques due to reversion are absent from the control selfings. Recombinants can be detected even in intracistronic crosses using this procedure. This spot test is useful for verifying the composition of multiple mutants constructed by recombination.

2.2 SELECTION OF MUTANTS

2.2.1 Temperature sensitive (ts) mutants

Temperature dependence of ϕ X wt growth. Traditionally ϕ X experiments have been conducted at a growth temperature of 37°C . The wild-type virus can, however, reproduce over the temperature range from 25°C to 42°C . Table 1 summarizes data from several experiments in which efficiency of plaque formation, burst size, and kinetic parameters of phage growth were measured as a function of temperature during the course of infection of several ϕ X sensitive host strains. Efficiency of plaque formation is fairly constant from 25°C to 42°C , although burst size is significantly reduced at the extremes of this range. The rate of the infective process, as measured by the time of onset of phage maturation and by the time of lysis, increases with increasing temperature up to 37°C . The infection proceeds very nearly twice as fast at 37°C or 40°C as at 30°C .

Selection of ϕ X ts mutants. The ts mutants were selected from nitrous acid treated preparations of ϕ X wt inactivated to a surviving fraction of approximately 10^{-3} . These survivors were plated out on C to give less than 50 survivors per plate and the plates incubated at low temperature (25°C or 30°C). In some "mutant hunts" the plates were incubated until all plaques were clearly visible (5-10 hours). Each plaque was then stabbed with a sterile pin and transferred by stabbing to two plates which were pre-seeded with C. One of these was incubated at the low and one at high temperature (37°C or 40°C). A small fraction (1-2%)

of the plaques produced no clear spot when transferred to high temperature. The corresponding clear spot from the low temperature plate was picked with a sterile piece of glass tubing and the phage eluted in a tube containing 1-2 ml of 0.05 M sodium tetraborate. Replating verified that the majority of these represented ts mutants which could form plaques at the low but not the high temperature.

In some "mutant hunts" a plating procedure which allows visual selection of ts mutants was used (86). Plates containing the mutagenized phage were incubated at 30°C for 3 hours and then transferred to 40°C for 2-3 hours. Under this incubation regime ts mutants produce small plaques with a characteristic "crisp" edge. Only plaques with this morphology were picked and spot tested as above. About 10% of the plaques tested appeared to be ts in the spot test, and most of these were verified to be real ts mutants on subsequent replating. Again, 1-2% of the phage surviving the nitrous acid treatment were recovered as ts mutants.

Table 2 lists the selection procedures used for isolation of $\emptyset X$ ts mutants. Tests performed with unmutagenized $\emptyset X$ stocks (lysates from crosses which were being screened for double mutant recombinants) indicate that the fraction of spontaneous ts mutants usually present is 10^{-3} or less, suggesting that the bulk of the mutants selected were actually induced by the mutagenic treatment. Reversion frequencies in stocks of the mutants indicate that none of them are multiple ts mutants.

Properties of $\emptyset X$ ts mutants. The ts mutants reproduce much like wt at low temperature (25°-30°C), although many have a reduced burst

size and form plaques which are smaller than wt at 30°*C*. There is considerable variability in the temperature at which mutant growth is shut off. Some mutants form no visible plaques at 37°*C* or above, others form plaques at 37°*C* but not at 40°*C*, and a few can form plaques up to 40°*C* but make no plaque or very minute plaques at 42°*C*. Burst size measurements indicate that differences in phage yield at high temperature are responsible for these differences in plating properties.

The mutants in general adsorb to sensitive bacteria at high temperature. They go into eclipse, as measured either by the free phage or the intracellular phage assay. Even if the phage are allowed to adsorb at 30°*C*, either in the presence of KCN or in starvation buffer, phage growth is still blocked when the infected cells are transferred to growth medium at high temperature.

The phage particles produced by $\emptyset X$ ts mutants at 30°*C* are quite stable at temperatures high enough to completely block replication. Table 3 shows that several ts mutants are as stable as the wt during incubation at 46°*C* for times which are long compared to the latent period.

Taken together, these results indicate that the ts mutants exhibit their sensitivity to high temperature during the intracellular phase of the life cycle.

2.2.2 Amber (am) mutants

Selection of $\emptyset X^S$ su⁺ (am) strains. A technical difficulty arose in the selection of $\emptyset X$ am mutants because none of the bacterial strains

previously characterized as "permissive" is sensitive to $\emptyset X$ infection.

A suitable permissive strain was found by screening all available $\emptyset X^S$ strains for sensitivity to am mutants of phage λ (λ sus mutants). The strain discovered in this way, CR, is a recombinant between a permissive but $\emptyset X$ resistant strain of E. coli (CR34), and a $\emptyset X^S$ but restrictive strain of E. coli (C416). This recombinant carries an am suppressor from the CR34 parent (this is believed to be su_1^+) and retains the $\emptyset X^S$ locus of C416. The presence of the $su^+(am)$ locus was further demonstrated by plating am mutants of phages f1 and f2 on strain CR (F^+). Table 4 compares the efficiency of plating of various am mutants of phages λ , f1 and f2 on CR and on other su^+ and su^- strains. It is clear that CR carries a suppressor for such mutants. C cannot suppress λ sus mutants and hence is used as the restrictive host for $\emptyset X$ am mutants.

Selection of $\emptyset X$ am mutants. am mutants were also nitrous acid induced. $\emptyset X$ wt was treated with nitrous acid to give a surviving fraction of approximately 10^{-5} . The survivors were plated on CR to give a about 50 survivors per plate, and the plates incubated at 30°C . After all plaques were clearly visible (6-10 hours) each plaque was transferred with a sterile pin to two other plates, one pre-seeded with CR and the other with C. These plates were incubated at 30°C . A small fraction (about 0.3%) produced no clear spot when transferred to C. The corresponding clear spot from the CR plate was picked with a sterile piece of glass tubing and the phage eluted in 1-2 ml of 0.05 M sodium tetraborate. Replating verified that almost all of these were mutants which could form plaques on CR but not on CR 61 mutants, designated am1, am2, . . . , am61, were selected by this procedure.

Properties of ØX am mutants. In general the am mutants grow much like wt in CR at 30°C, although many have reduced burst size and form plaques which are smaller than wt. ØX am mutants adsorb and eclipse to C and are blocked at some later stage of infection. Reversion frequencies in stocks of these mutants indicate that none of them are multiple am mutants.

A few of the mutants turn out to be ts as well as am in phenotype. That is, they form plaques on CR but not C at 30°C, and do not form plaques on either strain at 40°C. In order to determine whether both phenotypes are a consequence of a single mutation, the reversion properties of several such am, ts mutants were studied. Each such mutant was plated in high concentration under two plating conditions: 1) on C at 30°C, to select reversions to am⁺, 2) on CR at 40°C to select reversions to ts⁺. All revertants were then assayed under both plating conditions to determine whether reversion from the unselected phenotype had occurred. Table 5 shows the result of such an analysis of several am,ts mutants. It can be seen that some of the mutants appear to be single site events since selection for reversion from one of the two phenotypes often results in simultaneous reversion from the other. Three mutants (am8,9,23) have been found which show independent reversion of the two phenotypes and are most easily interpreted as containing a ts mutation at a site different from the am mutation. Revertants which are still am but are ts⁺ have been isolated for use in complementation tests with ts mutants and for mapping.

Because the permissive and restrictive strains used in the selection of $\emptyset X$ am mutants (CR and C) are not closely related it seems likely that they differ in many characters other than the presence and absence of a su^+ locus specific for the triplet UAG. For this reason we have performed experiments designed to establish that $\emptyset X$ am mutants are true am mutants. Spheroplasts of bacterial strains isogenic except for the presence or absence of well characterized suppressors of the nonsense triplet UAG (isolated by Garen, 106) were exposed to DNA extracted from a number of $\emptyset X$ am mutants. This experiment is feasible because the $\emptyset X$ resistance of these strains resides only in their cell walls. Table 6 shows the results obtained in such an experiment. It is clear that the block to phage production in the su^- strain is strongly suppressed in one or more of the su^+ strains for all the mutants tested except am 3. The actual degree of suppression by the various suppressors appears to vary greatly from one mutation to another. Presumably this reflects differences in the ability of the mutant protein to function when different amino acids are incorporated in response to UAG. am3 produces phage quite normally even in the su^- strain because the only phage function affected by this mutation is lysis of the host (see Part 3). The spheroplast assay measures phage production, but does not readily permit assay of lysis.

Having identified a number of $\emptyset X$ am mutants as genuine UAG mutants, other $\emptyset X^s, su^+$ strains could be identified by their ability to plate these mutants. In this way it was found that Shigella paradysenteriae strain Y6R and E. coli HF4714 carry am suppressors. Strain Y6R was used by

Tessman (107) as the permissive strain for the isolation of su mutants of phage S13. It seems likely, therefore that the S13 su mutants are also genuine UAG mutants. It should be noted that am3 as well as other lysis mutants tested form plaques on Y6R and HF4714. This suggests that these also are suppressed by the am suppressor, and not by some other suppressor in strain CR.

Recently a set of strains isogenic except for the absence or presence of various su⁺ mutations has been isolated in the $\emptyset X^S$ strain WWU (108). The suppressors have been characterized by their pattern of suppression of a number of T4 am mutants. $\emptyset X$ am mutants which were suppressed by Garen's am suppressors are able to form plaques on one or more of the WWU am suppressor strains as well. These strains have been used to prove conclusively that lysis mutants, such as am3, are genuine am mutants.

In the process of screening $\emptyset X$ am mutants for the ability to form plaques on various suppressor strains, two anomolous mutants (am22,32) were found. These are unable to form plaques on Y6R. They form plaques on HF 4714, but they also form plaques on another $\emptyset X^S$ recombinant (HF 4714.1) from the same cross which is unable to suppress genuine am mutants. Although they are not believed to be lysis mutants, they are able to reproduce in Garen's su⁻ strain. Table 7 compares the ability of $\emptyset X$ wt, genuine am mutants, and an anomolous am mutant to reproduce in various hosts. These results seem consistent with the idea that CR carries some suppressor in addition to the am suppressor, and that this suppressor is absent from C and Y6R, but present in K12. The nature

of this suppressor and of the suppressible mutations has not been investigated.

2.2.3 Opal (op) mutants

Selection of a $\emptyset X^s$ su⁺ (op) strain. A strong suppressor for the opal triplet UGA has recently been reported (104). As usual, this suppressor was isolated in a $\emptyset X^r$ strain. It was therefore necessary to introduce the $\emptyset X^s$ locus by recombination. The suppressor strain, CAJ 70, was cured of its F-lac by treatment with mitomycin C, by P. Boistard. The resulting F⁻ strain was then used as recipient in a cross with the $\emptyset X^s$ Hfr, HF 4711. The cross may be represented as follows:

$F^- \text{ lac}^- \text{ su}^+_{\text{UGA}} \text{ str}^s \emptyset X^r (\lambda) \times \text{Hfr-J2 str}^r \emptyset X^s \text{ ade}^- \text{ met}^- \text{ arg}^-$.

The cells were mated in KG broth, then two hours later were diluted and allowed to segregate recombinants for 5 hours. It is believed that the $\emptyset X^s$ locus lies near xyl, and hence not far from str. Therefore str^r prototrophic recombinants were selected by plating on minimal plates in the presence of streptomycin. These recombinants were spot tested for sensitivity to $\emptyset X$, and the presence or absence of the su⁺_{UGA} was determined by spot testing with T4 op mutants. In this way a strain was found which was $\emptyset X^s$ and would suppress T4 op mutants. The strain retained the λ prophage from the CAJ 70 parent. A nonlysogenic, λ^s , strain was isolated after an inducing dose of ultraviolet light. This strain, designated CIT 103, is F⁻ $\emptyset X^s$ T4^s λ^s lac⁻ (deletion) su⁺_{UGA} str^r. It does not carry an am suppressor since it will not plate $\emptyset X$ am mutants.

Selection of $\emptyset X$ op mutants. Mutants of $\emptyset X$ were isolated which could form plaques on CIT 103, but not on C. $\emptyset X$ wt was treated with nitrous acid to give a surviving fraction of 3×10^{-5} . Plates were pre-seeded with a layer of agar containing C. After this layer had hardened, a second agar layer containing CIT 103 and approximately 100 surviving phage was poured on top of the first layer. Plates were incubated at 30°C until all plaques were clearly visible (about 6 hours). Turbid plaques were picked with pins and transferred to two plates, one pre-seeded with C, the other with CIT 103, which were then incubated at 30°C . A few of these did not form a clear spot on C, but did on CIT 103. The spots were picked with sterile glass tubing and eluted in 2 ml of 0.05 M sodium tetraborate. Essentially all of these were mutants which form plaques on CIT 103 and form either no plaques or very faint plaques on C. Approximately 0.2% of the surviving phage were recovered as mutants. Some 20 mutants (designated op1, op2, ..., op22) have been isolated in this way.

Properties of $\emptyset X$ op mutants. A number of the op mutants have been tested for the ability to form plaques on the am suppressor strain HF 4714. This strain is not able to suppress T4 op mutants (opC6 and opC15). The ability of 10 $\emptyset X$ op mutants to form plaques on strains CIT 103, HF 4714, and C is shown in Table 8. Apparently 9 of the 10 mutants are suppressed by one or more suppressors present in CIT 103, but absent from HF 4714. Presumably they respond to the su_{UGA}^+ , although experiments with isogenic strains are necessary to prove this point. The degree of leakiness in strain C is variable. 5 of the mutants form no visible plaques on C, 3

form very faint plaques, and 1 forms essentially normal plaques. One of the mutants forms plaques on HF 4714 and is, therefore, presumably not a true op mutant.

2.2.4 Host range mutants

A number of mutants of $\emptyset X$ have been isolated which differ from the wt in their host specificity for the initial phase of the infection (attachment, eclipse, and injection).

Host mutants. Mutants of strain C which are resistant to infection by $\emptyset X$ wt are readily obtained. If 10^6 $\emptyset X$ wt are plated in an agar layer along with the usual number of C plating bacteria, several thousand colonies will develop during overnight incubation at $37^\circ C$. When these are purified by reisolation of single colonies and tested as plating bacteria for $\emptyset X$ wt essentially all such strains are resistant to infection (efficiency of plaque formation of $\emptyset X$ wt is less than 10^{-5}). C₁ was isolated in this way. These strains are usually altered in their ability to attach or eclipse $\emptyset X$ wt, and it is possible to select host range mutants which can form plaques on some $\emptyset X$ wt^r strains derived in this way (see below).

Host strains are available which, although able to plate $\emptyset X$ wt, do so with reduced efficiency, due to alterations in attachment or eclipse. CR is such a strain. It is in some cases possible to isolate restricted host range mutants which are unable to form plaques on such strains.

The attachment and plating properties of $\emptyset X$ wt and a previously characterized host range mutant, $\emptyset X$ H_aH_b, with strains C, CR, and C₁

are shown in Table 9. C₁ is clearly altered in its ability to irreversibly attach either wt or H_aH_b when the infection is carried out in liquid culture. Although CR shows a reduced efficiency of plating of wt H_aH_b plates with normal efficiency on this strain. This result is paralleled by the attachment measurements which show that although wt attaches somewhat less efficiently to CR than to C, H_aH_b attaches with high efficiency in both cases. In fact, H_aH_b attaches more efficiently than wt to both C and CR. The failure of H_aH_b to attach to C₁ was surprising. Since H_aH_b forms plaques on C₁, it must be able to attach fairly efficiently under the conditions of the plaque assay.

Selection of host range mutants. Spontaneous extended host range mutants were isolated by plating a large number of wt on a resistant strain such as C₁ and then selecting the few plaques which were formed.

10^5 - 10^7 $\emptyset X$ wt were plated in an agar layer along with mixed indicator bacteria (90% C₁ and 10% C). A much higher yield of host range mutants is obtained in this way than if C₁ alone is used as plating bacteria, presumably because this procedure allows one cycle of growth in C and allows the expression of mutants which were present as phenotypically mixed particles in the wt stock. Plaques were picked, purified by replating, and plated on C and C₁ to confirm their extended host range. Mutants were selected by this procedure at 30°C and at 37°C.

Mutants which can form plaques on CR/ $\emptyset X$ were isolated in the same way, using a mixture of 90% CR/ $\emptyset X$ and 10% HF 4714 as plating bacteria.

It is of interest that not all wt resistant mutants of the host can be used for the isolation of extended host range mutants. Also, if

a number of resistant strains are isolated some are sensitive, and some are resistant to previously selected extended host range mutants of $\emptyset X$.

Restricted host range mutants were selected from a preparation of wt which was inactivated to a survival of 4×10^{-4} with nitrous acid. The phage were plated on C and individual plaques were picked with pins and spot tested on C-22, C_G, and C. In this way 5 mutants were isolated, which could form plaques on C, but were unable to do so on C-22, C_G, or both. The plating patterns of these mutants are shown in Table 10.

Properties of $\emptyset X$ host range mutants. Extended host range mutants which are selected to form plaques on C₁ or CR/ $\emptyset X$ generally do so with an efficiency about 0.1 times as great as on C or HF 4714. In order to increase the efficiency of plating 10% sensitive cells (C or HF 4714) are routinely added to the resistant plating bacteria. The wt forms no visible plaques on such a mixture, and the host range mutants plate as efficiently as on wt-sensitive cells (presumably because one cycle of infection in C releases some 100 progeny which then form a plaque on C₁).

Extended host range mutants selected to plate on C₁ appear to invariably be ts in phenotype as well. Such mutants are ts when assayed either on C₁ or on C. They seem to be somewhat leaky at 40°C , sometimes forming minute plaques, but form no plaques at 42°C . One series of such mutants, designated tsh 1, tsh 2, ..., tsh 32, was selected by the above procedure at 30°C . The block to phage growth at high temperature does not seem to be simply a block in attachment, as might be expected for a host range mutant. Even if phage are attached at 30°C in the presence

of 0.005 M KCN, the phage yield is still ts following dilution away from KCN. The phage yield as a function of temperature is shown for a mutant of this type, tsh 6, and for wt in Table 11.

The restricted host range mutant hr 1, has been useful as a marker in some experiments since it is unable to form plaques on CR. This gives it a host specificity opposite to that of ØX am mutants, so that it is possible to selectively assay a mixture of an am-ts mutant and hrl.

2.2.5 Other types of mutants.

It would be useful to have as many types of conditional lethal mutants as possible, in order to increase the probability that mutants in all the phage genes can be detected. Many types of missence conditional lethals can be imagined, similar in nature to the ts and the pH-sensitive mutations, but hopefully differing in their exact specificity. For example, a preliminary investigation of the feasibility of isolating mutants which are sensitive to the presence of denaturing solvents in the bacterial growth medium has been carried out. It was hoped that mutations which affect different components of protein stability, such as hydrogen bonding, non-polar interactions, etc., might show differential sensitivity to various solvents during in vivo replication of the phage. Sensitivity to a particular solvent might then indicate a much more specific kind of alteration in protein structure than temperature-sensitivity.

Dimethylsulfoxide (DMSO), ethylene glycol (EG), and urea have been used. The wt phage can form plaques with reasonable efficiency when

1.0 ml of DMSO, EG, or 9 M Urea is added to the usual plating mixture of top agar, C plating bacteria, and phage. This result is demonstrated in Table 12.

$\emptyset X$ wt, inactivated by nitrous acid to a survival of about 10^{-5} , was plated on C at 30°C . Individual plaques were picked and spot tested on plates containing the above amounts of DMSO, EG, and Urea, at 30°C . In this way a series of 19 solvent sensitive (ss) mutants was isolated. Their plating properties are shown in Table 13.

The ss mutants have not been characterized in detail. It appears that most, if not all, mutants isolated in this way are also ts in phenotype. It is not yet known what fraction of mutants isolated for the ts phenotype are ss as a result. This situation is probably similar to the case of ts and pH-sensitive mutants of phage λ . Campbell (91) found that both sensitivities could be conferred by a single mutation.

2.2.6 Construction of multiple mutants.

Multiple mutants have been constructed for several reasons:

1) for use in three factor crosses for mapping purposes, 2) for use in certain physiological experiments, and 3) in order to facilitate large scale preparation of a mutant by combining it with a lysis defective mutant. For all of these purposes it was necessary to construct defined multiples from previously characterized single mutants. This is done by recombination. However, because of the low frequencies of recombination observed with $\emptyset X$ (see Part 2.4), this is much more tedious than with other phages.

am-ts double mutants. The two mutants are crossed by the usual procedure and the frequency of wt recombinants is determined to give an estimate of the frequency of am-ts recombinants expected. The lysate from the cross is then plated on su^+ cells (CR or HF 4714) at 30°C . Plaques are picked with pins and spot tested on su^- cells (C) at 30°C , su^+ cells at 40°C , and su^+ cells at 30°C . If a clear spot is formed only on su^+ cells at 30°C it is picked, the phage eluted in 0.05 M sodium tetraborate, and then replated to confirm that it is both am and ts.

In order to reduce the number of plaques which need be spot tested the plates were sometimes pre-seeded with an agar layer containing C plating bacteria, which was then overlaid with agar containing su^+ cells and phage from the cross lysate. The plates were incubated for about 3 hours at 30°C and then another 2 to 3 hours at 40°C . Under these plating conditions am mutants form turbid plaques, and ts mutants form small plaques with crisp edges. Only small turbid plaques with crisp edges were picked and spot tested as described above.

In constructing am-ts double mutants in which the am mutant is defective only in lysis of the host, it was possible to very strongly select for the am mutation by recycling the cross lysate at low multiplicity in an su^- host at 30°C . Anti- ϕX serum was added after adsorption in order to remove non-am phage as they were released by lysis. The cells were then thoroughly washed to remove the serum and lysed artificially with lysozyme and EDTA. Essentially all the phage released were am. The phage were then plated on su^+ cells and incubated for 3 hours

at 30°*C*, and then shifted to 40°*C* for another 2-3 hours. Small plaques with crisp edges are picked and spot tested as described above.

In all cases the am-ts mutants were backcrossed to the two parental mutants, in order to show that wt recombinants are not produced, thereby confirming that the double mutants had the intended composition. This was done either by the quantitative crossing procedure or by the recombination spot test. Such testing is necessary to exclude the possibility that the am-ts isolated actually pre-existed in one of the parental stocks.

Multiples containing extended host range mutants. It has been possible to construct several types of multiples containing host range markers. These include am-h, ts-h, and am-ts-h multiples. These are relatively easy to get since it is possible to select for the h marker by plating on C₁, or on CR/0X if an am mutation is present. The am and ts markers are identified by spot testing as described above, and the various plating procedures described may be used to help identify them. These multiples have been selected both by recombination of previously isolated h mutants, and by selection of spontaneous h mutants present in stocks of various am, ts, and am-ts mutants.

TABLE 1

Growth of $\emptyset X$ wt as a Function of Temperature

Temp (°C)	e.o.p		Burst Size		Min. Latent Period (minutes)	
	<u>C</u>	<u>CR</u>	<u>C</u>	<u>C_G</u>	<u>C</u>	<u>C_G</u>
18	<0.01	<0.01				
25	0.65	0.26		10		45
30	0.76	0.46	250	30	30	28
33				50		19
37	1.00	0.53	186	50	13	19
40	0.87	0.65	75	5	15	20
42	0.94	0.53		5.6		
45				0.4	No Burst	

Efficiencies of plating are normalized to the value obtained with C at 37°C. In the experiments measuring burst size, the m.o.i. of C = 6.6 and the m.o.i. of C_G = 3.1.

TABLE 2

Selection Procedures for $\emptyset X$ ts Mutants

<u>Series of Mutants</u>	<u>Selection</u>		<u>Selection Host</u>	<u>Source of Mutants</u>
	<u>Temp. (°C)</u>	<u>Low</u> <u>High</u>		
<u>ts</u> X	25	37	<u>C</u> <u>G</u>	nitrous acid treated <u>wt</u>
<u>ts</u> 1-22	25	37	<u>C</u> <u>G</u>	nitrous acid treated <u>wt</u>
<u>ts</u> 23-72	30	40	<u>C</u>	nitrous acid treated <u>wt</u>
<u>ts</u> 73-111	30	40	<u>C</u>	nitrous acid treated <u>wt</u>
<u>ts</u> 113	30	40	<u>C</u>	spontaneous—found among revertants of <u>am</u> 3
<u>ts</u> 114-127, 129-131	30	40	<u>C</u>	revertants of <u>am</u> 18, <u>am</u> 35-spontaneous
<u>ts</u> 128	30	40	<u>CR</u>	selected by a temperature shift procedure

The actual selection procedures are described in the text.

ts 113, 114-127, and 129-131 were found among revertant plaques from am mutant stocks plated on C at 30°C. ts 128 was found by A. J. Shafer by a procedure which enriches for mutants which release phage following a shift from high to low temperature at late times of infection. It was originally found in the combination am 9 ts 128, and the am was removed by reversion.

TABLE 3

Thermal Stability of ϕ X ts Mutant Phage Particles at 46°C

<u>ϕX strain</u>	<u>Time of Incubation at 46°C</u>	<u>0 min</u>	<u>55 min</u>	<u>105 min</u>	<u>195 min</u>
<u>wt</u>		1.0	0.88	0.67	0.62
<u>ts</u> ✓		1.0	1.3	1.0	0.95
<u>ts</u> 4		1.0	1.0	0.71	—
<u>ts</u> 8		1.0	1.2	2.3	1.0
<u>ts</u> 9		1.0	1.3	0.87	0.75

Phage were diluted into 2.5 ml of melted top agar in tubes which were incubated at 46°C. One tube of each mutant was assayed immediately by adding E_G plating bacteria, pouring on an agar plate, and incubating at 30°C. Additional tubes were assayed at the time intervals listed. The numbers tabulated are surviving fractions, i.e. the ratio of infective phage in a particular sample to that present before heating.

TABLE 4

e.o.p. of am Mutants of Phages λ , f1, and f2 on CR and other Bacterial Hosts

Phage Strain	Bacterial Host				
	su- <u>C</u>	su+ CR	su+ <u>C600</u>	su+ <u>K37</u>	su- <u>K38</u>
$\emptyset X$ wt	1.0	0.9	0	0	0
λ wt	1.0	0.07	1.0		
λ sus 7	10^{-4}	0.02	1.0		
MS2 wt		0.37		1.0	1.1
f2 su-1		0.55		1.0	0.002
f1 su-18		~ 1.0		1.0	$\sim 10^{-4}$

An F+ derivative of CR was used for the assays of male specific phages (MS2, f1, and f2). The numbers tabulated are efficiencies of plating, relative to C for $\emptyset X$, relative to C600 for λ , and relative to K37 for the male specific phages.

TABLE 5

Plating Properties of Revertants from Temperature-Sensitive ϕ X am Mutants

<u>Revertants from ϕX</u>	<u>Selection Conditions</u>	<u>C, 30°C</u>	<u>Form Plaques on C, 40°C</u>	<u>Plaques on CR, 30°C</u>	<u>CR, 40°C</u>	<u>Number Tested</u>
am 8	<u>C, 30°C</u>	3	0	3	0	3
am 8	<u>CR, 40°C</u>	0	0	3	3	3
am 9	<u>C, 30°C</u>	1	0	1	0	1
am 9	<u>CR, 40°C</u>	0	0	3	3	3
am 14	<u>C, 30°C</u>	3	3	3	3	3
am 14	<u>CR, 40°C</u>	2	2	3	3	3
am 16	<u>C, 30°C</u>	3	3	3	1	3
am 16	<u>CR, 40</u>	3	3	3	3	3
am 23	<u>C, 30°C</u>	3	0	3	0	3
am 23	<u>CR, 40°C</u>	0	0	3	3	3

The number of revertants tested which were able to form plaques under the stated conditions is tabulated. Revertants were selected by plating high concentrations of phage from stocks of the mutants listed, under the "Selection Conditions" listed in the Table.

TABLE 6

Phage Production by ϕ X am Mutant DNA in Spheroplasts of Garen's su
and su⁺ Bacteria

<u>ϕX strain</u>	<u>Cistron</u>	<u>su</u> ⁻	<u>Bacterial Strain</u>			
			<u>S26</u>	<u>S26 RFE</u>	<u>S26 RID</u>	<u>S26 R4C</u>
<u>wt</u>	-	0.5	1.0	1.5	1.3	
<u>am 3</u>	I	1.3	1.0	0.9	0.6	
<u>am 9</u>	III	0.002	1.0	17.0	<0.001	
<u>am 23</u>	III	<0.001	1.0	1.8	0.03	
<u>am 16</u>	IV	0.003	1.0	1.2	0.3	
<u>am 10</u>	V	0.008	1.0	2.2	0.4	
<u>am 33</u>	VI	0.008	1.0	1.4	0.5	

The numbers tabulated are phage yields relative to those obtained from the su₁⁺ host. The assay was performed as described in materials and methods.

TABLE 7

Growth of "true" and "anomolous" ϕ X am Mutants in Various Hosts

ϕ X strain	Description	Bacterial Strain																	
		C	su-	CR+	su+	Y6R	su+	HF 4714	su+	HF 4714.1	su-	WWU	su-	U6-44	su+	S26	su-	S26	su+
wt	wild-type	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
am 33	true am	-	+	+	+	+	+	-	-	-	+	-	+	-	-	+	+	+	+
am 3	true am in lysis gene	-	+	+	+	+	+	-	-	-	+	-	+	+	+	+	+	+	+
am 32	anomolous am	-	+	-	-	+	+	-	+	-	-	+	-	+	+	+	+	+	+

"+" indicates that plaques are formed on a bacterial strain, except in the case of S26 and S26 RIE where it indicates that phage are synthesized following infection of spheroplasts with DNA. "--" indicates that no plaques were formed, or that no phage were made in spheroplast. "su⁺" and "su⁻" refer to the presence or absence of a true amber (UAG) suppressor in the strain.

TABLE 8

Plating Properties of ϕX op Mutants

ϕX strain	Bacterial Strain		
	<u>CIT</u> 103	<u>C</u>	<u>HF</u> 4714
<u>wt</u>	+	+	+
<u>am</u> mutants	-	-	+
<u>op</u> 1	+	-	-
<u>op</u> 2	+	f	-
<u>op</u> 4	+	f	-
<u>op</u> 5	+	f	-
<u>op</u> 6	+	-	-
<u>op</u> 7	+	(+)	-
<u>op</u> 8	+	-	-
<u>op</u> 9	+	-	-
<u>op</u> 10	+	f	+
<u>op</u> 11	+	-	-

"+" indicates the formation of normal plaques, "(+)" indicates almost wild-type plaques, "—" indicates that no visible plaques are formed, and "f" refers to the formation of very faint plaques. All strains were assayed by the normal agar layer procedure.

TABLE 9

The Effect of Phage and Bacterial Mutations on ϕX Attachment and Plaque Formation

<u>ϕX strain</u>	<u>Fraction Unattached</u>			<u>Relative e.o.p.</u>		
	<u>C</u>	<u>CR</u>	<u>C_1</u>	<u>C</u>	<u>CR</u>	<u>C_1</u>
<u>$H_a H_b$</u>	0.003	0.005	0.97	1.0	1.0	0.7 ± 0.3
<u>wt</u>	0.035	0.12	1.00	1.0	1.6	10^{-6}

Relative e.o.p. is the ratio of phage titer to that obtained with C plating bacteria. Although the efficiency of plating of ϕX $H_a H_b$ varies slightly in different experiments, it is quite reproducible within an experiment.

TABLE 10

Plating Properties of ϕ X Restricted Host Range (hr) Mutants

<u>ϕX strain</u>	<u>Bacterial Strain</u>			<u>C</u>
	<u>C-22</u>	<u>C_G</u>	<u>C_{abc}</u>	
<u>wt</u>	+	+	+	+
<u>hr 1</u>	-	+	+	+
<u>hr 2</u>	-	+(s)	+(s)	+(s)
<u>hr 3</u>	+(s)	-	+(s)	+(s)
<u>hr 4</u>	+(s)	-	+(s)	+(s)
<u>hr 5</u>	-	-	+(s)	+(s)

"+" indicates the formation of plaques, "-" indicates that no plaques are formed, and "(s)" indicates that the plaques formed are small.

TABLE 11

Burst Size of ϕ X tsh 6 and ϕ X wt as a Function of Temperature

<u>ϕX strain</u>		
<u>Temperature</u>	<u>wt</u>	<u>tsh 6</u>
30° ^o C	250	172
37° ^o C	186	131
40° ^o C	75	3.4
42° ^o C	5.6	0.2

Strain C was infected at 30°^oC by the cyanide-block procedure.

m.o.i. of wt = 6.6, m.o.i. of tsh 6 = 2.0. Growth was initiated by a 10^4 -fold dilution into KC broth at the specified temperatures. The numbers tabulated are burst sizes.

TABLE 12

Plating of ϕ X wt in the Presence of Various Solvents

<u>Volume of Solvent</u>	<u>DMSO</u>	<u>SOLVENT</u>	<u>UREA</u>
		<u>EG</u>	
0	1.0	1.0	1.0
0.1	1.0	1.0	1.0
0.2	1.2	0.83	0.86
0.5	1.0	1.1	0.80
1.0	0.61	0.93	0.92
2.0	+	+	won't gel

The plaque assay was performed in the usual manner except that the stated volumes of dimethylsulfoxide (DMSO), ethylene glycol (EG), or 9 M urea (UREA) were added to the melted agar before plating. C plating bacteria was used and the plates were incubated at 30° C. The numbers tabulated are efficiencies of plating compared to that obtained without any added solvent. "+" indicates that plaques were formed, although the bacterial lawn was very faint so that counting was not possible.

TABLE 13

PLATING PROPERTIES OF ss MUTANTS

ØX	30°C	40°C	DMSO	EG	UREA
<u>ss</u> 5,7	+	-	-	-	-
<u>ss</u> 3,9,13 14,18	+	-	+	+	-
<u>ss</u> 4,15,16	+	-	+	+	(-)
<u>ss</u> 2	+	+	+	+	-
<u>ss</u> 12	+	min	+	+	-
<u>ss</u> 17	+	min	+	+	(-)
<u>ss</u> 1	+	-	+	(-)	(-)
<u>ss</u> 8,11	+	-	(-)	+	(-)
<u>wt</u>	+	+	+	+	+

Plating conditions are described in the text.

A "+" indicates that plaques of normal size are formed, a "min" indicates minute plaques, and "-" indicates that plaques are not formed (some of these mutants are slightly leaky in the presence of urea, so that a few minute plaques are formed or the lawn is chewed, usually at the edge of the plate). The symbol "(-)" represents partial sensitivity to a solvent. This partial sensitivity results in plaque formation over a fraction of the plate.

2.3 COMPLEMENTATION2.3.1 General properties of complementation between mutants of $\emptyset X$

Mixed infections. In order to optimize the conditions for complementation between conditional lethal mutants of $\emptyset X$, conditions of infection were chosen which permit several parental phage particles to produce progeny in the majority of phage-infected cells. The results of Yarus and Sinsheimer (61) indicated that the infection of cells in the exponential phase of growth by the cyanide-block procedure would be optimal for this purpose. The result of a single burst experiment in which a mixture of four different mutants of $\emptyset X$ was used to infect C is shown in Fig. 1. The infection was performed under conditions which are permissive for all the mutants involved. In agreement with the findings of Yarus and Sinsheimer, the majority of infected cells produce mixed yields of progeny phage. The results suggest, however, that some limit to participation does exist even in KCN synchronized infection of broth grown cells. It should be noted that the total multiplicity used in this experiment is higher than that used by Yarus and Sinsheimer (61). It is assumed that these conditions of infection will also permit several infecting parental phage to contribute to essential phage functions other than the synthesis of progeny single-stranded DNA.

Phage production by complementation. Phage yields from mixed infections with several pairs of $\emptyset X$ conditional lethal mutants performed under restrictive conditions are shown in Table 14. The phage yield produced by each mutant alone under the same conditions of infection is

also shown. The phage yields in such experiments are usually normalized to the number of phage produced in a control sample which is infected with $\emptyset X$ wt. This control is treated in the same manner as the complementation experimental samples in all other respects. This procedure facilitates the comparison of results from different experiments, since at the temperature that complementation is usually measured, 40°C , burst size is quite strongly temperature dependent.

It is clear from the results presented in Table 14 that complementation can produce large increases in phage yield compared to infection with one mutant alone under restrictive growth conditions. However, such complementation is not perfect with $\emptyset X$, at least under the experimental conditions used. Complementation phage yields are generally in the range from 0.1 to 0.5 of the wt yield. Phage yields produced in infections with single mutants are usually 0.01 of the wt yield or less, although certain mutants may produce 0.02-0.03 of the wt phage yield.

It was of interest to determine the reason for the low phage yields in the complementation situation as compared to wt infection. Experiments were performed to determine whether only a fraction of the infected cells produce complementation yields, or whether the burst size produced by complementation in single cells was inherently low. In complementation between am mutants it is possible to directly measure the number of cells in which complementation occurs by plating the multiply infected su⁻ cells, before lysis, on su⁺ indicator bacteria. The results of such an experiment are shown in Table 15. Nonpermissive (C) cells infected with a single am mutant are usually unable to form plaques on permissive

indicator since they do not release progeny phage. Cells infected with certain mutants were able to form plaques on a permissive indicator with an efficiency of approximately 0.1. These infective-centers must release very small bursts of phage (compare the phage yield measurements of Table 18) and presumably result from leakiness of the particular mutants. The majority of the infected cells produced complementation yields when mixedly infected with complementing mutants. The fraction of cells able to form a plaque on permissive indicator varied with the pair of complementing mutants in the range 0.69 to 0.95. This result indicates that low total phage yields in complementation experiments are due to low bursts of phage released by the majority of the multiply infected cells.

In order to obtain more information concerning phage yields produced by complementation, a single burst experiment was performed. C was multiply infected with ts 4 and ts \vee h (a double mutant which is ts and has an extended host range which allows it to form plaques on C₁). The infection was performed in KCN according to procedure 1 (see Materials and Methods). Following adsorption and removal of unadsorbed phage with anti- ϕ X serum the infected cells were diluted into KC broth and distributed into single burst tubes prewarmed to 40° C so that almost all tubes contained either 0 or 1 infected cells. The tubes were incubated until completion of lysis (1 hour) at 40° C , and then assayed on C at 30° C . The distribution of burst sizes obtained in this way is shown in Fig. 2. The distribution contains many very small bursts and is quite broad. An average burst size can be calculated from the single burst data by

dividing the total number of phage found in single-burst tubes by the number of bursts (the number of bursts is calculated as m times the number of single-burst tubes, where e^{-m} is the fraction of tubes containing no phage). The burst size obtained in this way is 13.5. This value is in close agreement with that obtained in mass culture from a 40°C growth tube diluted from the same infection as the single-burst experiment. In the mass culture 12.2 phage were produced per infected cell. The agreement in these results indicates that essentially all the infected cells produced some phage in the complementation experiment.

The reason that complementation between $\emptyset X$ mutants results in burst sizes lower than w_t is not known. It seems possible that geographical separation of the infecting particles might result in incomplete mixing of phage coded proteins within the infected cell. Some results of phenotypic mixing experiments which bear on this point will be presented in Part 3 of this thesis.

Symmetric and asymmetric complementation. It is of interest to know whether both infecting mutants are rescued by complementation. In order to determine this it is necessary to be able to distinguish the two mutants by their plating properties. This can be done in various ways. If one of the two mutants is labelled with an extended host range mutation, then h and h^+ plaques can be distinguished by plating on a mixed indicator composed of 5 parts of C and 1 part of C_1 (h plaques are clear and h^+ plaques are turbid on such a mixed indicator). If one of the mutants is am and the other is ts, two procedures may be used. Plaque

types may be distinguished by double-layer plating (bottom layer C-su⁻; top layer HF 4714-su⁺ with phage). On such plates am plaques are turbid and ts plaques are clear. The am and ts phage may also be distinguished by selective plating of the am mutant on su⁺ indicator at 40°^oC (assuming the am mutant is not also ts in phenotype) and of the ts mutant on su⁻ indicator at 30°^oC.

By using these plating procedures it has been possible to show that complementation between $\emptyset X$ mutants is usually symmetric, with both parental phage types represented with nearly equal frequency among the progeny. The results of several such complementation experiments are shown in Table 16. Some cases of asymmetric complementation have been observed. These will be discussed below (Part 2.3.2) in connection with the particular cistrons concerned.

The bacterial site and complementation. As previously mentioned, it seemed advisable to use conditions of infection which permit the replication of a large number of parental $\emptyset X$ particles within a single infected cell for the purposes of routine complementation tests. On the other hand, it was of general interest to determine whether complementation could occur under conditions which strongly limit the "participation number" by limiting the number of bacterial sites (61). For this reason experiments were performed using starved cells, in order to see whether complementation can occur in such cells, and whether any relationship exists between occupancy of a bacterial site and the ability to perform various phage induced functions.

Since even with starved cells two parental phages can replicate in a significant fraction of the population, single-burst experiments were performed. Cells were starved for 90 min according to the procedure of Denhardt and Sinsheimer (69) and then multiply infected with a mixture of $\emptyset X$ ts \checkmark h and ts 9 in the presence of starvation buffer. Un-adsorbed phage were removed with anti- $\emptyset X$ serum and the infected complexes were diluted into KC broth and distributed into two groups of single-burst tubes, one incubated at 30°C and the other at 40°C . The single-burst tubes were incubated for 75 min, then chilled and assayed by plating the entire contents of each tube on a mixed indicator containing 5 parts of C to 1 part of C₁, at 30°C (ts \checkmark h plaques are clear and ts 9 plaques are turbid under these conditions). The results of two such experiments, one using strain C and the other strain C_s are shown in Table 17. Although the multiplicities of infection are high enough that almost all cells adsorb both types of mutant phage particles, approximately half of the infected cells yield pure bursts which contain phage of only one genotype when development is allowed to proceed at the permissive temperature. At the restrictive temperature, however, essentially all the phage produced by complementation are released in mixed bursts. In the infection of starved C no pure bursts were produced at 40°C , and the numbers of mixed bursts produced at 30°C and 40°C were approximately equal. In the infection of starved C_s a few pure bursts were observed at 40°C (4 out of 22), however these were very small bursts (average burst size = 5.0) and represent only 5% of the phage produced at the restrictive temperature. It seems reasonable to

suppose that these few phage which were produced in pure bursts at 40°^oC resulted either from failure to sample both genotypes, simply because of the small numbers of phage matured within these cells, or from leakiness of the ts mutants involved rather than complementation. Mass culture measurements, performed at the same time, indicated that phage yield was increased 10-fold by complementation, over the level observed in selfings of the two mutants (complementation produced a 50-fold increase in the infection of C). In this C_s experiment complementation was not as efficient as in C since only approximately one fifth as many mixed bursts were produced at 40°^oC as at 30°^oC.

These results suggest that attachment to a bacterial site is necessary in order for one $\emptyset X$ genome to rescue another by complementation, in much the same way that site attachment is necessary for the production of single-stranded DNA. Cells infected by phage of two genotypes, but with only one genotype attached to a site (as judged by the production of only one type of single-stranded DNA at 30°^oC), could not produce any significant phage yields by complementation at 40°^oC. Strictly speaking, this result indicates the necessity of the bacterial site only for the performance of the functions defective in the mutants ts ✓ h and ts 9. These mutations are in cistrons III and IV respectively. Evidence that these cistrons code for the production of protein components of the phage coat will be presented in Part 3 of this thesis.

An experiment was also performed to determine whether an infecting phage must occupy a site in order to provide the phage induced lytic function. This experiment made use of a $\emptyset X$ mutant, am 3 (cistron I),

which is not able to lyse su^- host cells although progeny phage are matured within the infected cell (see Part 3 of this thesis). The experimental procedure was modeled after the participation experiments originally performed by Dulbecco with phage T2 (113). Strain C was starved and a series of cultures was infected. Each received an equal low multiplicity (0.23) of wt. The multiplicity of am 3 was varied from 0 to 12. Adsorption was performed at 30°C by the starvation-block procedure, and unadsorbed phage were removed by incubation with anti- ϕX serum. The infected cells were assayed, before burst, on two different indicators. Assay on C (su^-) measures the number of infected cells which produced wt progeny phage and lysed the host, since wt but not am 3 can form plaques on C. Assay on CR (su^+) measured the number of infected cells which lysed, whether or not the progeny contained any wt phage. The difference in these values (titer on CR - titer on C) would be a measure of the number of cells which were lysed by the wt without producing any progeny phage which contain wt DNA. The results of this experiment are shown in Figure 3. The fraction of wt phage which produce an infected cell able to plate on su^+ and su^- cells is plotted as a function of the multiplicity of the competing phage, am 3. The results of the assays on su^+ and su^- indicators are identical within the limits of experimental error. This means that no significant number of wt phage are able to lyse the host cells unless they also produce progeny phage (and therefore presumably occupy a bacterial site). The number of sites per bacterial cell can be calculated from such data, according to the method used by Dulbecco (113) and by Yarus and Sinsheimer (61).

Such calculations assume that those wt phage which occupy a site are selected randomly from all the phage infecting a cell, and that the distribution of infecting phage among the bacteria is also random.

Figure 3 includes theoretical curves obtained by assuming 1, 2, or 3 sites. The data are in reasonable agreement with a value of 2 sites per cell.

It would appear from these results that under conditions of limited participation the infecting phage which are able to produce progeny single-stranded DNA are also the only phage able to supply other phage functions in a complementation experiment. This has been demonstrated for the functions of cistrons I, III, and IV, which supply a protein necessary for the lysis function and two of the phage coat proteins, respectively. In terms of the site theory this means that it is necessary for the infecting genome to occupy a site in order to produce phage coded proteins (at least in sufficient quantity and in appropriate form to provide function by complementation). At least two different types of models can be proposed to explain this requirement for the site: 1) It may be necessary for DNA to be associated with a site on the bacterial membrane in order to be transcribed into messenger RNA, or in order for the messenger RNA to be translated into protein. In other words, a proper association with the site might really be an absolute requirement for virus coded protein synthesis. 2) Alternatively, the requirement for a site in order to produce function may be quantitative in nature. It appears to be necessary for a parental RF-DNA to occupy a site in order to replicate (see General Introduction). Perhaps all RF molecules can serve

as templates for messenger RNA synthesis and the bulk of viral protein synthesis is normally directed by the large number of progeny RF molecules free in the cytoplasm. Under this assumption an RF molecule which was unable to replicate because it did not occupy a site would be expected to produce a subnormal amount of virus coded proteins. Observable complementation might, however, require a fairly normal quantity of viral gene product, which could only be produced by the progeny of RF molecules associated with sites.

The ability of mutants in cistron VI, which are unable to replicate RF, to provide other phage functions by complementation argues against the second model. It seems likely that RF of these mutants is not replicated even in a mixed infection, since such mutants are effectively not rescued by complementation. The results of Tessman (51) using mutants in the analogous S13 cistron show that the mutant RF does not replicate to produce infective progeny RF. Evidence exists which suggests that production of phage-induced proteins increases with increasing multiplicity of infection, in situations where RF replication is blocked. Lindqvist has observed a multiplicity dependence of the rate of lysis by mutants in cistron VI (personal communication). Levine finds that the quantity of cistron VI protein produced in infected rep cells likewise increases with increasing multiplicity of infection (personal communication). It would be interesting to study the effect of multiplicity on the production of some phage-coded protein by parental RF molecules, under conditions where the number of bacterial sites is strongly limited. Such experiments might clearly distinguish between the two proposed models.

2.3.2 Cistrons

Complementation experiments have identified seven complementing groups of mutants among the $\emptyset X$ am and ts mutants. Table 18 shows phage yields obtained in tests between all pairwise combinations of representatives of each of these groups. If available, results with an am mutant in each cistron are presented in order to exclude the possibility of intragenic complementation, since am mutants do not show this phenomenon (85). A list of mutant strains assigned to the seven cistrons on the basis of complementation tests is shown in Table 19. Mutations to the am codon have all been identified in all cistrons except II and VII, and ts mutations in all except I and V. Recently two op mutants have been assigned to cistron VII.

In proving that two mutants lie in different cistrons it is important that the complementation test involves at least one mutant of the nonsense type (since a pair of missense mutants may sometimes give intragenic complementation). Cistrons II and VII could not be unambiguously distinguished on the basis of am and ts mutants alone (because of the lack of am mutants). It should be noted that these groups are clearly different than any of the other cistrons (I, III, IV, V, VI) since intragenic complementation should not occur between an am and a ts mutant. Physiological data concerning the mutants of cistrons II and VII (see Part 3) suggest that different phage functions are affected. Furthermore, complementation tests between these mutants, su mutants of the closely related phage S13, and am mutants of $\emptyset X$ selected in Hayashi's laboratory, indicate that II and VII are really distinct cistrons (Part

2.2.3). This is supported by the finding of $\emptyset X$ op mutants (which are presumed to be nonsense mutants) in cistron VII which do complement ts mutants in cistron II.

The absence of complementation between representatives of the same cistron is demonstrated by data presented in Table 20. Recombination experiments (section 2.4) have demonstrated the identification of mutants at a number of different sites within a single cistron.

Two exceptional am mutants have been found which fail to complement other am mutants in either cistron IV or VI. These appear to be polar mutants. Their properties will be discussed in a separate section (Part 2.5).

Asymmetric complementation. Mutants in cistron VI are unusual in that they are rescued very poorly, if at all, by complementation. Results of complementation tests involving am mutants in cistron VI, in which the two parental genotypes can be distinguished in the progeny, are presented in Table 21. The complementation yields in each case are composed almost entirely of the non-VI mutant. The yield of the cistron VI am mutant is not enhanced in the complementation situation above the level, due to leakiness, which is observed in infections with the mutant alone.

The cistron VII mutant ts 41D also exhibits asymmetric complementation. This mutant is rescued only slightly, if at all, in mixed infections with mutants defective in cistrons I, II, III, IV, and V, although it is able to rescue these mutants by complementation. On the other hand, ts 41D is rescued in a mixed infection with cistron VI am

mutants. The cistron VI mutant, as usual, is not rescued. The results of such complementation experiments are presented in Table 22. It seems likely, however, that the mutant ts 41D is in fact a double mutant containing a ts mutation in cistron VII, and a very leaky ts mutation in some other cistron. This idea is based on the observation that such a leaky ts mutant (which forms minute plaques at 40°*C*) is recovered from ts 41D in recombination experiments (see Part 2.4). It seems likely that the asymmetric complementation observed with ts 41D is somehow a result of the presence of this second mutation. The two op mutants which fail to complement ts 41D are rescued by complementation, supporting the idea that the asymmetry is a special property of ts 41D rather than cistron VII.

The basis for the phenomenon of asymmetric complementation is not yet known. Models can be proposed, which involve such things as non-diffusible gene products. These possibilities will be discussed more fully after the data concerning the cistron functions are presented in Part 3 of this thesis.

The *am* mutants of Hayashi. Recently a set of am mutants of $\emptyset X$ have been selected and classified into cistrons in Hayashi's laboratory (personal communication). These mutants fell in six cistrons, designated by the letters A through F. In order to determine how these cistrons correspond to those defined in this laboratory, complementation tests between representatives of the two sets of cistrons have been performed. The results are presented in Table 23. The cistrons A through F correspond to our cistrons II through VII as summarized in Table 25. No

new cistrons were identified among this set of mutants. The discovery of am mutants in cistrons II and VII should prove quite useful in physiological experiments concerning the functions of these cistrons.

2.3.3 Complementation with the related phage S13. Conditional lethal mutants of some other single-stranded DNA phages have been studied. These include the filamentous phage M13 (115) and the spherical phage S13 (107). A number of types of evidence indicate that S13 and $\emptyset X$ are closely related (see Introduction). It has not been possible to detect complementation between $\emptyset X$ and M13 (Knippers, personal communication). $\emptyset X$ and S13, on the other hand, appear to complement quite well.

Representative mutants in the $\emptyset X$ cistrons I through VII have been tested against am mutants in S13 cistrons I, II, IIIa, IIIb, IV, V (no representative of the recently reported cistron VI (114) was available). The complementation pattern obtained in these tests is displayed in Table 24. This pattern has several interesting features. Firstly, mutants in S13 cistron I showed only extremely low levels of complementation in tests with any of the $\emptyset X$ mutants. The reason for this is not known. Both S13 cistron I mutants tested adsorbed poorly under the conditions of the complementation experiment and it seems possible that those mutants (which have been characterized as coat protein mutants (107)) are not able to initiate infections under the conditions used. Also, several mutants showed normal levels of complementation in certain tests, and low but significant levels in others. These low complementation levels appear to be cistron specific rather than site specific since two representatives of each S13 cistron were tested and

in each case gave similar results. The low levels obtained with certain pairwise combinations of $\emptyset X$ and S13 mutants could be explained in several ways. Perhaps the most interesting possibility is that low complementation reflects a partial incompatibility in function between a $\emptyset X$ gene product and the S13 analog of a second gene product. These are presumed to be two gene products which must interact in some way during a successful infection. In the complementation situation, in which one gene is defective in S13 and the second in $\emptyset X$, the infection becomes dependent upon a $\emptyset X$ -S13 gene product interaction, which might in certain cases not be as efficient as the homologous interactions.

TABLE 14

COMPLEMENTATION BETWEEN ϕ X MUTANTS

ϕ X Mutants	Cistrons	BS	BS(wt)	Relative yield	Experiment
<u>ts</u> 4 x <u>am</u> 33	II x VI	58	90	0.64	
<u>ts</u> 4 (SELF)	II	2.0	90	0.02	1
<u>am</u> 33 (SELF)	VI	2.2	90	0.02	
<hr/>					
<u>am</u> 10 x <u>am</u> 33	V x VI	24	120	0.20	
<u>am</u> 10 (SELF)	V	0.03	120	2×10^{-4}	2
<u>am</u> 33 (SELF)	VI	0.03	120	0.003	
<hr/>					
<u>ts</u> 4 x <u>am</u> 10	II x V	12.5	105	0.13	
<u>ts</u> 4 (SELF)	II	0.094	105	0.001	3
<u>am</u> 10 (SELF)	V	<0.01	105	$<10^{-4}$	

C was infected as described in Materials and Methods, procedure 2 (Exp. 1 was unsynchronized, with adsorption and growth carried out at 40°C; Exp. 2 and 3 were KCN synchronized, adsorption was at 30°C, growth at 40°C, and unadsorbed phage were removed with serum). The burst size (phage produced per infected cell) of the complementation experiment, infections with each of the mutants alone (SELF), and a wt control included in the same experiment, are tabulated. The relative yield (ratio of phage yield to that of the wt control) is also listed.

TABLE 15

COMPLEMENTATION BETWEEN ϕ X MUTANTS: INFECTIVE CENTERS MEASUREMENTS

	<u>am</u> 3(I)	<u>am</u> 9(III)	<u>am</u> 16(IV)	<u>am</u> 10(V)	<u>am</u> 33(VI)
<u>am</u> 3(I)	<0.01	0.95	0.75	0.78	0.91
<u>am</u> 9(III)		0.09	0.80	0.88	0.69
<u>am</u> 16(IV)			<0.01	0.76	0.86
<u>am</u> 10(V)				<0.01	0.95
<u>am</u> 33(VI)					0.12

Infection of C at 30°C was performed as described in Materials and Methods (procedure 1). Infective centers were plated on CR at 30°C immediately after dilution from KCN and serum. The fraction of infected cells which produce a plaque is tabulated. (This value is calculated as the ratio of the number of infective centers in an experimental culture to the number of infective centers in the wt - infected control culture.)

TABLE 16

SYMMETRIC COMPLEMENTATION BETWEEN ØX MUTANTS

ØX Mutants	Cistrons	Yield h	Yield h ⁺	Total Yield
<u>ts</u> ✓ <u>h</u> x <u>ts</u> 4	III x II	0.083	0.084	0.17
<u>ts</u> ✓ <u>h</u> x <u>ts</u> 9	III x IV	0.049	0.045	0.094
<u>ts</u> 4 <u>h</u> x <u>ts</u> 9	II x IV	0.13	0.055	0.19
<u>ts</u> ✓ <u>h</u>	III	0.002	--	0.002
<u>ts</u> 4	II	--	0.008	0.008
<u>ts</u> 4 <u>h</u>	II	0.006	--	0.006
<u>ts</u> 9	IV	--	0.006	0.006

C was infected as described in Materials and Methods (procedure 1).

Incubation was carried out at 40°C after dilution from KCN and serum.

Progeny phage were assayed on mixed indicator (5 parts C plus 1 part

C₁) at 30°C. Under these plating conditions h mutants form clear

plaques and h⁺ phage form turbid plaques. Phage yields are expressed

as a fraction of the yield from a wt control infection.

TABLE 17

SINGLE BURST COMPLEMENTATION IN STARVED CELLS

Host	Temp (°C)	Number of Bursts			Average Burst Sizes		
		Mixed	Pure		Total	Mixed	Pure
			<u>ts</u>	<u>Y</u>	<u>h</u>	<u>ts</u>	<u>9</u>
<u>C</u>	30	11	11	6	100	146	70.5
<u>C</u>	40	12	0	0	12.3	12.3	--
<hr/>							
<u>C_s</u>	30	42	21	11	81.6	114.4	38.4
<u>C_s</u>	40	18	2	2	18.9	22.0	5.0

Cells (C or C_s) were starved for 90 min in SB (pH 7.5) at 37°^oC (69). Phage were adsorbed in SB (moi (ts Y h) = 3.4, moi (ts 9) = 4.2 for the infection of C; moi (ts Y h) = 5.0, moi (ts 9) = 4.0 for the infection of C_s). Unadsorbed phage were removed by incubation with anti- ϕ X serum after a 10 min attachment period at 30°^oC. The infected cells were diluted in KC broth at 0°^oC, and growth was initiated by distribution of 0.5 ml aliquots into several hundred tubes which were preheated to 30 and 40°^oC. The tubes were chilled after 75 min incubation and then the entire content of each tube was assayed on mixed indicator (C plus C₁) at 30°^oC to determine the genetic composition of each burst. The fraction of tubes containing bursts was small in all cases (C, 30°^oC - 28 out of 200; C, 40°^oC - 12 out of 200; C_s, 30°^oC - 74 out of 400; C_s, 40°^oC - 22 out of 400).

TABLE 18

COMPLEMENTATION BETWEEN ϕX MUTANTS IN SEVEN CISTRONS

	<u>am</u> 3(I)	<u>ts</u> 4(II)	<u>am</u> 9(III)	<u>am</u> 16(IV)	<u>am</u> 10(V)	<u>am</u> 33(VI)	<u>ts</u> 41D(VII)
<u>am</u> 3(I)	0.017	1.80	0.35	0.96	0.68	1.00	0.53
<u>ts</u> 4(II)		0.010	0.068	0.21	0.13	0.64	0.57
<u>am</u> 9(III)			0.001	0.17	0.13	0.11	0.37
<u>am</u> 16(IV)				0.003	0.29	0.46	0.037
<u>am</u> 10(V)					<0.001	0.21	0.15
<u>am</u> 33(VI)						0.012	0.16
<u>ts</u> 41D(VII)							0.001

Complementation yields (and selfing yields) are expressed as a fraction of the yield obtained in a wt control included in the same experiment. The values presented here are taken from several experiments, and the values tabulated for the selfings are averages of the values obtained in the different experiments involving a particular mutant.

TABLE 19
CISTRON ASSIGNMENTS

CISTRON	MUTANTS
I	<u>am</u> 3, 6, 20, 24, 26, 27, 29, 34
II	<u>ts</u> 4, 28, 29, 30, 34
III	<u>am</u> 9, 23, 22*, 32* <u>ts</u> 1, 8, 10, 33, 36, 41, 49, 53, 66, 73, 74, 49, 81, 83, 83, 87, 106
IV	<u>am</u> 14, 16, 18, 35 <u>ts</u> 6, 9, 65, 68, 70, 77, <u>95</u> , <u>108</u> <u>ts</u> 116 through 127, 129
V	<u>am</u> 10, 42
VI	<u>am</u> 8, 30, 33, <u>41</u> , <u>50</u> <u>ts</u> 128
VII	<u>ts</u> 41D <u>op</u> 6, 9

The $\emptyset X$ mutants assigned to each cistron by complementation experiments are listed. Underlined mutants are only tentatively assigned. The am mutants indicated by "*" are anomalous am mutants (see Part 2.2) in the sense that they do not respond to well characterized am suppressors, although they exhibit the same host specificity used for the isolation of true am mutants.

TABLE 20

COMPLEMENTATION TESTS BETWEEN am MUTANTS IN THE SAME CISTRON

A. CISTRON I MUTANTS

ØX	COMPLEMENTATION WITH		
	SELF	<u>am</u> 3	<u>am</u> 6
<u>am</u> 3	0.01	0.01	0.02
<u>am</u> 6	<0.001	0.02	<0.001
<u>am</u> 20	0.006	0.003	0.003
<u>am</u> 24	0.002	0.001	0.002
<u>am</u> 26	0.003	0.008	0.004
<u>am</u> 27	0.002	0.003	0.002

B. CISTRON VI MUTANTS

ØX	COMPLEMENTATION WITH		
	<u>am</u> 8	<u>am</u> 30	<u>am</u> 33
<u>am</u> 8	0.002	0.005	0.003
<u>am</u> 30		0.005	0.005
<u>am</u> 33			0.002

Complementation yields are expressed as a fraction of the yield obtained in a wt control performed in the same experiment.

TABLE 21

ASYMMETRIC COMPLEMENTATION OF A CISTRON VI MUTANT (am 33)

OX	CISTRON	SELF	SELF am 33	YIELDS IN COMPLEMENTATION WITH am 33			TOTAL YIELD
				YIELD am 33	YIELD ts		
ts 4	II	0.02	0.03	0.03	0.61	(0.64)	
am 9	III	0.004	0.015	0.009	(0.073)	0.082	
am 16	IV	0.003	0.015	0.012	(0.12)	0.13	
ts 41D	VII	0.001	0.004	0.002	0.17	0.16	

Complementation yields are expressed as a fraction of the wt control yield. These data are derived from several experiments; the yields obtained in selfings in each experiment are tabulated. am 33 among the progeny was scored selectively by plating on CR (su⁺) at 40°C (since the other mutants, including am 16 and am 9, are ts). ts 4 and 41D were assayed selectively by plating on C (su⁻) at 30°C. Total yield measurements were made by plating on CR (su⁺) at 30°C. The values in parentheses are calculated from the other, measured, values.

TABLE 22

COMPLEMENTATION OF CISTRON VII MUTANTS

ΦX	CISTRON	SELF	COMPLEMENTATION WITH			COMPLEMENTATION WITH		
			YIELD	YIELD	TOTAL	YIELD	YIELD	TOTAL
<u>am</u>		<u>ts</u>	<u>am</u>	<u>ts</u>	<u>am</u> or <u>ts</u>	<u>op</u>	<u>op</u>	<u>op</u>
<u>am</u> 3	I	0.003, ---	0.57	0.062	0.53	---	---	---
<u>ts</u> 4	II	0.005, 0.01	---	0.69	0.57	0.013	0.029	(0.042)
<u>am</u> 9	III	<0.001, 0.001	0.33	0.039	0.37	0.019	0.027	(0.046)
<u>am</u> 16	IV	0.007, 0.001	(0.030)	0.007	0.037	0.074	0.079	(0.15)
<u>am</u> 10	V	<0.001, 0.005	(0.13)	0.018	0.15	0.18	0.10	(0.28)
<u>am</u> 33	VI	0.004, 0.006	0.002	0.17	0.16	0.006	0.12	(0.13)
<u>ts</u> 41D	VII	---, 0.009	---	---	---	0.001	0.002	(0.003)
none (SELF)		---	---	---	0.001	0.001	---	<0.001 (<0.001)

Complementation yields are expressed as a fraction of the wt control yield. In the tests with ts 41D selective assays of am 3, 9 (a ts⁺ derivative), and 33 were performed by plating on HF4714 (su⁺) at 40°C. The yield of ts 41D was selectively assayed (except in the test with ts 4) by plating on G (su⁻) at 30°C. Total yield measurements were performed by plating on HF4714 (su⁺) at 30°C. In the tests with op 6, a selective measurement of op 6 was performed by plating on GII 103 (su-op⁺) at 40°C. The complementing mutant was selectively assayed by plating on HF4714 (su-am⁺) at 30°C. Values in parentheses were obtained as sums or differences of measured values.

TABLE 23

COMPLEMENTATION WITH THE ϕX MUTANTS OF HAYASHI

	none (SELF)	<u>am</u> N-1(A)	<u>am</u> H-210(B)	<u>am</u> H-90(C)	<u>am</u> H-81(D)	<u>am</u> H-57(E)	<u>am</u> H-116(F)
None (SELF)	--	<u><0.001</u>	<u><0.001</u>	<u><0.001</u>	<u><0.001</u>	<u><0.001</u>	<u><0.001</u>
<u>am</u> 3(I)	1.4	3.1	2.8	5.1	3.2	2.5	3.6
<u>ts</u> 4(II)	0.017	<u>0.047</u>	0.30	1.8	0.13	0.10	0.16
<u>am</u> 9(III)	<u><0.001</u>	0.028	0.052	0.044	0.090	0.023	<u><0.001</u>
<u>am</u> 16(IV)	<u>0.001</u>	0.92	<u><0.001</u>	0.13	0.49	0.13	0.98
<u>am</u> 10(V)	<u><0.001</u>	0.41	0.27	0.082	<u><0.001</u>	0.065	0.25
<u>am</u> 33(VI)	<u>0.004</u>	1.08	0.052	<u>0.004</u>	0.38	0.14	0.42
<u>ts</u> 41D(VII)	0.023	0.67	0.83	0.59	0.67	<u>0.018</u>	0.67

Phage yields are expressed as a fraction of the wt control yield. Underlined values indicate the absence of significant complementation. The high yield obtained in the selfing of am 3 indicates some spontaneous lysis of am 3 infected cells in this experiment. The complementation results still suggest that am 3 complements groups A through F.

TABLE 24

COMPLEMENTATION BETWEEN $\emptyset X$ AND S13

S13 CISTRON AND MUTANT							
	I	II	IIIa	IIIb	IV	V	
none	<u>am</u> 39	<u>am</u> 86	<u>am</u> 43	<u>am</u> 66	<u>am</u> 100	<u>am</u> 15	
-	-	<u><0.001</u>	<u>0.004</u>	<u><0.001</u>	<u><0.001</u>	<u>0.001</u>	<u>0.046</u>
-	<u>wt</u>	1.00	0.67	0.53	0.59	0.83	0.51
I	<u>am</u> 3	<u>0.001</u>	<u>0.006</u>	0.10	0.10	0.064	0.12
II	<u>ts</u> 4	<u>0.004</u>	<u>0.004</u>	0.16	0.027	<u>0.003</u>	0.14
<u>$\emptyset X$ CISTRON</u>	<u>III</u>	<u>am</u> 9	<u>0.001</u>	0.19	<u><0.001</u>	0.043	0.26
<u>AND MUTANT</u>	<u>IV</u>	<u>am</u> 16	<u><0.001</u>	<u>0.001</u>	0.23	0.26	0.20
V	<u>am</u> 10	<u><0.001</u>	<u><0.001</u>	0.073	0.039	0.043	0.010
VI	<u>am</u> 33	<u>0.003</u>	<u><0.001</u>	0.17	0.23	0.33	<u>0.003</u>
VII	<u>ts</u> 41D	<u>0.001</u>	<u>0.003</u>	0.16	0.43	0.60	0.20

Phage yields are expressed as a fraction of the wt control yield. Underlined values indicate the absence of significant complementation.

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TABLE 25

HOMOLOGOUS CISTRONS OF $\emptyset X$ AND S13

$\emptyset X$ (CIT)	$\emptyset X$ (UCSD)	S13
I	?	V
II	A	IIIb
III	F	IIIa
IV	B	II
V	D	?
VI	C	IV
VII	E	I
?	?	VI

This Table lists the corresponding cistrons defined by the mutants described in this thesis (CIT), the mutants isolated in Hayashi's laboratory (UCSD), and the mutants of the related phage S13 isolated by Tessman. This Table is deduced from the results presented in Tables 23 and 24 except for the assignment of S13 cistron I which is based on the results of tests between the $\emptyset X$ (UCSD) mutants and the S13 mutants. All the assignments are consistent with the results of these $\emptyset X$ (UCSD) x S13 tests (Hayashi, personal communication).

Figure 1. Single Bursts - Infection with Four Genotypes.

C was infected at 30° C in 0.003 M KCN with four distinguishable $\emptyset X$ strains (wt-moi=3.0; ts Y-moi=3.2; ts Y h-moi=3.5; H_aH_b-moi=2.4). After a 10 min adsorption period unadsorbed phage were removed by anti- $\emptyset X$ serum and the infected cells were diluted and distributed into single-burst tubes at 30° C . After 1 hr incubation the single-burst tubes were chilled. The entire content of each tube was then assayed on mixed indicator (C plus C₁), incubated for 2 to 3 hrs at 30° C and then incubated at 40° C (see Materials and Methods) in order to distinguish the four genotypes by plaque morphology. 23 bursts were found among 100 tubes at one dilution, and 36 bursts among 300 tubes at a higher dilution. The numbers of bursts containing 1,2,3, and 4 genotypes are plotted. At the multiplicities employed, the fraction of bursts in each class to be expected on the basis of random infection, with no limit to the participation number are: 1 genotype - 5×10^{-6} ; 2 genotypes - 0.014; 3 genotypes - 0.182; 4 genotypes - 0.804.

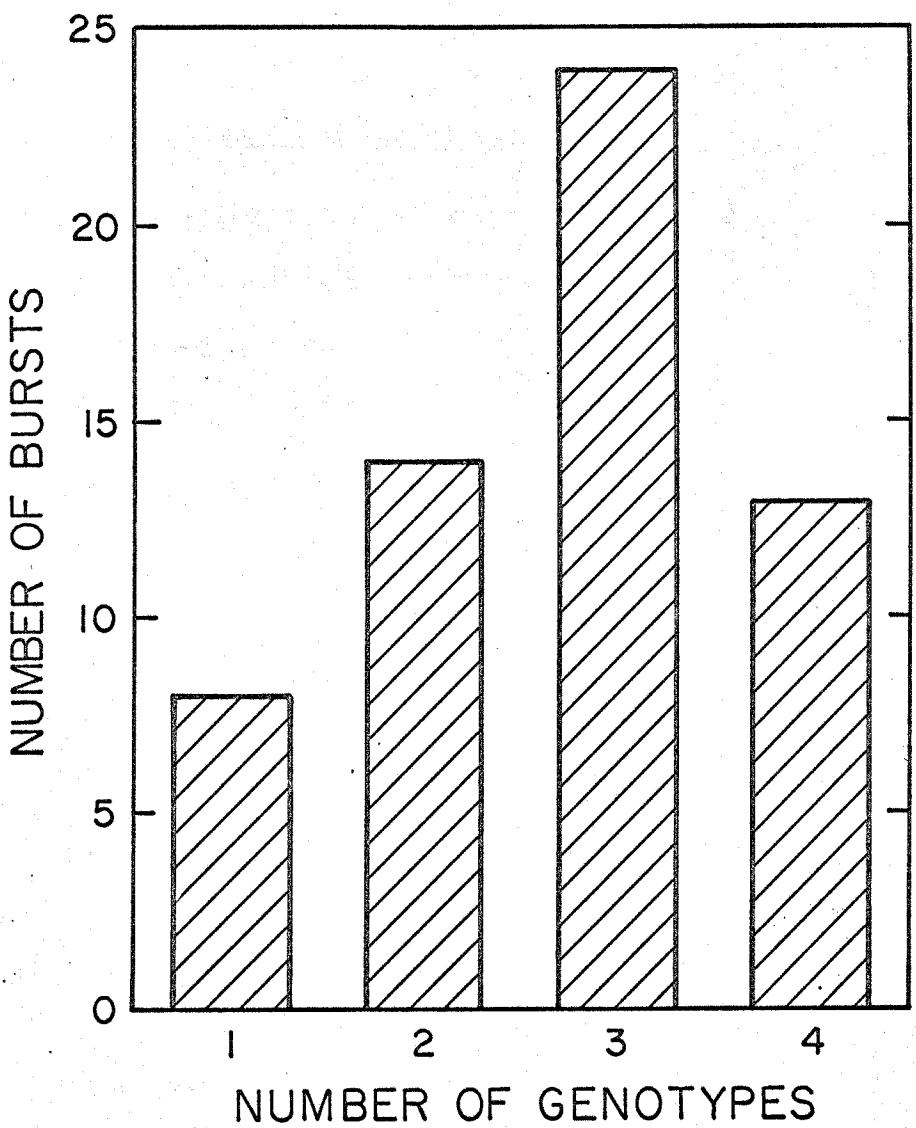


Figure 2. Complementation Yields in Single Bursts.

The experiment was performed as described in the text. 54 bursts were found among 194 tubes at one dilution, and 30 bursts among 200 tubes at a higher dilution. The number of bursts is plotted for each increment in burst size.

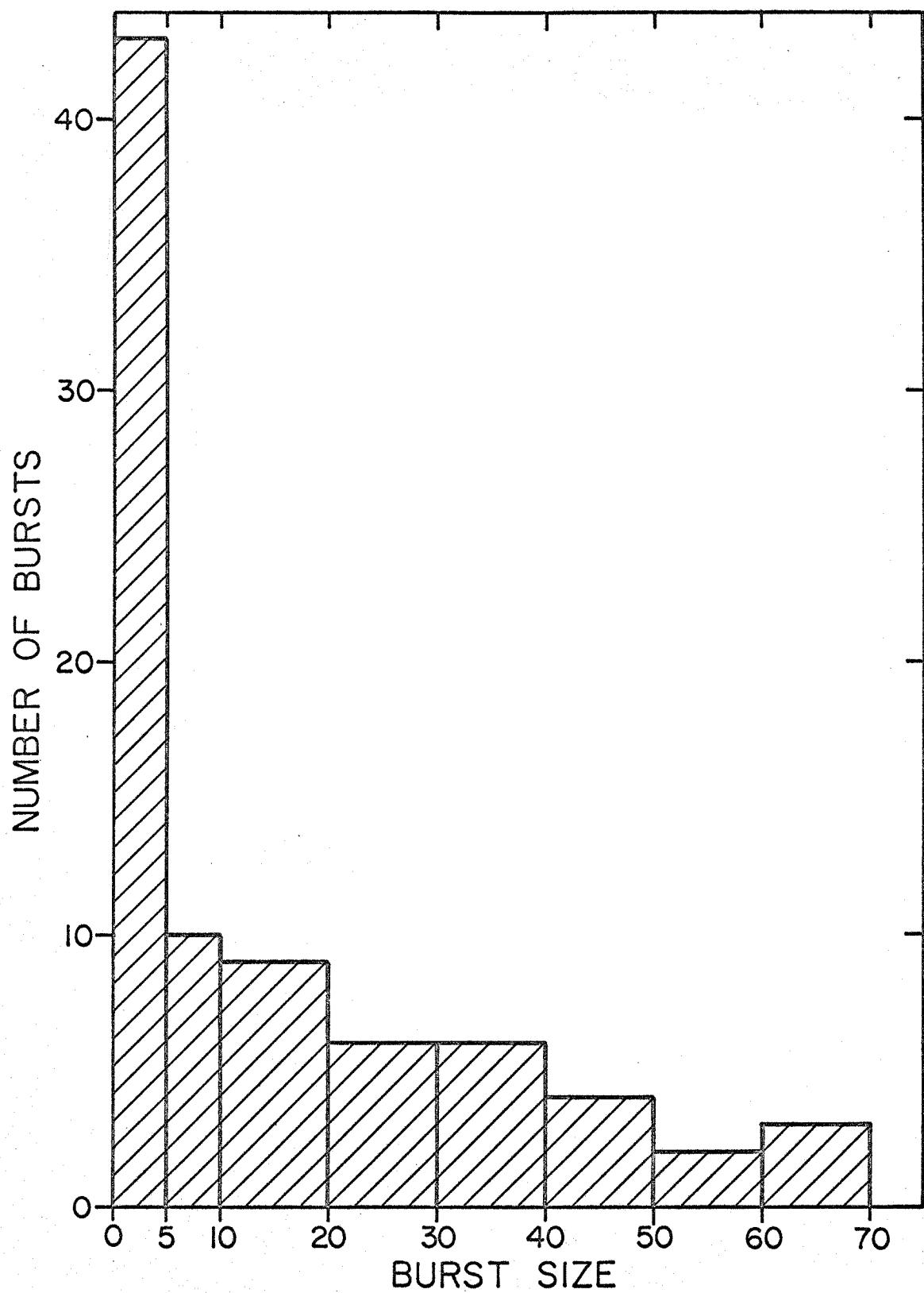


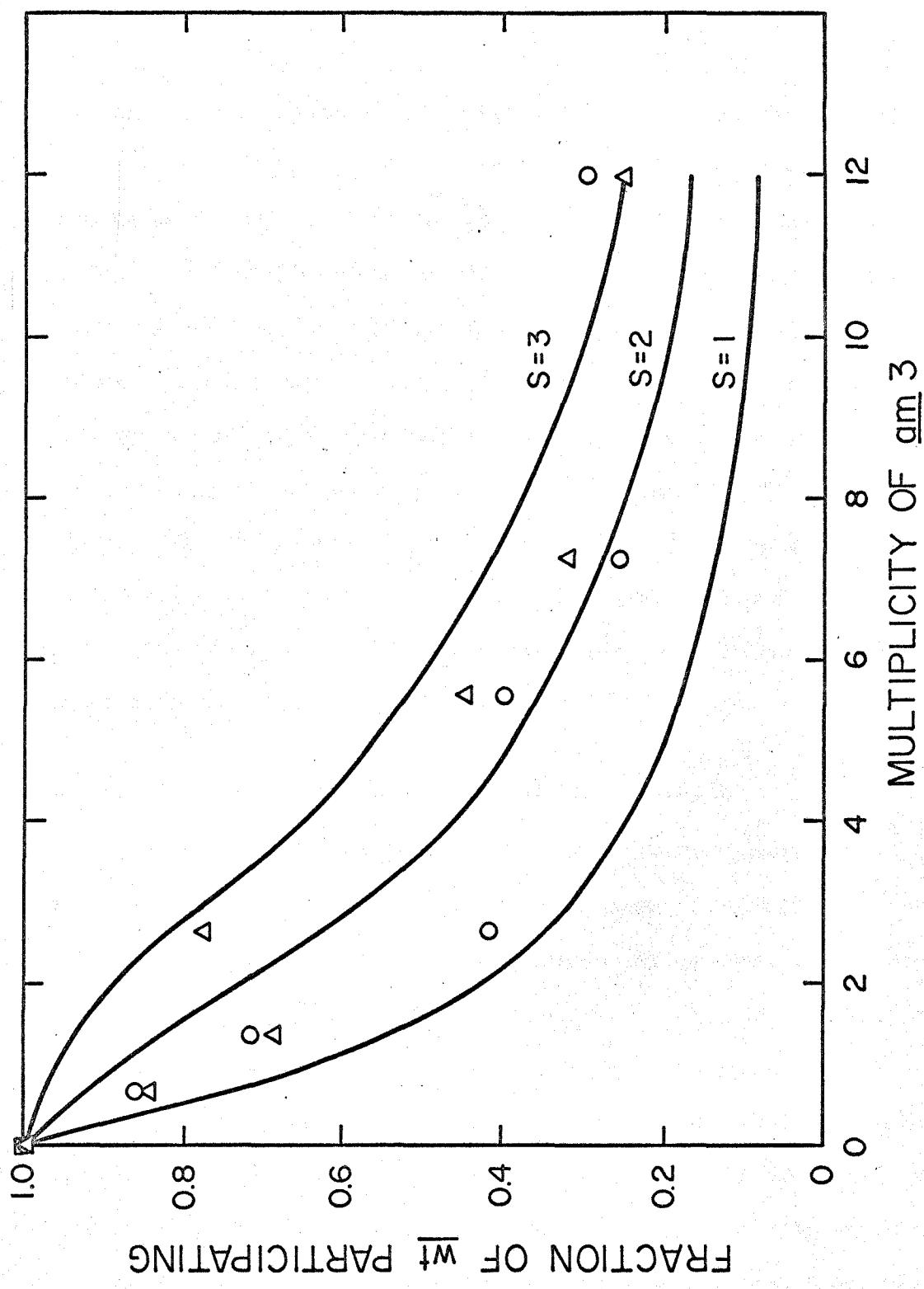
Figure 3. Participation Number for Lysis Function.

The experimental procedure is described in the text.

0 - Infective center titer on CR. (This measures the number of cells which lyse.)

Δ - Infective center titer on C. (These cells produce wt progeny and lyse.)

The solid curves are calculated (61, 113) on the basis of 1, 2, and 3 sites per cell.



FRACTION OF WT. PARTICIPATING

2.4 RECOMBINATION

Conditional lethal mutants are useful for the study of genetic recombination in $\emptyset X$ since they provide a selective system for the detection of recombinants. This is important because recombination frequencies are very low in crosses of $\emptyset X$ mutants (81). A cross between two conditional lethals (of the types am x am, am x ts, and ts x ts) at different genetic sites results in the formation of wt recombinants. These wt recombinants can be selectively assayed in the presence of a large excess of parental types simply by plating on C(su⁻), at 40° C (restrictive for ts mutants). The fraction of wt recombinants among the progeny from a cross is a measure of the recombination frequency between the two mutant sites. (The reciprocal, double mutant, recombinant cannot be readily detected.)

2.4.1 General properties of recombination in $\emptyset X$

Results of the cross ts \checkmark (III) x ts 9 (IV) are presented in Table 26. The wt recombinants are produced at a level (about 5×10^{-4} of the progeny) which is quite significantly above the background of revertants measured in selfings of the two mutants. The Table shows that recombinants arise in small clones (recombinant phage per infective center yielding at least one recombinant) of approximately 2 recombinant particles. Crosses are normally performed under conditions which are permissive for both of the parental types, in this case at 30° C . If, however, the infected cells are incubated at 40° C the phage yield

is reduced approximately 15-fold due to imperfect complementation of these mutants. Incubation of the infected cells under restrictive conditions also results in a slight increase in the observed recombination frequency, by about a factor of 2.

An experiment has been performed to measure the distribution of clone sizes in a cross of these same two ts mutants. Mixedly infected cells were diluted into single burst tubes so that the majority of tubes would not contain any infective centers which produced wt recombinant phage (although at this dilution all tubes will contain infective centers which yield parental types only). The entire content of each tube was then selectively assayed for wt recombinants. The result of this experiment is shown in Fig. 4. The experimental data fit the "Luria Distribution" (117). This is the distribution which is normally associated with genetic alterations which occur at random within an exponentially replicating population. The same experimental result has been obtained by Denhardt and Silver (118) who also described how this result can be interpreted in terms of a model in which only the RF at the bacterial site is replicating.

2.4.2 Mapping

Two factor crosses. Crosses have been performed in all pairwise combinations between a set of 17 mutants, including representatives of all seven cistrons. The results of this experiment are summarized in Tables 27 A, B, and C. Several general conclusions may be drawn from such data: 1) The recombination frequencies observed in intracistronic crosses are lower than for intercistronic crosses (with the exception

of the pair of cistron VI mutants tested which for unknown reasons give a very high value) as would be expected (see Table 27A, 27C). 2) It does not appear that such data can provide a reliable ordering of the cistrons on a genetic map. 3) Certain mutants give consistently low or high values when compared with the recombination frequencies observed with the majority of mutants (am 10(V) gives low values, and both am 33(VI) and am 30 (VI) give high recombination values). One source of variation might result from differences in burst sizes of the mutants, resulting in a variation of allele ratios in different crosses. (The recombination frequency should be highest for a recombining pool which contains the two parental types in equal numbers, other factors being equal.) Allele ratios were not measured in the progeny from the crosses.

In spite of these problems it is possible to make some inferences concerning the $\emptyset X$ map on the basis of such two factor cross data. The data presented in Table 27B show that the host range mutant tsh 6 is more closely linked to ts 41D(VII) than to mutants in any other cistron, suggesting that the tsh mutation is in cistron VII. This is reasonable in view of the homology between $\emptyset X$ cistron VII and S13 cistron I, since S13 host range mutants fall in this cistron (107). The data in Table 27C show that cistron V is considerably more closely linked to cistron I than to any other cistron, suggesting that they should map contiguously.

Another observation resulting from this two factor cross data concerns the apparent complexity of the ts 41D(VII) lesion. Crosses of this mutant with mutants in other cistrons resulted in two plaque types

when wt was selectively scored by plating on C at 41°C. There were large, presumably true wt, plaques and there were also small plaques. The simplest interpretation of this result is that ts 41D is a double mutant consisting of a ts mutation in one cistron (VII) and another mutation, possibly a quite leaky ts mutation, at another site. This second mutation can then segregate in crosses, giving rise to two plaque types (true wt and phage carrying only the second mutation of ts 41D). The recombination frequencies tabulated are calculated from total plaque counts and therefore presumably give the total recombination frequencies between the non-leaky ts in cistron VII and other markers. In view of the complexity of ts 41D, another mutant in cistron VII should be used to define that gene in physiological experiments. The recently characterized mutants op 6 and 9 could be useful for this purpose.

Three factor crosses. Some three factor crosses of the type am-ts x ts have been performed. The progeny from these crosses were plated at 40°C in order to select for ts⁺ recombinants. These recombinants were assayed both on CR (su⁺) and on C (su⁻) in order to determine the proportions of am and am⁺ recombinants. This allele ratio for the unselected marker should be relatively independent of factors which could alter the absolute recombination frequency between the two ts markers.

Results of such three factor crosses are presented in Table 28. In each case the two reciprocal configurations (am-ts a x ts b, and am-ts b x ts a) show a reversal in the relative frequencies of am and am⁺ alleles among the ts⁺ recombinant, as would be expected. It should be noted that in the case of each pair of ts mutants both am and am⁺

recombinants are produced, in both configurations. This observation indicates a high degree of negative interference. For example, in the case of the crosses between ts 7 and ts 79 (both in cistron III) it is reasonable to assume that the am 3 mutation is an outside marker, since both ts mutants are in the same cistron. Therefore one of the recombinant types (presumably the minority type) must involve at least two crossovers, one between the two ts mutations and one outside this region. These recombinants derived from double crossovers form a significant fraction of the ts⁺ recombinants (about 20% in cross 8, Table 28). It appears that double crossovers are at least 100 times more frequent among a selected recombinant class than total ts⁺ recombinants among the population as a whole (the highest frequencies of wt recombinants observed in two factor crosses are approximately 2×10^{-3}). Such a high level of negative interference may result from circularity of the genetic map, since circularity imposes a requirement for even numbers of crossover events. The present data are insufficient to establish or exclude circularity.

It is possible to infer an ordering (II-III-I-IV) from the data presented in Table 28, if several assumptions are made. We assume that when the am 3 mutation is an outside marker the majority recombinant arises from a single crossover (or, in the case of a circular map, that the second crossover falls outside of the shorter arc joining the three mutant sites). When the am 3 site falls between the two ts mutations, both am and am⁺ recombinants can arise from single crossovers. We assume that in such a situation the two recombinant types will be produced

in proportion to the lengths of the two intervals ts a-am 3 and am 3-ts b. In the case of crosses between the two cistron III mutants (7 and 8, Table 28) am 3(I) must be an outside marker since ts Y and ts 79 are both in cistron III. We can therefore infer the order ts 79 - ts Y - am 3. In the crosses involving the mutants ts Y (III) and ts 9(IV) both recombinant classes are equally frequent (crosses 9 and 10). This indicates that am 3 (I) is an internal marker in this case, located very near midway between the two ts sites. We infer the order ts Y - am 3 - ts 9 (or III-I-IV). The crosses between ts 79(III) and ts 9(IV) (crosses 11 and 12) involve the same cistrons, so that the am 3(I) site must also be an internal marker in this case. The proportions of am and am⁺ recombinants indicate that the interval ts 79 - am 3 must be greater than the interval am 3 - ts 9. Since the intervals ts Y - am 3 and am 3 - ts 9 are approximately equal (see above), we can conclude that the distance ts 79 - am 3 is greater than ts Y - am 3, which implies the order ts 79 - ts Y - am 3. This is the same result that was derived independently from crosses between ts Y and ts 79. The crosses between ts 4(II) and both the ts(III) mutants (crosses 1 through 4, Table 28) give similar results for the two cistron III mutants, as should be the case. The proportions of recombinant types are consistent either with the order II-III-I (am 3(I) an outside marker) or with the order III-I---II (am 3(I) internal) in which the interval III-I is shorter than the interval I---II. The observed frequencies of recombination suggest that ts 4(II) is more closely linked to ts 79(III) than to ts 9(IV) (see also the two factor recombination data of Table 27). The order II-III-I

is therefore probably correct. This is consistent with the results of the crosses between ts 4 (II) and ts 9(IV) (crosses 5 and 6, Table 28). The composite order resulting from these arguments is:

ts 4(II)-ts 79(III)-ts ✓ (III)-am 3(I)-ts 9(IV).

A tentative map of the $\emptyset X$ genome is presented in Figure 5. This map has been constructed to be consistent with the following:

- 1) The three factor cross data discussed above.
- 2) The two factor cross data suggest that cistron V is most closely linked to cistron I, and that cistron VII is closely linked to III (see Table 27).
- 3) The data concerning a polar am mutant which is defective both in cistron IV and cistron VI function, but is located in cistron IV (see Part 2.5) indicate that cistrons IV and VI are adjacent, and that the polar site is near the proximal end of IV (the end furthest from VI).
- 4) The order of the cistrons is identical with the order of the homologous cistrons of the closely related phage S13 (119) with the assumption that $\emptyset X$ cistron V (group D of Hayashi) corresponds to cistron VI (the most recently identified cistron) of S13 (see Table 25). The S13 map is based on a greater number of three factor crosses, and is therefore probably quite reliable. The map has also been drawn as a circle because of the evidence that the S13 map is circular (119).

TABLE 26

RECOMBINATION BETWEEN TWO $\emptyset X$ ts MUTANTS

CROSS	REC-IC	REC-30	REC-40	REC-BS	BS-30	BS-40
<u>ts</u> 7 (III) x <u>ts</u> 9 (IV)	4.7×10^{-2}	4.9×10^{-4}	9.1×10^{-4}	1.6	150	11
<u>ts</u> 7 (III) (SELF)	4×10^{-6}	2×10^{-5}	--	40	84	0.06
<u>ts</u> 9 (IV) (SELF)	1×10^{-5}	1×10^{-5}	1×10^{-4}	60	48	0.73

Strain C was infected at 30°C in the presence of 0.003 M KCN with a moi of 5 plaque formers of each parent (or 10 in the case of the selfings). Growth was initiated by diluting 100-fold into fresh KC broth, at 30°C and also at 40°C . At the same time, a sample was diluted into anti- $\emptyset X$ serum and incubated to remove unadsorbed phage, then diluted further and plated to assay infective centers at 30°C and 40°C . After 50 min incubation the diluted growth tubes were chilled and then assayed by plating at 30°C and 40°C . This Table lists the frequency of wt recombinants (REC - the titer at 40°C divided by the titer at 30°C) among the infective centers (IC), the progeny from the 30°C growth tube (30), and from the 40°C growth tubes. (For the selfings these numbers presumably represent the frequencies of revertants rather than recombinants.) "REC-BS" indicates the number of wt progeny in the 30°C growth tube produced per infective center which forms a plaque at 40°C . "BS-30" and "BS-40" refer to burst sizes (total progeny per infected cell) in the 30°C and 40°C growth tubes.

Table 27. TWO FACTOR CROSSES

Frequencies of the wt recombinant are tabulated in units of 10^{-4} recombinants per progeny phage. Crosses were performed in HF4714(su⁺) at 30°C , total progeny were assayed under these same conditions, and wt recombinants were selectively assayed on C(su⁻) at 41°C .

A. Close linkage within a cistron. The frequencies of recombination between two cistron III mutants and two cistron IV mutants, in all pairwise combinations, are tabulated. The data show that intracistronic frequencies of recombination are lower than intercistronic frequencies.

B. Close linkage of ts 41D(VII) and tsh 6. This table shows recombination frequencies between ts 41D(VII), tsh 6, and a representative of each cistron. The results show that tsh 6 is more closely linked to ts 41D than to the representative of any other cistron, and that it is next most closely linked to V and III. ts 41D is most closely linked to V, tsh 6, and III (it should be kept in mind that am 10(V) gives low values in all crosses (Table 27C)). These results indicate that tsh 6 is probably in cistron VII.

C. Linkage between the seven cistrons. Crosses were performed between am 3 and 27 in cistron I, ts 4 and 28 in cistron II, am 9 and ts 79 in cistron III, am 16, 18, and 35, and ts 9, 116, and 124 in cistron IV, am 10 in cistron V, am 30 and 33 in cistron VI, and ts 41D and tsh 6 in cistron VII, in all pairwise combinations. The values tabulated here are averages of all crosses involving a particular pair of cistrons. In the case of intracistronic crosses, the values listed

are averages of those crosses between resolvable sites within the cistrone. The order of the cistrons along the axes of this Table is believed to be the map order (obtained by cutting the circular map of Figure 5 between cistrons VI and II).

TABLE 27A

		CISTRON AND MUTANT			
		III <u>am</u> 9	III <u>ts</u> 79	IV <u>am</u> 16	IV <u>ts</u> 9
III	<u>am</u> 9	0.01	0.18	3.0	5.4
III	<u>ts</u> 79		<0.01	2.1	5.6
IV	<u>am</u> 16			0.02	0.72
IV	<u>ts</u> 9				≤0.02

TABLE 27B

CISTRON	MUTANT	<u>ts</u> 41D	<u>tsh</u> 6
I	<u>am</u> 3	2.5	8.7
II	<u>ts</u> 4	1.9	5.3
III	<u>am</u> 9	0.63	3.9
IV	<u>am</u> 16	1.8	6.1
V	<u>am</u> 10	0.1	1.6
VI	<u>am</u> 33	3.8	13
VII	<u>ts</u> 41D	<0.01	0.62
(VII)	<u>tsh</u> 6	0.62	0.2

TABLE 27C

	II	III	VII	V	I	IV	VI
II	--	1.6	3.3	1.2	3.3	5.6	9.2
III		0.18	1.5	0.75	6.7	5.7	8.1
VII			0.62	0.86	3.7	4.8	8.7
V				--	0.20	1.4	2.1
I					0.42	4.1	7.1
IV						0.87	9.7
VI							22

TABLE 28

THREE FACTOR CROSSES

Crosses were performed in CR(su⁺) at 30°C, the total progeny assayed on CR at 30°C, and the ts⁺ recombinants were selectively assayed at 40°C on CR (su⁺) and C(su⁻) to determine the ratio of am to am⁺ among them (the values were corrected for differences in efficiency of plating on the two strains, using data from a wt control included in the same experiment and plated at the same time). For each cross the fraction of ts⁺ progeny which are am and am⁺ (am 3 or wt) are listed. The majority class is indicated, and the total frequency of ts⁺ recombinants is tabulated in units of 10^{-4} ts⁺ recombinants per progeny phage.

TABLE 28
THREE FACTOR CROSSES

CROSS	ts ⁺ RECOMBINANTS				frequency x 10 ⁴
	wt	am 3	Majority type	ts ⁺	
1. <u>ts</u> 4(II)- <u>am</u> 3 x <u>ts</u> ✓ (III)	.30	.70	<u>am</u>		3.6
2. <u>ts</u> ✓ (III)- <u>am</u> 3 x <u>ts</u> 4(II)	.93	.07	<u>wt</u>		2.9
3. <u>ts</u> 4(II)- <u>am</u> 3 x <u>ts</u> 79(III)	.37	.63	<u>am</u>		1.5
4. <u>ts</u> 79(III)- <u>am</u> 3 x <u>ts</u> 4(II)	.70	.30	<u>wt</u>		1.2
5. <u>ts</u> 4(II)- <u>am</u> 3 x <u>ts</u> 9(IV)	.22	.78	<u>am</u>		5.9
6. <u>ts</u> 9(IV)- <u>am</u> 3 x <u>ts</u> 4(II)	.70	.30	<u>wt</u>		4.5
7. <u>ts</u> ✓ (III)- <u>am</u> 3 x <u>ts</u> 79(III)	.91	.09	<u>wt</u>		0.8
8. <u>ts</u> 79(III)- <u>am</u> 3 x <u>ts</u> ✓ (III)	.23	.77	<u>am</u>		0.7
9. <u>ts</u> ✓ (III)- <u>am</u> 3 x <u>ts</u> 9(IV)	.49	.51	--		6.1
10. <u>ts</u> 9(IV)- <u>am</u> 3 x <u>ts</u> ✓ (III)	.54	.46	--		4.2
11. <u>ts</u> 79(III)- <u>am</u> 3 x <u>ts</u> 9(IV)	.22	.78	<u>am</u>		3.4
12. <u>ts</u> 9(IV)- <u>am</u> 3 x <u>ts</u> 79(III)	.68	.32	<u>wt</u>		3.1

Figure 4. Clone Size Distribution of $\emptyset X$ Recombinants.

Strain C was infected at 30°C in the presence of 0.003 M KCN with a moi of 5 plaque formers of each parent (ts and ts 9). Unadsorbed phage were removed by anti- $\emptyset X$ serum. The infected cells were then diluted and distributed into single-burst tubes at a concentration such that most tubes produced no wt recombinant phage. After 40 min incubation at 30°C the single-burst tubes were chilled. They were assayed by plating the entire contents of each tube on C at 40°C . 11 bursts of recombinants were found among 191 tubes. The fraction of these clones of size greater than or equal to N is plotted versus the clone size, N (in a log-log plot). The solid line is the expected result for a "Luria distribution" (117).

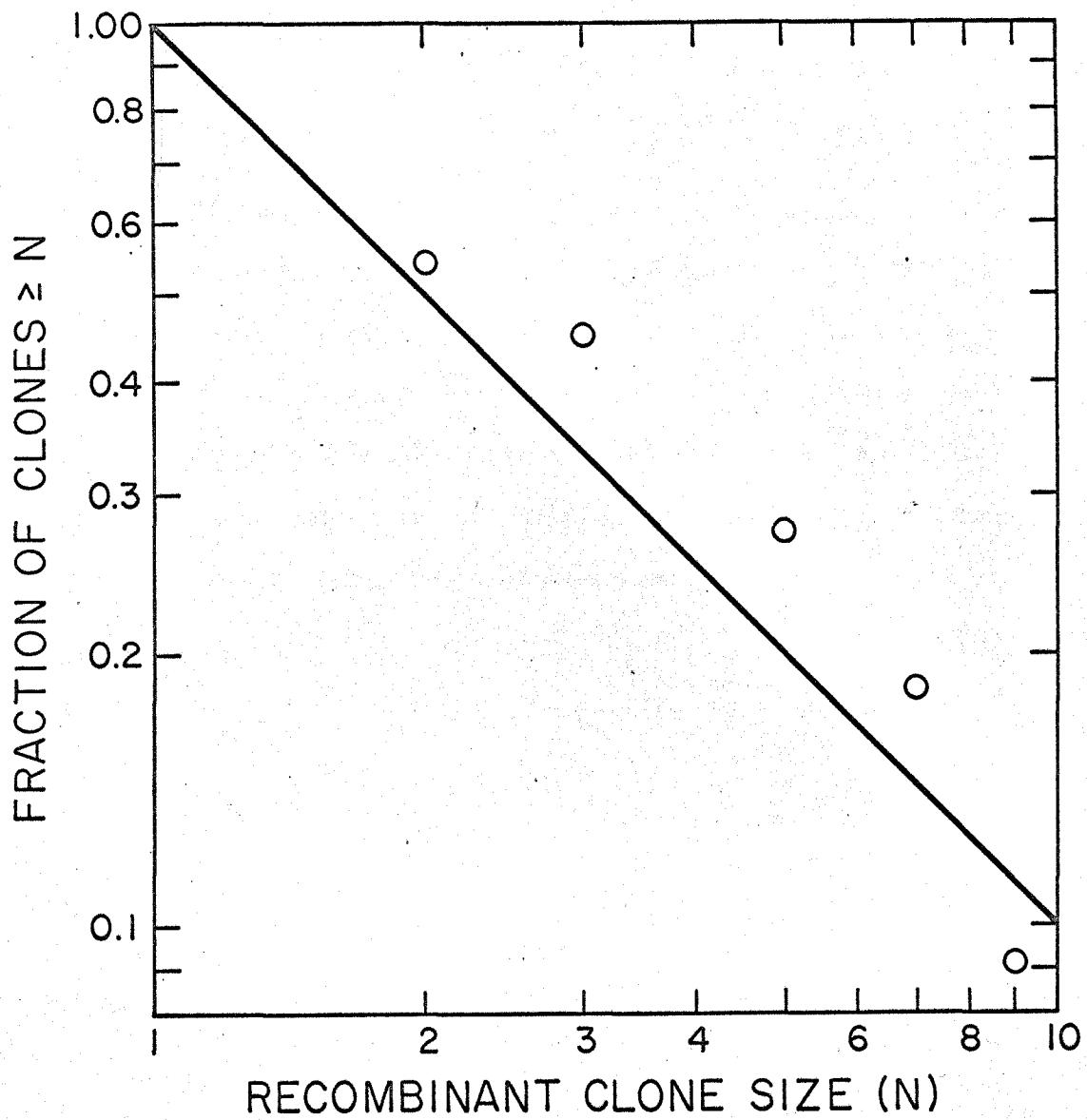
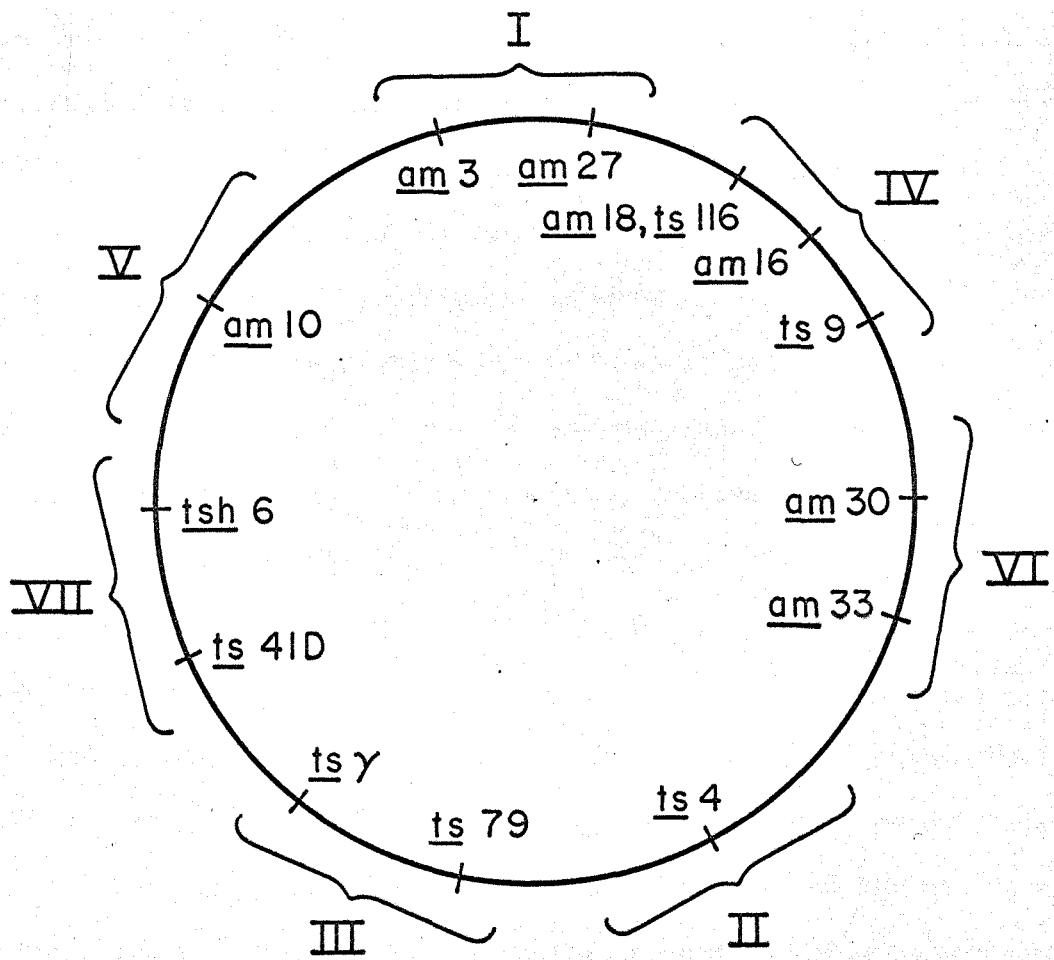


Figure 5. Map of the ØX Genome.

This tentative map is constructed as described in the text. The order of the cistrons is believed to be correct. There is evidence for the order of the sites within cistron III, and preliminary evidence for the ordering within cistron IV (see Part 2.5). All other sites indicated are resolvable by recombination, but the intracistronic orders are not known. Distances are not drawn to scale.



2.5 POLARITY OF TRANSCRIPTION2.5.0 Introduction.

The available information concerning the transcription of ϕ X mRNA (see General Introduction) can be simply summarized. 1) The mRNA is transcribed from an RF template and is complementary to the (-) strand. 2) The mRNA molecules are large and presumably polycistronic.

It seems reasonable to assume that both mRNA synthesis and translation into ϕ X specific proteins proceed in the usual way, starting at the 5' end and proceeding toward the 3' end of the mRNA molecule. Thus the order of translation of the ϕ X cistrons into protein molecules by ribosomes moving along the mRNA molecule, the order of transcription of the cistrons into mRNA from the RF, and the order of the cistrons along the viral DNA strand (in the 5' to 3' direction), are expected to be identical.

The phenomenon of "polarity", which has been studied in several genetic systems (see, for example, 120,121), provides genetic evidence both for the existence of polycistronic messengers and for the direction of their translation. A polar mutation is an alteration of one gene which eliminates or reduces function of both the altered gene and one or more adjacent (distal) genes. It is believed that the pleiotropic effects of such mutations result from interruption of the directional translation of polycistronic messengers. Polar mutations may be either nonsense or frameshift mutations. Studies of the polar effects of non-sense mutations (am and oc) show that the strength of the polarity ob-

served (i.e. the degree of reduction of distal functions) is greatly dependent on the site of the nonsense mutant. In general the polar effects are strongest for the most proximal nonsense mutations and become weaker for mutations nearer the distal end of a gene.

An am site in $\emptyset X$ cistron IV has been identified, which, in addition to cistron IV function also lacks cistron VI function. A description of the properties of mutants at this site follows.

2.5.1 A Polar Mutant of $\emptyset X$

Two independently isolated $\emptyset X$ am mutants do not complement representatives of either cistron IV or VI. These unusual mutants are designated am 18 and am 35. Their complementation behavior is illustrated by the data presented in Tables 29 and 30. Table 30 also reiterates the fact that am mutants in cistrons IV and VI do complement with normal efficiency, and therefore represent genes for different protein chains. Physiological characterization of the defects present in mutants of cistrons IV and VI also demonstrated that two separate functions are affected. Cistron IV codes for a protein which is a structural component of the phage coat, whereas the protein coded by cistron VI functions in the replication of $\emptyset X$ RF (see Part 3 of this thesis).

One possible explanation of the pleiotropic effect of these mutants would be that they are double am mutants, composed of one am mutation in cistron IV and one in cistron VI. Both mutants revert spontaneously, indicating that this interpretation is not correct. Stocks of am 18 and am 35, produced in the usual way, contain revertants which will form plaques on an su host (C) at frequencies which

are typical for normal am mutants, usually about 1 revertant per 10^5 infective phage.

The two mutants appear to be at the same site. An upper limit on the fraction of wt recombinants among the progeny of the cross am 18 x am 35 is 2×10^{-5} (this limit was set by the number of revertants already present in the phage stocks).

Which cistron is mutated? Another explanation of the pleiotropic effect of am mutants at this site would be that the mutation is actually in one of the cistrons, but exhibits a polar effect on the other. It has been possible to identify which cistron contains this presumed polar mutant, due to a particularly fortunate set of circumstances.

If revertants of either am 18 or am 35 are selected by plating large numbers of phage on C at 30°C , it is found that a significant fraction of these revertants are ts mutants. This was discovered by stabbing revertant plaques with a sterile pin and transferring phage to two plates pre-seeded with C. One was then incubated at 30°C and the other at 40°C . Out of 25 revertants from am 18, 9 were found which are ts. Similarly, out of 25 revertants from am 35, 8 were ts. These ts strains form plaques at 30°C , but not at 40°C , on either C (su⁻) or CR(su⁺). The original am mutants from which they are derived form plaques with equal efficiency at both temperatures on CR, although the plaques are somewhat smaller at the high temperature. This temperature-sensitivity of revertants from am mutants is not frequent among $\emptyset X$ am mutants. Revertants from a number of other $\emptyset X$ am mutants were tested (these are am 3, 6, 20, 22, 24, 26, 27, 29, 30, 32, 33, 34, 41, 42, 50),

but no ts derivatives were found. Similar situations in which am mutants may revert to produce a ts mutant have been reported in other phage (93).

Because of the nature of the am mutation it is difficult to imagine any mechanism which could produce a revertant by mutation outside of the altered triplet. It is presumed that these ts revertants from am 18 and am 35 represent mutations from the am nonsense triplet (UAG) to some other triplet which is different from that present at this site in the wt. The result is a missense mutant which happens to be ts.

It is therefore expected that the ts revertants will behave as a normal ts mutant at the same site as the initial am. In particular it should be possible to assign these ts revertants to either cistron IV or VI. The complementation behavior of such revertants is shown in Table 30. They behave as would be expected for a ts in cistron IV. These results indicate that the polar site is in cistron IV, and that transcription proceeds from cistron IV toward cistron VI.

2.5.2 Mapping the polar site

The model proposed above, in order to account for the properties of am 18 and am 35 and their ts derivatives, has several consequences with respect to mapping of the various mutants involved: 1) We would expect that the ts derivatives of the polar mutants would not recombine with the mutants from which they were derived, or with each other, to give wt. 2) All these mutants at the presumed polar site should map in cistron IV, that is they should be more closely linked to other mutants in cistron

IV than to mutants in cistron VI. 3) In analogy with other systems in which polar mutants have been found, we would expect the polar site to be located near the proximal end of cistron IV (the end farthest from VI).

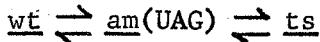
Crosses between the ts revertants and the am polar mutants from which they are derived show that they are either mutant at the same site, or very closely linked. In one such cross, ts 124 x am 35, an upper limit of 1×10^{-6} can be set on the fraction of wt recombinants among the progeny. The highest recombination frequencies observed in crosses between $\emptyset X$ mutants (about 2×10^{-3}) are approximately 2×10^3 times this great. Under the assumption that recombination frequency is proportional to separation of the mutant sites along the length of the DNA molecule, we could set an upper limit of $5.5 \times 10^3 / 2 \times 10^3$, or approximately 3 nucleotides to the separation of the mutant sites in am 35 and ts 124. Two factors, circularity of the map and high negative interference, which complicate the relation between physical separation and recombination frequency, would both act to decrease the value of the upper limit. The result of this calculation is in agreement with the idea that the ts reversions occur within the same triplet as the initial am mutants. (It is of course possible, however, that some type of positive interference exists, which manifests itself only at very short range, causing us to underestimate the limit on the separation of the mutants.)

Similarly, ts revertants derived from the two independently isolated am mutants at the polar site (am 18 and am 35) do not recombine with each other to give wt.

The recombination data presented in Figure 6 demonstrate that mutants at the polar site are considerably more closely linked to two mutants in cistron IV (am 16 and ts 9) than to a mutant in cistron VI (am 33). This agrees with the complementation data which place the polar site in cistron IV. Recombination frequencies obtained in such two factor crosses are not adequate to unambiguously determine the relative location of the polar site within cistron IV.

2.5.3 Codons and amino acid substitutions at the polar site.

\emptyset X wt, the polar am mutants, and their ts revertants would be expected to contain three different triplets at a single codon position in cistron IV. All the mutational events



probably occur by single base changes. The fact that nitrous acid induced mutation to a polar am has been observed twice makes it seem unlikely that this change involves a double mutation. Spontaneous reversion of the am mutant both to the ts mutant and to what appears to be wt provides evidence that these also occur by single mutational steps. It has also been possible to select for spontaneous mutation from a ts at this site to the am triplet. Several stocks of ts 116 (a revertant from am 18) were grown from single plaques in the standard way (see Materials and Methods) except that CR(su⁺) was used as a host. Revertants from ts 116 were selected from these stocks by plating on CR at 40°C. Of 4 ts⁺ revertants tested, all were am (in the sense that they formed plaques on CR but not on C at both 30°C and 40°C). It

has not yet been determined whether these am mutants possess the properties of the original polar am mutant from which they are derived.

There are nine triplets which differ by a single base change from the am triplet (UAG) and can therefore be considered as possible sequences for the wt or ts codons at this site. It is possible to attempt to identify which codon produces the ts phenotype by growing the am mutant at 30°C and 40°C in various suppressor strains which cause insertion of different amino acids in response to the UAG triplet.

Table 31 lists the codons which differ from UAG by a single base change, the amino acids which they code for, and the suppressor strains which cause the insertion of these amino acids, if any.

DNA from am 18 and am 35 was used to infect spheroplasts of su⁻, su₁⁺, su₂⁺, su₃⁺, and su₅⁺ strains (106,122) at 30°C and 40°C. The results, presented in Table 32, indicate that phage production is not temperature-sensitive in these strains (or at least no more so than ØX wt). It therefore seems unlikely that the triplet present in the ts revertants codes for the insertion of an amino acid specified by one of these suppressors (serine, glutamine, tyrosine, or lysine). This leaves the triplets GAG (glutamic acid) UUG (leucine), and UGG (tryptophan) as possible ts codons.

Preliminary experiments suggest that the am triplet is induced to revert both to wt and to ts by growth in the presence of BUdR, indicating that both of these mutations are transitions. This would imply that the wt contains the triplet CAG which specifies glutamine (since glutamine at this site does not produce temperature-sensitivity) and

the ts revertant contains UGG which codes for tryptophan (see Table 31). Further mutagenic studies should unambiguously define the various triplets present at this polar site.

2.5.4 Physiological basis of the polar effect.

Physiological studies of the polar am mutants show that they are actually blocked in cistron VI function by all available criteria. They exhibit the asymmetric complementation typical of cistron VI mutants. They show delayed lysis (see Part 3.2). The mutant am 35 shows greatly inhibited RF synthesis, just as cistron VI am mutants do, and is blocked in the synthesis of the chloramphenicol resistant cistron VI protein. The ts revertant, ts 124, on the other hand, synthesizes cistron VI protein at 40°*C*, and is not blocked in RF replication at 40°*C*, indicating that its defect is not in cistron VI (A.J. Levine, personal communication).

These results support the idea that polar mutants at this site are defective in cistron VI function because they do not synthesize the cistron VI protein, as would be expected for a classical polar mutation of the type described in the introduction to this section.

Recently a polar effect has been reported in the closely related phage S13 (123). In complementation tests between S13 mutants in cistrons IIIa and IIIb, the progeny yields are greatly reduced if the mutant in cistron IIIa is an am mutant (the su mutants of S13 are probably amber-type mutants) rather than a ts mutant. The nature of the IIIb mutant used did not affect the level of complementation observed. This result

was interpreted to mean that am mutants in IIIa are defective in IIIb function because they are polar mutants. It was therefore argued that the S13 genome is translated in the direction from cistron IIIa toward cistron IIIb.

Cistrons IIIa and IIIb of S13 are homologous to $\emptyset X$ cistrons III and II respectively (Part 2.3, Table 25). The direction of translation implied by the S13 studies (from III toward II) and by the studies of the $\emptyset X$ polar site described here (from IV toward VI) are opposite.

This situation can be explained in various ways:

- 1) Perhaps different regions of the genome of $\emptyset X$ (and S13) are actually translated in different directions. This would seem to require that mRNA is synthesized from both strands of the RF, although no $\emptyset X$ mRNA complementary to the (+) strand has so far been detected.
- 2) Perhaps some mistake has been made in determining the homologous cistrons of $\emptyset X$ and S13. We plan to perform complementation tests between the $\emptyset X$ polar am mutants and S13 mutants. The polar am should fail to complement S13 mutants in cistrons II and IV (which correspond to $\emptyset X$ IV and VI). If this is found to be the case, it will eliminate a few links from the chain of arguments leading to the conclusion that the two observed polar effects act in opposite direction.
- 3) Perhaps $\emptyset X$ and S13 are translated in different directions, or the ordering of the homologous cistrons is actually not the same in the two phages so that it does not make sense to compare the directions of translation. The observed degree of complementation and recombination between the two phages makes it seem very unlikely that the structure and function of the two genomes is not homologous.

4) Perhaps one of these effects does not result from true polar mutations. It seems possible, for example, that an apparent polar effect could result from interaction of a protein fragment resulting from an am mutation with the product of another cistron, producing an inactive complex. Misleading polar effects unrelated to the mechanism of translation have been observed in the tryptophan synthetase system of E. coli (124). It should be noted that the products of the $\emptyset X$ cistrons homologous to S13 IIIa and IIIb (II and III) are both components of the spikes which protrude from the $\emptyset X$ coat (see Part 3.3).

The S13 polar effect apparently does not exhibit the phenomenon of site specificity usually associated with true polarity. The effect described here, on the other hand, is observed only for am mutants at a particular site within cistron IV. This fact, plus the physiological observations which show that the $\emptyset X$ polar mutant is blocked in the production of cistron VI protein, strongly suggests classical polarity operating at the translational level.

These results indicate that the $\emptyset X$ genome is translated in the direction from IV toward VI (at least locally). This same orientation should then correspond to the 5' to 3' polarity of the mRNA and of the viral DNA strand.

TABLE 29

COMPLEMENTATION OF A POLAR am MUTANT

MUTANT	SELF	am 18 (SELF)	YIELD x am 18
<u>am</u> 3 (I)	<u>0.014</u>	<u>0.010</u>	1.50
<u>ts</u> 4 (II)	<u>0.004</u>	<u>0.003</u>	0.42
<u>am</u> 9 (III)	<u><0.001</u>	<u>0.010</u>	0.11
<u>am</u> 16 (IV)	<u>0.003</u>	<u>0.003</u>	<u>0.005</u>
<u>am</u> 33 (VI)	<u>0.015</u>	<u>0.003</u>	<u>0.005</u>
<u>ts</u> 41D (VII)	<u>0.002</u>	<u><0.001</u>	0.030

Complementation yields are expressed as a fraction of the wt control yield in the same experiment. These data are derived from several experiments, so the yield obtained following infection with am 18 alone is given for each test, as well as the yield obtained by selfing the complementing mutant. Underlined values indicate the absence of significant complementation.

TABLE 30

COMPLEMENTATION OF POLAR am MUTANTS AND THEIR ts REVERTANTS

Complementation yields are expressed as a fraction of the wt control yield in this experiment. Underlined values indicate the absence of significant complementation. The test am 18 x ts 4 was included in this experiment to provide a control in which a polar am mutant does exhibit complementation.

A slight increase in the yield of the ts revertants ts 116 and 124 (3 to 4 fold) results from mixed infection with am 35. It is suggested that this results from a slight leakiness of am 35 with respect to synthesis of cistron IV product, but that this leakiness is not apparent from the selfing yield of am 35 since phage production is blocked also by the absence of cistron VI product.

TABLE 30

COMPLEMENTATION OF POLAR am MUTANTS AND THEIR ts REVERTANTS

	<u>am</u> 16(IV)	<u>am</u> 33(VI)	<u>am</u> 18(IV-VI)	<u>am</u> 35(IV-VI)	<u>ts</u> 116(IV)	<u>ts</u> 124(IV)	<u>ts</u> 4(II)
<u>am</u> 16(IV)	<u>0.003</u>	0.13	<u>0.005</u>	<u>0.006</u>	<u>0.003</u>	<u>0.006</u>	
<u>am</u> 33(VI)		<u>0.015</u>	<u>0.005</u>	<u>0.003</u>	0.16	0.13	
<u>am</u> 18(IV-VI)			<u>0.003</u>	<u>0.007</u>	<u>0.004</u>	<u>0.009</u>	0.42
<u>am</u> 35(IV-VI)				<u>0.002</u>	<u>0.021</u>	<u>0.030</u>	
<u>ts</u> 116(IV)					<u>0.008</u>	<u>0.014</u>	
<u>ts</u> 124(IV)						<u>0.008</u>	
<u>ts</u> 4(II)							0.004

TABLE 31

POSSIBLE SINGLE BASE CHANGES FROM UAG (AMBER TRIPLET)

Triplet	Amino Acid	Amber Suppressor	Mutation
CAG	Glutamine	su_2^+	TS
AAG	Lysine	(su_5^+)	TV
GAG	Glutamic		TV
UUG	Leucine		TV
UCG	Serine	su_1^+	TV
UGG	Tryptophan		TS
UAU	Tyrosine	su_3^+	TV
UAC	Tyrosine	su_3^+	TV
UAA	None (Ochre)	su^-	TS

This table lists all possible single base changes from the am triplet UAG. The amino acid coded by each triplet (125), the amber suppressor gene which causes insertion of this amino acid in response to UAG (106,122) and the nature of the mutation from UAG (TS = transition, TV = transversion) are also listed.

TABLE 32

SUPPRESSION OF POLAR am MUTANTS AT 30°C AND 40°C

ΦX	su ⁻		su ₁ ⁺		su ₂ ⁺		su ₃ ⁺		su ₅ ⁺	
	30	40	30	40	30	40	30	40	30	40
wt	5.46	1.55	1.45	0.18	1.00	0.22	2.55	0.25	0.22	0.022
<u>am</u> 18	0.021	0.057	1.63	1.00	1.00	1.00	0.55	0.33	0.072	0.036
<u>am</u> 35	0.018	0.034	1.69	0.44	1.00	0.58	0.55	0.22	0.13	0.083

Spheroplasts of Garen's suppressor strains were infected at 30°C and 40°C with DNA from ΦX wt, am 18, and am 35. The relative titers of phage produced, normalized to the phage titer produced in su₂⁺ spheroplasts at 30°C, are tabulated.

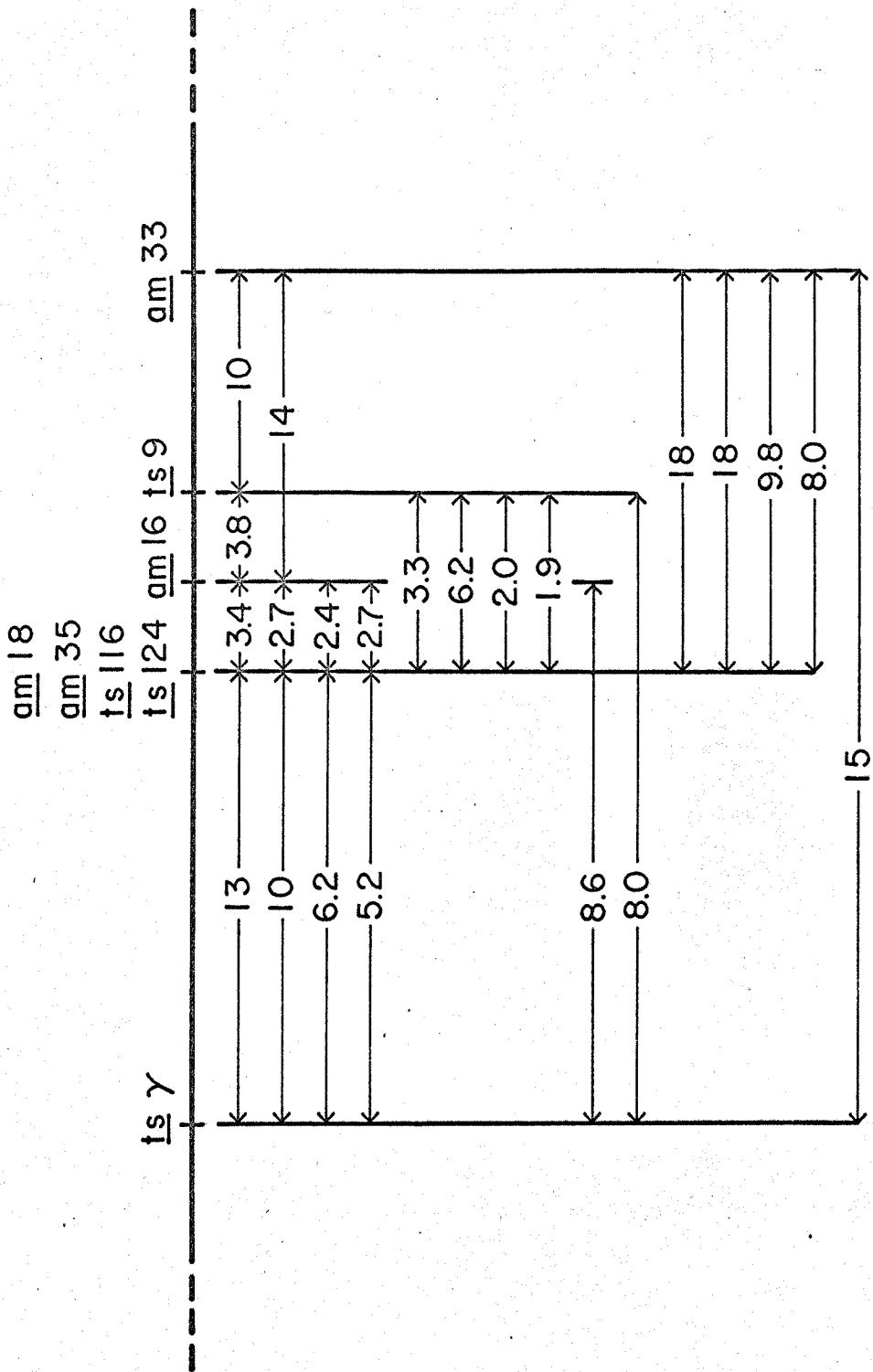
Figure 6. Recombination of Mutants at the Polar Site.

The frequencies of wt recombinants are listed in units of 10^{-4} recombinants per progeny phage. The four mutants at the polar site are not resolvable by recombination. Recombination values of mutants at the polar site are listed in the order, from top to bottom, am 18, am 35, ts 116, ts 124. Note that am and ts mutants at the polar site give recombination frequencies which differ by about a factor of two, with all other markers.

VII

IV

III



3. PHYSIOLOGICAL STUDIES OF ϕ X MUTANTS

3.0 INTRODUCTION

The purely genetic studies presented in Part 2 of this thesis demonstrate that the $\emptyset X$ genome codes for at least seven proteins which are essential for the normal life cycle of the virus. In order to investigate the nature of the functions performed by these proteins, a different approach is necessary.

When a conditional lethal mutant infects a cell under restrictive conditions, the resulting "abortive infection" is blocked at a step which requires a functional product of the gene carrying the mutation in order to proceed. There are a large number of experimentally definable stages in the $\emptyset X$ infective process. (See, for example, the list of $\emptyset X$ functions listed in Table 1 of the General Introduction.) A number of $\emptyset X$ conditional lethals have been assayed for their ability to perform certain key steps in the infection: RF replication, SS synthesis, serum blocking protein (SBP) synthesis, phage maturation, and lysis of the host. These studies have allowed us to identify the phage cistrons which are required for performance of each function.

In the identification and study of the $\emptyset X$ cistrons which code for structural components of the phage coat, it has proven useful to investigate alterations in the physical properties of the mature phage particles produced by mutations. For these studies we have used phage produced under permissive conditions, and have measured such properties as thermal stability and electrophoretic mobility. A change in either of these parameters produced by a mutation provides evidence that the mutant gene codes for a protein which is a component of the phage particle.

The following sections will present the results of these physiological studies of the $\emptyset X$ mutants along with our interpretation of the functions of the $\emptyset X$ gene products.

In addition to the results presented here, a number of people have contributed to our understanding of the defects present in $\emptyset X$ conditional lethal mutants. S.G. Krane and L.L. Greenlee have studied the production of SBP in cells infected by $\emptyset X$ ts mutants. C.E. Dowell, B.H. Lindqvist, A.J. Levine, and R. Knippers have all made studies of DNA synthesis in mutant infected cells. M.H. Edgell and I have collaborated in studies of the cistrons which code for components of the phage coat. These results will be discussed in relation to those to be presented here.

3.1 MATERIALS AND METHODS

Media, phage and bacterial strains, and general biological procedures are described either in Part 2.1 of this thesis or in the publications reproduced in Parts 3.2 and 3.3.

3.1.1 Large scale phage preparations

The following procedure was used to prepare large quantities of purified phage for work with the phage coat proteins. 200 liters of KC broth, in a 300 liter fermentor, were inoculated with 2-3 l of strain C at a concentration of approximately 5×10^8 cells/ml. 50 ml of a 1:200 dilution of antifoam (Dow Chemical) was also added. The cells were grown to 2×10^8 cells/ml at 37°C with vigorous aeration ($11 \text{ ft}^3/\text{min}$). Enough of the lysis-defective mutant $\emptyset\text{X am 3}$ was added to give a multiplicity of 5. The infected cells were incubated with aeration for another 2 to 3 hr. and then chilled to 10°C . The infected cells were then collected using a Sharples continuous flow separator. The cells (which contain essentially all the phage produced) were then resuspended in 500 ml of 0.05 M sodium tetraborate solution by means of a Servall Omnimixer, and lysed by treatment with lysozyme and EDTA. Egg white lysozyme was added to give a concentration of 0.5 g/l, and EDTA to give a concentration of 0.4% (these were added in solution, in a total of 250 ml of 0.05 M sodium tetraborate). The cells were allowed to stand 30 to 60 min at room temperature. The lysate was then extremely viscous (so that the use of scissors was necessary for sampling). The material was sonicated in 50-100 ml aliquots using a Branson Sonifier,

model S 75. Approximately 2 to 3 min at full power (given in bursts of 30-45 sec, followed by pouring the lysate in order to judge the viscosity and homogeneity of the material) was sufficient to reduce the viscosity to that "strings" did not form while pouring. Particulate debris was removed by centrifugation at 9,000 rpm in the Servall GSA rotor. The supernatant was collected. Residual phage were eluted from the pellet by resuspension in 200 ml of 0.05 M sodium tetraborate followed by resonication. Debris was again removed by centrifugation and the supernatants combined. The volume at this point was usually about 600 ml. Solid CsCl (Trona Chemicals - American Potash & Chemical Corp., Los Angeles, California) was added to give the lysate a density of 1.36 to 1.38 g/ml. The lysate was then centrifuged at 25,000 rpm for 18 to 24 hr in a Spinco 30 rotor. An opalescent band of phage was then visible in the lower portion of the centrifuge tubes. The band was removed by inserting the needle of a syringe through the wall of the tube into the phage band and withdrawing the phage. This material was then directly rebanded (it is already in CsCl at the proper density) in a 40 rotor, and phage bands again collected with a syringe.

At this point the main contaminants present are low molecular weight materials which do not band sharply in CsCl. These are removed by passage through a column of G200 Sephadex, or by sedimentation in a sucrose gradient (following removal of CsCl by dialysis).

The yield from 200 l has usually been about 0.5 g of $\emptyset X$ am 3 phage particles. The phage are approximately 30% active. The fraction of revertants from am 3 is usually about 10^{-5} . The spectral characteristics

are those of purified phage (3), and electrophoretic analysis has shown the preparations to be quite free of contaminating protein. One very faint E. coli protein band can occasionally be seen when ten times the normal quantity of phage are electrophoresed. The amount of contamination by E. coli protein is probably well below 1%.

Essentially the same method has been applied to the purification of the coat mutants H_aH_b and ts 79. The double mutants am 3 H_aH_b and am 3 ts 79 were constructed and used for infection as described, except that in the case of ts 79 the infection was carried out at 30°*C*.

3.1.2 Purification of ts 4 defective particles

The double mutant am 3 ts 4 was constructed in order to make use of a procedure similar to the one described above, but modified in the following ways. Two 10 l cultures were infected, one at 40°*C* for 2 hr, and a control culture at 30° for 4 hr. Growth was stopped by pouring the infected cultures into buckets of crushed ice, in order to avoid any appreciable time near 30°*C* for the defective particle culture. The cells were collected by centrifugation in the cold, and lysed in the cold. The lysates were banded twice in CsCl then dialysed and sedimented in a sucrose gradient. The resulting yields were low (about 1 mg from each culture) compared to those obtained with am 3. The infectivity of the particles produced at 40°*C* was 2×10^{-3} of the infectivity of phage produced at 30°*C*.

3.1.3 Serum blocking power (SBP) measurements.

SBP measurements were performed as described by Krane (46).

3.1.4 Procedure for spike removal

One volume of 8 M urea was added to one volume of phage in 0.05 M sodium tetraborate. This solution was incubated at 30°C for three hours and then chilled. The solution was then dialysed against 100 volumes of cold 0.05 M sodium tetraborate for 2 hr. The material was then sedimented in a 5 to 20% sucrose gradient if a separation of spike protein and capsids was desired.

3.1.5 Electron microscopy

Grids were prepared with a support film of 3% parlodion. In some cases a carbon film was deposited over the parlodion. The material to be examined (phage or subviral particles) was diluted to about 10^{12} particles/ml in 0.05 M sodium tetraborate solution and applied to the grid surface for approximately 1 min. The grid was briefly washed with 0.15 ammonium acetate and then floated on a 1% solution of uranyl acetate for 1 min. Excess stain was removed with a filter paper and the grids were allowed to air dry. The micrographs were taken with a 60 or 80 KV beam on a Phillips model 200 electron microscope.

3.1.6 Thermal stability measurements

The following procedure was used to measure the rate of heat inactivation of $\emptyset X$ mutants. Each mutant to be tested was diluted to 1×10^6 infective particles/ml in 0.025 M Tris buffer pH 8.1. The host range mutant hl-3 (which has a rate of heat inactivation identical to the wt) was added to give an approximately equal concentration. This

mixture was sampled to determine the ratio of the two genotypes before heating, and then heated at 60° C. Samples were removed and diluted for assay at room temperature after 10 and 30 min. of heating. All samples were then assayed on a mixed indicator which gives clear plaques with the host range mutant and turbid plaques with the am or ts mutant being tested. A mixture of 5 parts C to 1 part C₁ was used for ts mutants, and 5 parts CR to 1 part C₁ was used for tests of am mutants. All plates were incubated at 30° C. The surviving fraction of each genotype after 10 and 30 min. of heating was determined, and the rate of heat inactivation was calculated (assuming a 1 hit process). The ratio of the rate constant for the mutant being tested to that observed for h1-3 in the same tube was used as a measure of relative heat stability.

3.1.7 Gel electrophoresis of phage proteins

Whole phage or subviral particles were disrupted for electrophoresis by mixing the sample with urea to give a concentration of 9 M. Mercaptoethanol was added to a final concentration of 5 to 10%. The sample was then left at 30° C for at least 3 hr. 0.05 to 0.10 ml of this material was then applied to a 4% acrylamide gel which contained 9 M urea.

pH 3.8 gels contained 4 g acrylamide, 0.11 g N,N'-methylenebisacrylamide (BIS), 54 g urea, 1.2 ml 5 N KOH, 26.6 ml acetic acid (glacial), 0.57 ml N,N,N',N'-tetramethylethylenediamine (TEMED), plus enough water to give 100 ml of solution. The electrode buffer for use with these gels contained 28.1 g glycine and 3.06 ml acetic acid per 1.

The gel was polymerized by adding 7 mg of ammonium persulfate per ml of solution.

pH 9.5 gels contained 4 g acrylamide, 0.11 g BIS, 54 g urea, 1 ml 6 N HCl, 4 g Tris (base), 0.03 ml TEMED, plus enough water to give 100 ml of solution. This solution is kept frozen until just prior to use. The gel was polymerized by addition of 2 mg of ammonium persulfate per ml of solution. The electrode buffer for this gel contained 3 g Tris and 14.4 g glycine per 1.

Samples were electrophoresed at 4 ma in the pH 3.8 system and at 1 ma in the pH 9.5 system. The gels were stained with 0.25% coomassie brilliant blue R250 in 1 part acetic acid; 5 parts methanol; 5 parts water. The gels were destained electrophoretically in 15% acetic acid.

3.1.8 Radioactive labeling and analysis of ϕ X DNA synthesis

Host DNA synthesis was blocked with mitomycin C (using an HCR⁻ host), newly synthesized DNA was labeled by incorporation of tritiated thymidine, DNA was extracted and analysed by zone sedimentation in a preformed CsCl gradient at neutral pH by the methods described by Lindqvist and Sinsheimer (67,109).

3.1.9 $^{32}P^{15}ND$ ϕ X am 3

Preparations of this material were the generous gifts of J.E. Newbold.

3.2 A LYSIS GENE - CISTRON I

ϕX induced lysis of the host cell is the first phage induced function to be discussed here for two reasons: 1) Mutants in one of the ϕX cistrons (cistron I) have an especially clearcut and specific effect on the lysis function. Lysis is blocked although all the steps leading up to and including phage maturation proceed normally. The results with mutants in some other cistrons, to be discussed later, are more difficult to interpret. 2) Mutants of this type have proved technically very useful in the study of other phage functions. In the studies of mutants in other cistrons frequent use has been made of double mutants which contain an am mutation in cistron I and another mutation, usually ts, in the cistron under study. When lysis is blocked in this way, the time period available for study is greatly increased, and large pools of the intermediates produced by the mutant accumulate.

3.2.1 Lysis in ϕX wt infection.

Because of the small genetic content of ϕX it was not clear, a priori, that the lytic process was under the direction of specific ϕX genes. In fact, it has been suggested that ϕX release from an infected cell precedes the lytic event (73).

We have published an experiment which was designed to show that the host cell lysis observed in ϕX -infected cultures is actually the phage's normal mechanism for escape from the infected cell. This publication is reproduced on the following three pages.

Denhardt and Sinsheimer (69) have obtained further evidence that the release of ϕX from infected cells is accompanied by cellular dissolution.

LETTERS TO THE EDITOR

Kinetics of Bacteriophage Release by Single Cells of ϕ X174-infected *E. coli*

The mechanism of release of ϕ X174 by infected *E. coli* is of interest since its understanding must result in the discovery of either: (1) a phage-specific lytic enzyme (Streisinger, Mukai, Dreyer, Miller & Horiuchi, 1961; Jacob & Fuerst, 1958) which would be the first example of a ϕ X-specific enzyme; or (2) a novel method of phage release. Recently some unsuccessful attempts to detect a lytic enzyme in the mature phage particle and in infected cells have been reported (Fujimura & Kaesberg, 1962; Eigner, Stouthamer, Van der Sluys & Cohen, 1963). It has been suggested (Eigner *et al.*, 1963) that release of ϕ X precedes cellular dissolution. Denhardt (1963, in preparation) has shown that even when the infection process is synchronized by infection in the presence of 0.003 M-KCN or by infection of starved cells phage release occurs over a period of time longer than the minimum latent period. These results suggested the possibility that ϕ X might be released slowly from single infected complexes, as is the case with certain animal viruses (see, for example, Dulbecco & Vogt, 1954), rather than in bursts occurring at the time of lysis.

We have developed a method for observing phage release from single infected cells. Such experiments show that essentially all the mature phage is released by a single complex in a burst occupying less than 30 seconds. The spread in time of phage release by a mass culture is due to a dispersion in the times of single bursts. Reinvestigation of the relation between phage release and lysis indicates that the burst of phage release and the lytic event probably coincide.

Synchronization of infection is obtained by infection of starved *E. coli* C (BTCC no. 122) with purified ϕ X174 at a multiplicity of 0.1 (Denhardt, 1963, in preparation). The following operations are performed in a room maintained at 37°C. Growth is initiated by dilution into KC broth (Sinsheimer, Starman, Nagler & Guthrie, 1962). One ml. of an appropriate dilution in KC broth is placed on a 25 mm type HA Millipore filter (Millipore Filter Corp., Bedford, Mass.). This filter has a pore size of 0.45 μ . It passes no bacteria as measured by colony formers, but quantitatively passes ϕ X174 with about 90% of the phage recovered in the initial filtration and the remaining 10% recoverable by repeated washing of the filter. The infected cell sample is washed a few times with 1 ml. portions of KC broth to remove any unadsorbed phage. Starting before the end of the latent period, washes of KC broth are added and pumped through the filter at regular time intervals and collected in individual tubes. Each wash consists of two 1 ml. portions. Time intervals between the initiation of each wash are 30 seconds or 60 seconds. After completion of one wash the first 1 ml. of broth for the next wash is placed on the filter to ensure that the sample does not dry out between washes. The washings collected in this way each contain the phage released during the preceding time interval. If the sample contains a large number of infected cells, then titrations of the successive washes yield a direct

measure of the time derivative of the growth curve. If the sample contains only one or a few infected cells, release kinetics for the single cells may be obtained by plating the entire contents of each 2 ml. wash (using 2.5 ml. of 1.5% top agar).

Figure 1, curve a, shows the results of such an experiment in which the sample contains 1.6×10^3 infected cells. A much clearer picture of the time course of phage release is obtained than is possible by differentiation of conventional growth curves

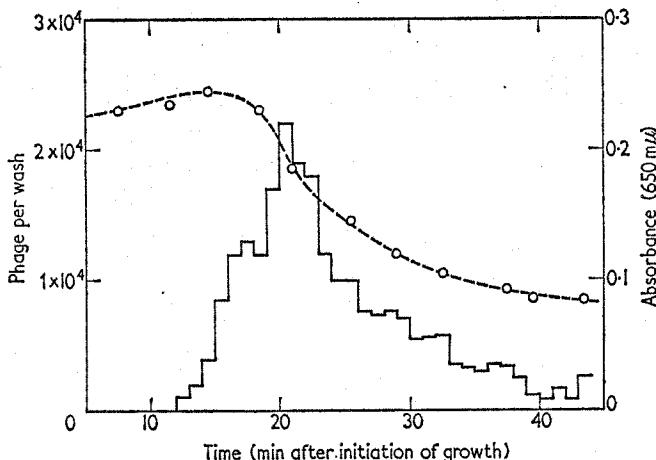


FIG. 1. Kinetics of phage release and lysis in mass infections by ϕ X174: — experiment a, phage release; - - - experiment b, absorbance at 650 m μ .

(Adams & Wassermann, 1956). Maximal rate of phage release occurs during the twenty-first minute after initiation of growth and 90% of the phage is released by about 35 minutes. A growth curve obtained by integrating these data is in good agreement with curves from conventional one-step growth experiments. The burst size obtained by dividing total phage released during the course of the experiment by the number of infected cells is 1.4×10^2 . The burst size of the same infection, measured on a fraction transferred to a separate growth tube and then assayed at the end of the experiment, is 1.8×10^2 . These results indicate that phage growth and release under the conditions of the filtration experiment are normal.

Figure 2 shows the results of two separate experiments in which the expected number of infected cells in each sample is 3. The phage are released in discrete bursts shorter than the intervals between washes, which is 30 seconds for experiment a and 60 seconds for experiment b. A small amount of trailing material follows the peaks and is the result of the kinetics of washing out of the filter apparatus. Such trailing shows that the burst occurs on the filter and that the infected cell does not pass through the filter and then burst in the collection tube.

Since there is no background of infected cells, it is possible to detect very early bursts. These are few and appear to be small bursts. For example, in the experiment shown in Fig. 1, curve a, 10 phage were released during the eleventh minute. Apparently 1 of the 1.6×10^3 infected cells burst at this time. Aside from these very early bursts, no correlation between time and size of individual bursts has been observed, but such a relation might be revealed by more extensive experiments. The reason for the spread in lysis times of individual cells is not known. It might be due either to some inherent randomness in the ϕ X lytic mechanism or to inadequate

synchronization. If the latter is true then the method described here allows the selection of a sample of phage produced synchronously.

Lysis may be followed by measurement of the absorbance at 650 m μ . Cells may be infected at a multiplicity of 5 so that lysis occurs after a single cycle of infection.

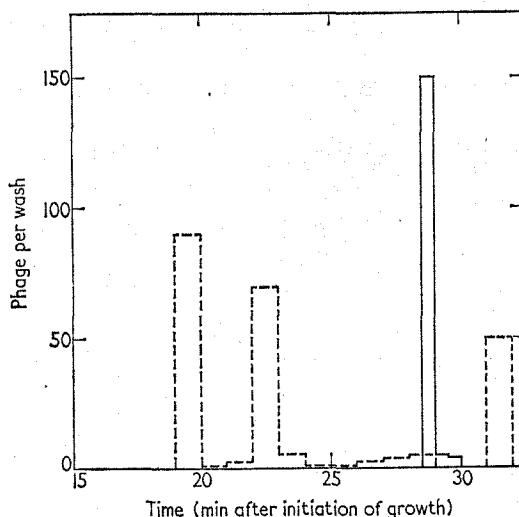


FIG. 2. Kinetics of phage release by single ϕ X174-infected cells: — experiment a; - - - experiment b.

Fig. 1, curve b, shows such an absorbance *versus* time curve, for comparison with the time course of phage release. In this experiment the infection had not been synchronized, but under these conditions adsorption is complete within 2 minutes (Sinsheimer *et al.*, 1962) and the latent period is not significantly affected. Maximum rate of lysis is seen to occur simultaneously with maximal phage release.

In a medium which favors rapid readsorption of released phage, the instantaneous phage titer will be proportional to the rate of phage release. It seems likely to us that the conclusion of Eigner *et al.* (1963) is a result of difficulties in the interpretation of a complex experiment which requires several cycles of growth to produce lysis, carried out in a medium which favors readsoption.

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3.2.2 ØX Mutants with altered lytic properties

The publication reproduced on the following pages describes the screening of ts and am mutants of ØX for altered ability to lyse the host under restrictive conditions of infection. The paper also presents detailed studies of ØX am mutants in one complementation group (cistron I), which do not produce cell lysis, but otherwise replicate normally in the restrictive host. Mutants in cistrons II and VI show reduced or delayed lysis, in addition to some defect which prevents phage maturation. Further evidence concerning the physiological basis of the altered lytic response in these cases will be presented following the publication.

J. Mol. Biol. (1966) 18, 429-447.

The Process of Infection with Bacteriophage ϕ X174

X. Mutations in a ϕ X Lysis Gene†

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The ability of conditional lethal mutants of phage ϕ X174 to induce host cell lysis during infection under restrictive conditions has been studied. We have found amber (*am*) and temperature-sensitive (*ts*) mutants which present a variety of alterations in the normal lytic process. In particular, there is a class of *am* mutants which do not produce cell lysis but otherwise replicate normally in the restrictive host. These mutants constitute a single complementation group. The existence of these mutants implicates a phage-coded protein in the lytic process. This protein is not an essential structural component of the phage, since normal phage particles are produced in the absence of lysis.

The growth of a particular mutant of this type, ϕ X *am3*, in the restrictive host, has been studied in detail. Phage maturation starts at approximately the same time and at the same rate as in a ϕ X wild type (*wt*) infection. Maturation of ϕ X *am3*, however, continues at this rate for about two hours. Burst sizes of 2 to 3×10^3 phage per cell are obtained. This is about ten times the normal ϕ X *wt* phage yield. Only 1 to 2% of the phage are released spontaneously. The rest remain intracellular until released by artificial lysis. The turbidity of an infected culture continues to rise after infection. This effect primarily represents elongation of the infected cells, without increase in cell number. Cells infected by ϕ X *am3* are not able to form a colony and do not undergo more than a single division.

We have used ϕ X *am3* to study temporal exclusion in ϕ X infection under conditions that are not complicated by lysis of the infected cells. ϕ X *wt* is excluded by prior infection with ϕ X *am3*. The superinfecting phage is blocked at a stage prior to synthesis of the molecule of the parental replicative form; its DNA remains an intact (infective) single strand.

We have also used ϕ X *am3* to study the effect of addition of chloramphenicol at various times during infection upon subsequent viral DNA synthesis. If chloramphenicol is added before initiation of progeny single-stranded DNA synthesis, the normal transition from replication of replicative form to single-stranded DNA formation does not occur; replication of the replicative form continues for at least 40 minutes. If chloramphenicol is added after the initiation of single-stranded DNA synthesis, such synthesis continues, at a lesser rate than normal, for 20 to 25 minutes; synthesis of replicative form is resumed for at least 40 to 50 minutes and its concentration may reach a level several-fold that normally observed.

1. Introduction

In a normal ϕ X infection, the host cell lyses about 20 minutes after infection and releases a burst of mature progeny phage (Hutchison & Sinsheimer, 1963; Markert & Zillig, 1965; Denhardt & Sinsheimer, 1965a). Because of the small genetic content of this phage, several investigators have tried to determine whether specific phage

† The previous paper in this series was Dowell & Sinsheimer (1966).

gene products are involved in the lytic process (as is the case with large phages: Streisinger, Mukai, Dreyer, Miller & Horiuchi, 1961; Jacob & Fuerst, 1958), or whether some novel process, as for example induced autolysis, is used in order to conserve genetic information. Some unsuccessful attempts to detect a lytic enzyme in the mature phage particle and in infected cells have been reported (Fujimura & Kaesberg, 1962; Eigner, Stouthamer, Van der Sluys & Cohen, 1963; Markert & Zillig, 1965).

Markert & Zillig (1965) used metabolic inhibitors to study ϕ X-induced host-cell lysis. They showed that synthesis of both DNA and protein following infection was essential for normal lysis, whereas formation of mature progeny phage or even mature progeny DNA was unnecessary. Most interestingly, they found that protein synthesis was required at a time later than the onset of coat protein production in order for lysis to occur. It still remained to be determined whether this synthesis was phage- or host-specific. Thirteen ϕ X temperature-sensitive (*ts*) mutants studied by Markert & Zillig (1965) all lysed normally at the restrictive temperature. Since no mutants showing defective lysis were found, the question of phage control of lysis remained open.

Denhardt & Sinsheimer (1965a) have described a mutant of ϕ X with a longer mean latent period than wild type. This mutant (ϕ X ρ^-) also shows reduced lysis when grown in concentrated culture, suggesting some degree of phage genetic control over the lytic process. However, the rather complicated behavior of this mutant, in particular the concentration dependence of its effect, makes it difficult to study. It is not clear whether the mutation directly affects a phage specific lytic factor or whether some more complex interaction analogous to lysis inhibition in T-even phages is involved.

We have found both amber (*am*) and *ts* mutations in ϕ X which directly affect the lytic process. We have studied in detail one of a group of *am* mutants which reproduces normally in the restrictive host but does not lyse the cells. This mutant has been used to study both temporal exclusion and viral DNA synthesis in ϕ X infection, in the absence of complications introduced by lysis.

2. Materials and Methods

(a) Media

KC broth contains 10 g Bacto Tryptone (Difco), 5 g KCl and 0.5 ml. 1 M-CaCl₂/l. of distilled water.

T3A medium. For 1 liter: dissolve 0.5 g NaCl, 8.0 g KCl, 1.1 g NH₄Cl, 12.1 g Trizma Base (Sigma Chemical Co.), 1.0 g KH₂PO₄, 0.8 g sodium pyruvate in 250 ml. of distilled water. Dissolve 2.7 g of amino acid mixture (special 20 natural L amino acid mixture, Nutritional Biochemicals Corporation) in 500 ml. distilled water. Mix these two solutions and add 1.0 ml. of 20% MgCl₂, 6 H₂O, 0.1 ml. of 1 M-CaCl₂, 2 H₂O, 20 ml. of 0.05 g/100 ml. adenine and 1.0 ml. of 0.16 M-anhydrous Na₂SO₄. After adjustment of the pH to 7.4, the volume is brought to 1 liter and the solution is autoclaved. After autoclaving, add 1 ml. of 0.1 mg/ml. FeCl₃, 6 H₂O and 20 ml. of sterile 10% glucose.

Bottom agar contains 10 g Tryptone, 2.5 g NaCl, 2.5 g KCl, 10 g agar and 1 ml. 1 M-CaCl₂/l. of distilled water. Ten ml. of bottom agar are used per plate.

Top agar contains 10 g Tryptone, 5 g NaCl and 8 g agar /l. of distilled water.

(b) *Escherichia coli* strains

C is the usual host for ϕ X used in this laboratory, BTCC No. 122.

C₁ is a spontaneous mutant derived from C which is resistant to ϕ X wild type.

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CR is a recombinant derived from a cross between the *E. coli* strains CR34 and C416. It was described by Denhardt & Sinsheimer (1965b).

THU was described by Stern, Sekiguchi, Barner & Cohen (1964) and was kindly supplied by Dr Cohen.

(c) ϕ X174 strains

wt is the ϕ X wild type characterized by Sinsheimer (1959).

ts mutants were isolated from a nitrous acid-treated preparation of *wt*. They will form plaques on C at 30°C but not at 40°C, whereas *wt* forms plaques at both temperatures.

am mutants were also induced by nitrous acid. They were selected to form plaques on CR but not on C, at 30°C. The *wt* forms plaques on both hosts. The mutants are called *am* by analogy with T4 *am* mutants (Epstein *et al.*, 1963). A technical difficulty arose in the selection of ϕ X *am* mutants because none of the bacterial strains previously characterized as "permissive" is sensitive to ϕ X infection. Therefore the permissive strain used in the system described here, CR, is a recombinant between a permissive but ϕ X-resistant strain of *E. coli* K12, and a ϕ X sensitive, but restrictive, strain of *E. coli* C. This recombinant carries an *am* suppressor from the K12 parent and retains the ϕ X sensitivity of C.

Experiments designed to establish that ϕ X *am* mutants are true "nonsense mutants", like T4 *am* mutants, have been performed. Spheroplasts of bacterial strains isogenic except for the presence or absence of well-characterized nonsense suppressors (Weigert & Garen, 1965; Garen, Garen & Wilhelm, 1965) were exposed to DNA extracted from a number of ϕ X *am* mutants. (This experiment is feasible because the ϕ X resistance of these strains resides only in their cell walls. ϕ X *am* mutants other than lysis mutants were used, as the lysis function cannot be assayed in spheroplasts.) Successful infections were obtained with DNA's from the ϕ X *am* mutants only when the spheroplasts carried a suppressor, indicating that these mutants are true nonsense mutants.

Details of the isolation and characterization of the *ts* and *am* mutants will be presented in another paper.

γh is an abbreviation for the double mutant ϕ X *ts* γh_1 . This is a spontaneous extended host-range mutant, selected to plate on C₁ at 30°C, which was isolated from a stock of the mutant ϕ X *ts* γ . This phage is useful as a biological marker in centrifugal experiments since γh phage particles and γh -DNA are physically indistinguishable from those of *wt* in such experiments. Both γh and *wt* may be assayed selectively in a mixture of the two. In such a mixture the γh is assayed by plating on C₁ at 30°C. (10% C is always added to the plating bacteria to improve the efficiency of plating. The *wt* does not make visible plaques on this mixture.) The *wt* is assayed on C at 40°C; at this temperature γh does not make plaques.

Any of these strains is available from the authors.

(d) Preparation of genetically pure mutant phage stocks

A reference stock of the mutant is plated out under permissive conditions to give less than 50 plaques per plate. A single, young plaque is picked with a piece of sterile glass tubing and blown into 30 ml. of sensitive cells (at a concentration of 1×10^8 /ml.) growing with aeration in KC broth. All lysates are made at 30°C. The *ts* mutants are grown on C and the *am* mutants on CR. The culture usually lysed in 2 to 3 hr. In KC broth at the cell concentration used, most of the infective phage released by lysis is readsorbed to cellular debris. Therefore the debris is spun down at 10 to 15×10^3 rev./min, and the supernatant fraction is discarded. The debris is resuspended in 5 ml. 0.05 M-sodium tetraborate in order to elute the adsorbed phage. After shaking for a few hours in the cold, the debris is spun down again. The supernatant fraction is the "stock". Such stocks typically have titers of 2 to 5×10^{10} plaque-forming particles/ml. A small fraction of the phage, usually between 10^{-5} and 10^{-7} , are revertants which will plate under restrictive conditions. This procedure of stock preparation is advantageous, because the phage are very stable in the final borate suspension, and because the stock is relatively free of nutrients and so can be used to infect starved cells (Denhardt & Sinsheimer, 1965a).

(e) $^{32}P^{15}ND$ - ϕX wt

This was the gift of Michael Yarus. The lysate was produced by the method described by Sinsheimer, Starman, Nagler & Guthrie (1962), and the phage was purified in the phase system used by Hall & Sinsheimer (1963).

(f) *Biological assays*

Plating bacteria are grown in KC broth with aeration to a concentration of about 5×10^8 /ml., then stored in the cold until used.

Plaque assays are performed by the agar layer technique. We use 2.5 ml. of top agar and 0.3 ml. of plating bacteria per plate.

Dilutions of phage or infected cells for plating are made through KC broth.

Free phage are measured after killing infected cells by shaking with chloroform. This procedure inactivates plaque formation by ϕX -infected cells even at late times when intracellular phage are present. In some experiments, unadsorbed phage are removed by incubating the infected cells for 5 min with anti- ϕX serum with a K of 2 min^{-1} . Cells are then diluted at least 10^4 -fold away from the serum before assay.

Intracellular phage are measured by lysing the infected cells with lysozyme and EDTA. To 1 ml. of infected cells in KC broth at 0°C are added 0.1 ml. of lysozyme solution followed by 0.1 ml. EDTA (solutions as in Guthrie & Sinsheimer, 1963). After incubation for 30 min at 0°C, the spheroplasts are disrupted by freezing and thawing 3 times in a dry-ice-acetone bath before plating.

Infective DNA is assayed by the spheroplast procedure of Guthrie & Sinsheimer (1963), modified by incubating the infected protoplasts at 30°C for 2 hr. In a mixture of phage resulting from infection by DNA from *wt*, γh and *am3*, the *wt* and γh are assayed selectively as described above (since C and its derivative C₁ are restrictive to *am3*). Also the total infectivity of *wt* plus *am3* is assayed by plating on CR at 40°C.

(g) *Synchronization of infection*

In some experiments synchronization of infection has been achieved by starvation-block or cyanide-block as described by Denhardt & Sinsheimer (1965a), except that phage adsorption was allowed to proceed for 10 to 15 min.

(h) *Complementation test*

To test the capacity of two phage mutants to reproduce through complementation, C is mixedly infected with a multiplicity of 3 to 5 of each of the two mutants. The infection is performed at a cell concentration of 2×10^7 /ml., in KC broth containing 0.003 M-KCN, at 30°C.

For convenience in performing a large number of tests simultaneously, the cyanide-block procedure is modified as follows. Phage are diluted into KC broth containing KCN. To 0.8 ml. of phage-KCN is added 0.2 ml. of C at a concentration of 1×10^8 /ml. in KC broth. After a 35-min adsorption period (at 30°C), the unadsorbed phage are removed with anti- ϕX serum. The cells are then diluted 10^4 -fold away from KCN and serum into fresh KC broth and incubated 60 min. In tests of *ts* mutants, this incubation is carried out at 40°C. In tests involving only *am* mutants, the incubation is performed either at 30 or 40°C. The culture is then assayed for progeny phage under permissive conditions. In the context of this paper, it is important to note that phage produced in such a complementation test will be assayed only if the lysis function is successfully performed. In this modified cyanide-block procedure, little if any phage synthesis occurs during the extended adsorption period, and *wt* phage yields after dilution are normal.

In each experiment the progeny yield of the mixed infection is compared to the yields obtained in selfings of the two parents, and to a *wt* infection performed in an identical manner.

In tests involving only *am* mutants, the infected C cells are sometimes plated on CR immediately after dilution from KCN and serum. The same controls are performed as in the progeny yield measurements.

(i) *Parental RF synthesis*

The procedures for infection with $^{32}\text{P}^{15}\text{ND}$ - ϕ X *wt*, lysis and phenol extraction of the infected cells, and equilibrium sedimentation of the extracted DNA in a CsCl density-gradient, differ only in trivial ways from those described by Sinsheimer, Starman, Nagler & Guthrie (1962) and by Burton & Sinsheimer (1965).

(j) *Lysis measurement*

Lysis is observed as a decline in the turbidity of an infected culture. Cells of the appropriate strain are multiply infected (usually a multiplicity of 3 to 5 is used), so that lysis will occur after a single cycle of virus growth. In some experiments the infection is synchronized by starvation-block or cyanide-block. (In the case of cyanide, growth is initiated by centrifugation and resuspension of the infected cells.) Growth is initiated at an infected cell concentration of 5×10^7 to 1×10^8 /ml. in KC broth at the appropriate temperature. In most experiments the turbidity of infected cultures is followed by measuring the absorbance at $650 \text{ m}\mu$ in a Bausch & Lomb Spectronic 20 colorimeter. In one experiment mentioned in the text, lysis was observed visually. In both the qualitative and quantitative experiments, the experimental samples are compared to control samples which have been infected with *wt* or are uninfected.

(k) *Cell counts*

These are made in a Petroff-Hausser counting chamber. Samples are either counted immediately or are stored at 0°C for at most a few minutes before counting.

(l) *Photomicrographs*

A drop of growing bacterial culture is spread on a glass microscope slide with the edge of a second slide. After a few seconds (for drying), a drop of melted top agar is placed on the slide and a cover slip immediately pressed down on top of this. After the agar has solidified, the bacteria thus immobilized in the plane of the slide surface are photographed with a Zeiss photomicroscope.

3. Results

(a) *ts Lysis mutants*

In our first attempts to find ϕ X mutants specific for a lysis function, we investigated lysis by *ts* mutants at the restrictive temperature (40°C). In a series of experiments performed in collaboration with Richard J. Levine, we studied the kinetics of lysis induced by 79 independently isolated ϕ X *ts* mutants. The majority of these showed lysis essentially identical to *wt*. (These included the mutants ϕ X *tsy*, ϕ X *ts9* and ϕ X *ts41* studied by Dowell & Sinsheimer (1966).) Of the 79 mutants tested, 17 were *ts* either in the extent or rate of lysis.

Figure 1(a) shows the lysis kinetics of one of the most extreme of these, ϕ X *ts4*. The *wt* lyses to the same extent at 30 and 40°C , and *ts4* is obviously *ts* with respect to lysis; even at 30°C , lysis is delayed and somewhat reduced. The inhibition of lysis at the restrictive temperature is not a consequence of poor attachment or injection of DNA at 40°C , since the infections were initiated at 30°C (using the starvation-block procedure). Also, as shown previously (Dowell & Sinsheimer, 1966), progeny single-stranded DNA is made in normal amount by *ts4* at 40°C . The initial kinetics at 40°C suggest that a small fraction of the *ts4*-infected cells lyses, but the majority of infected cells continue to grow after an initial retardation. This increase in cell mass following infection by certain ϕ X *ts* mutants has not been investigated further; however, a similar situation found with some ϕ X *am* mutants is discussed below.

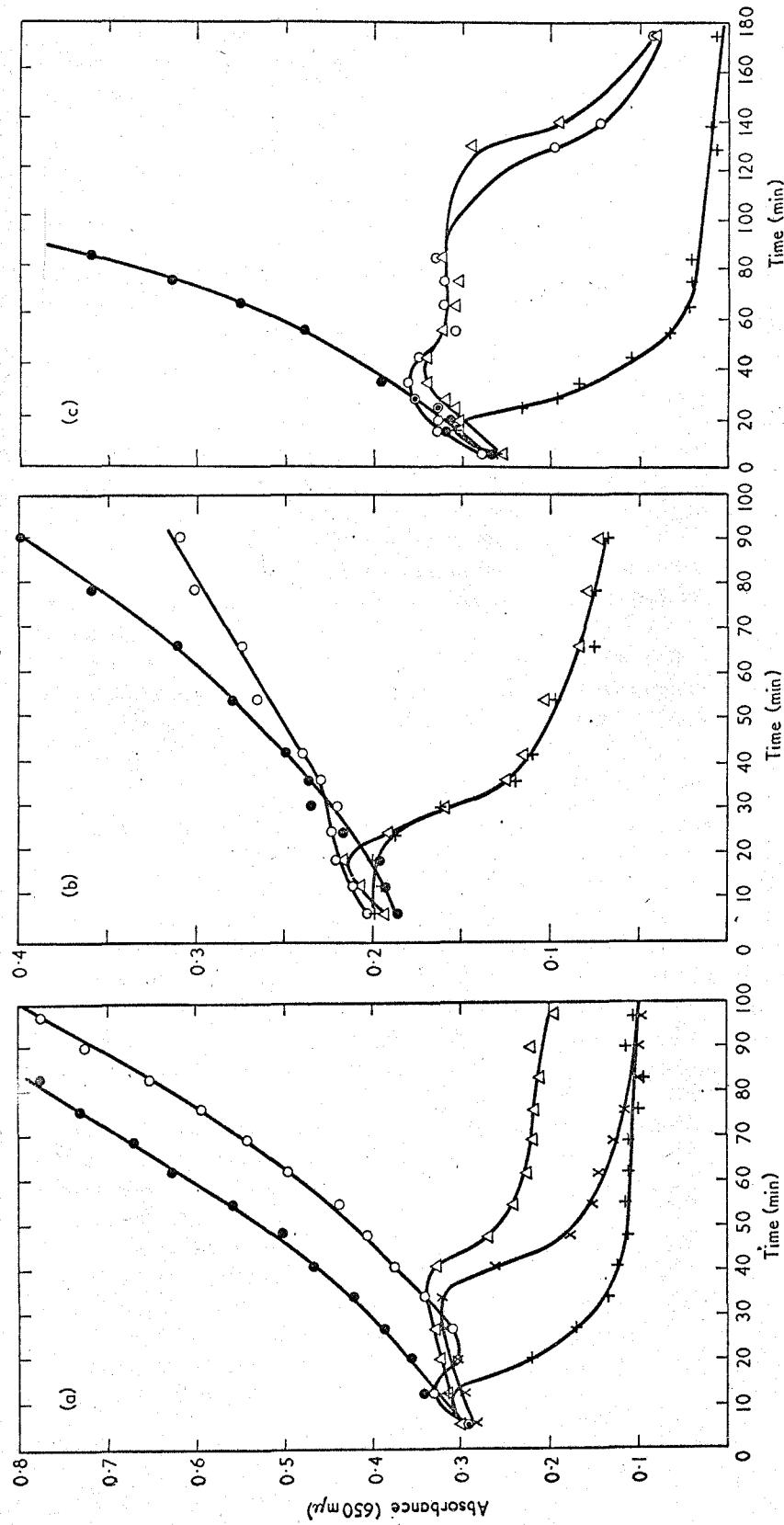


FIG. 1. Lysis induced by mutants of ϕX .

(a) Temperature-sensitive lysis induced by ϕX ts4. *E. coli* C was infected at 30°C using the starvation-block procedure. The culture was then divided and growth initiated at 30 and 40°C. The absorbance was measured as a function of time after initiation of growth, for cells infected by ϕX ts4 and ϕX wt. —○—○—, ϕX ts4, 30°C; —△—△—, ϕX ts4, 40°C; —×—×—, ϕX wt, 30°C; —●—●—, ϕX wt, 40°C.

(b) Lysis of the restrictive host, C, by ϕX am mutants. Cells were infected non-synchronously at 37°C. —○—○—, ϕX am3; —△—△—, ϕX am9; —×—×—, ϕX am18; —●—●—, ϕX am30; —△—△—, ϕX am18; —+—+—, ϕX am18; —●—●—, uninfected.

(c) Lysis of the restrictive host, C, by ϕX am mutants. Cells were infected non-synchronously at 37°C. —○—○—, ϕX am30; —△—△—, ϕX am18; —+—+—, ϕX am18; —●—●—, uninfected.

None of the 17 ϕX *ts* mutants with altered lysis properties made mature intracellular progeny phage at 40°C. This was determined by assay at 30°C after artificial lysis of the infected cells.

(b) *am lysis mutants*

We have performed similar studies of the lysis of the restrictive host *E. coli* C by ϕX *am* mutants. In a qualitative experiment, 17 *am* mutants were divided into three classes with respect to lysis and phage production in C. The infected cells were observed after 60 minutes at 30°C. We found five mutants that lysed normally but produced no mature progeny phage (class I), five which neither lysed normally nor produced progeny (class II), and seven which produced mature progeny although the infected cells did not lyse (class III). These results have been confirmed by quantitative lysis experiments with nine mutants, three of each class.

Figure 1(b) illustrates the lysis kinetics induced by one of the class III mutants, ϕX *am3*. The unsynchronized infection was performed in C at 37°C. An uninfected control, a *wt* control, and lysis induced by *am9* (class I) are shown for comparison. (Growth of the *am3* infected cells continues, although in partial analogy with the *ts4* situation it is depressed compared to *wt*.)

The class II mutants studied in quantitative lysis experiments lysed, but at later times than *wt*. Figure 1(c) shows the delayed lysis induced by two of these mutants (*am18* and *am30*).

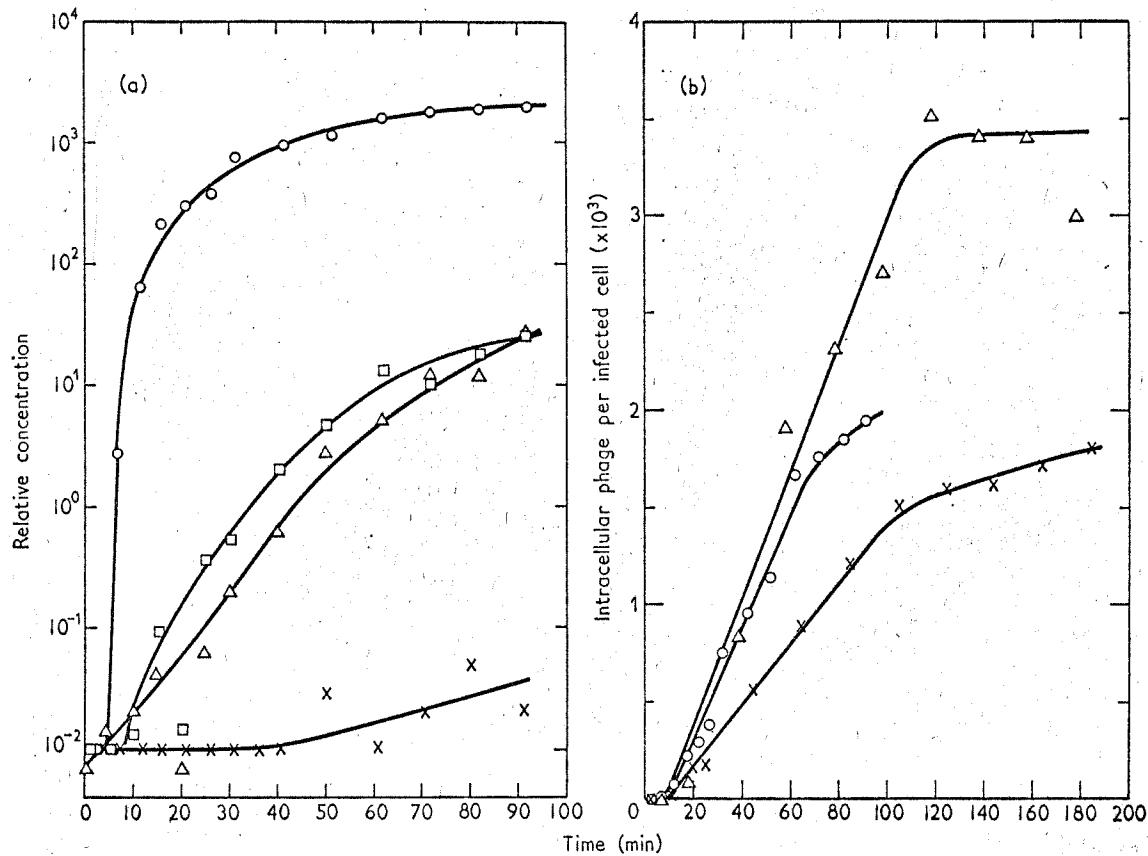
In subsequent experiments we have concentrated on class III mutations, because their effect appears to be specific for the lytic process; such mutants induce normal intracellular phage production but not lysis. Most of the work has been done using ϕX *am3*.

(c) *Growth of ϕX am3 in the restrictive host*

Figure 2 shows the time course of ϕX *am3* growth in C, the restrictive host. Figure 2(a) is the result of an experiment in which the infection was synchronized using the cyanide-block procedure. The cells were infected with a multiplicity of 2.4 plaque-forming particles per cell. Adsorption and eclipse were normal. In this experiment, one intracellular phage was produced per infected cell by about seven minutes after dilution from cyanide. By 20 minutes, the time of most rapid lysis in a ϕX *wt* infection, there were about 200 phage per cell. This is a normal phage yield for ϕX *wt* (Denhardt & Sinsheimer, 1965a). In ϕX *am3*, however, phage maturation continued, so that intracellular phage reached a level of 2×10^3 phage per cell after 90 minutes of growth (in the diluted culture). It is apparent that although large numbers of phage were matured within the cell, they were not released. Infected cells did not form plaques, as can be seen from the measurements of total infective centers at early times. At 30 minutes, only 10^{-3} of the phage produced had been released. By 90 minutes the fraction of free phage rises to 1.5×10^{-2} .

Although ϕX *am3*-infected cells continue to grow following infection, as judged by turbidity measurements (Fig. 1(b)), the infected cells do not form colonies. Further investigations of the growth of infected cells are described below.

Figure 2(b) shows linear plots of intracellular phage production in ϕX *am3*-infected C, and in infected *E. coli* THU, another restrictive host for ϕX *am* mutants. Although the final yields vary somewhat between experiments, in each case phage is matured at a nearly constant rate for approximately the first 100 minutes of infection. The phage maturation rates in the three experiments shown are 16, 29 and 33 phage per cell-minute.

FIG. 2. Growth of ϕX am3 in the restrictive host at 37°C.

(a) *E. coli* C at a concentration of 10^8 cells/ml. was infected using the cyanide-block procedure. A multiplicity of 2.4 plaque-forming ϕX am3 adsorbed and eclipsed. After a 10-min adsorption period, anti- ϕX serum was added to remove unadsorbed phage. Then the infected cells were diluted away from KCN and serum to a concentration of 2×10^3 infected cells per ml. Samples were taken at various times and plated as described under Materials and Methods. All phage samples were plated on CR. The data plotted have been normalized to the number of infected cells.

—○—○—, Intracellular phage; —□—□—, free phage; —△—△—, total infective centers; —×—×—, colony-forming bacteria.

(b) A linear plot of intracellular phage development. Data from 3 experiments are shown. The figures are normalized to the number of infected cells.

—○—○—, Data of Fig. 2(a) (multiplicity of 2.4); —×—×—, data from an experiment essentially identical but extended to later times (multiplicity of 2.2); —△—△—, production of ϕX am3 in THU. The medium was T3A supplemented with 5 μ g/ml. each of thymidine and uracil. The cells were infected non-synchronously at a concentration of 2×10^7 /ml. (multiplicity of 1).

The large burst sizes observed in single-step growth experiments described above have been confirmed in single-burst experiments. Figure 3(a) and (b) show distributions of burst size obtained under similar conditions with ϕX wt and with ϕX am3. The infection of C was synchronized with KCN. Growth was initiated by dilution into fresh KC broth. This culture was immediately distributed into single burst tubes which were incubated at 37°C for two hours before assay. The bursts of ϕX wt were plated directly, by adding a mixture of plating bacteria and melted agar to the

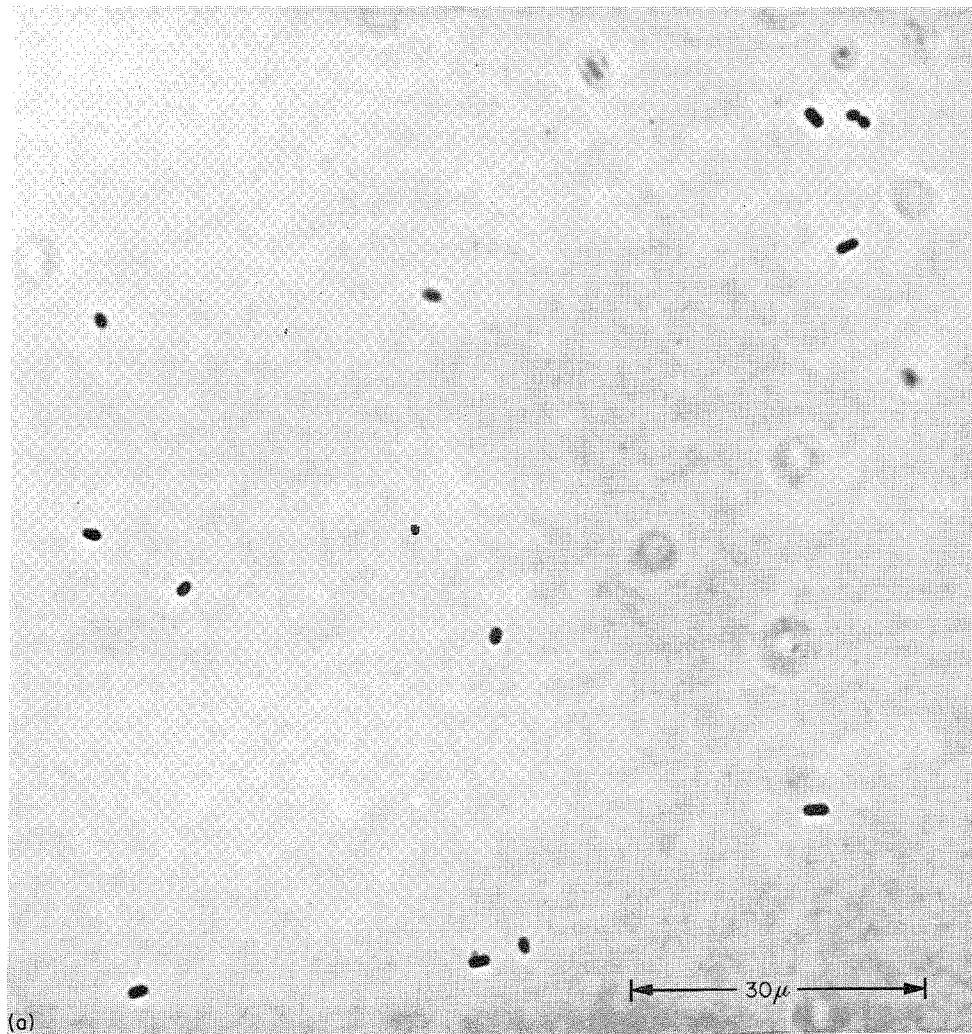


PLATE I(a)

Plate I. Effect of ϕ X *am3* infection on morphology of *E. coli* C. (a) Uninfected cells. (b) Cells infected for 2 hr by ϕ X *am3* at a multiplicity of 2.5.

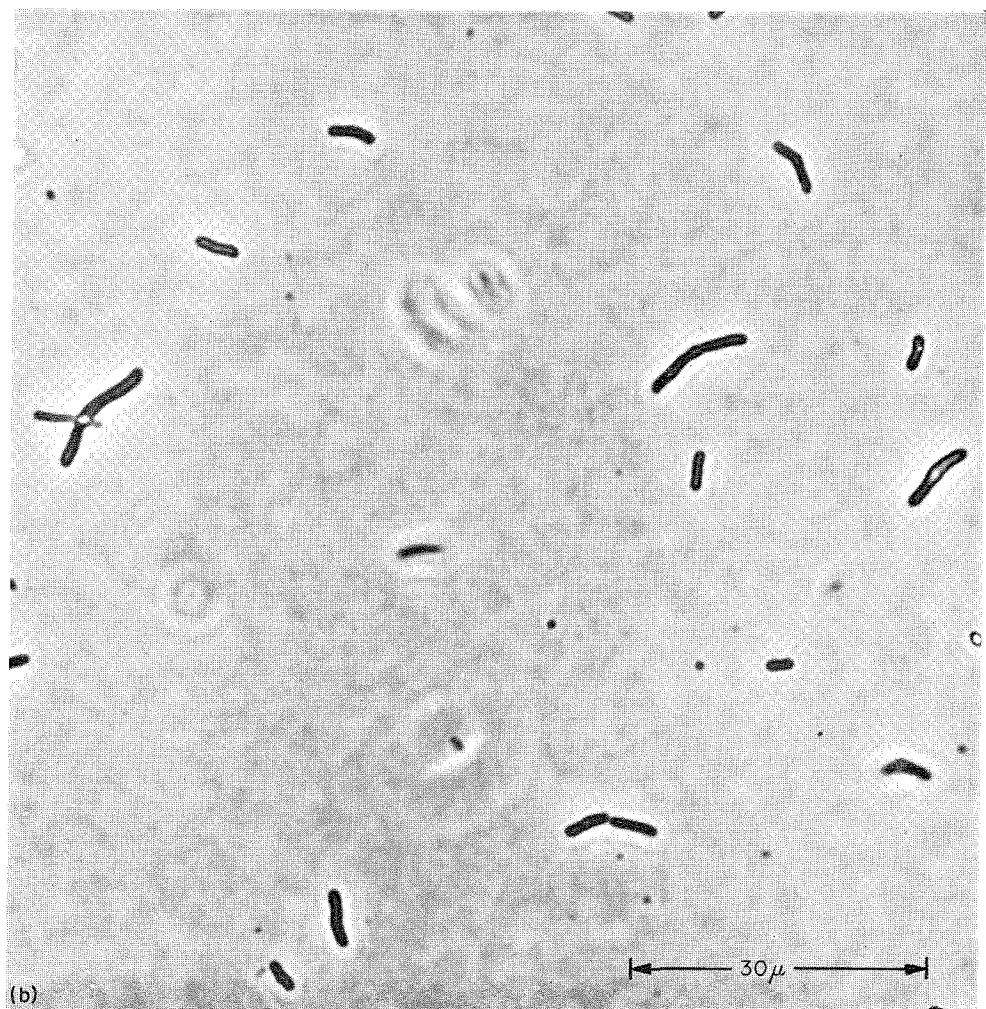


PLATE 1(b)

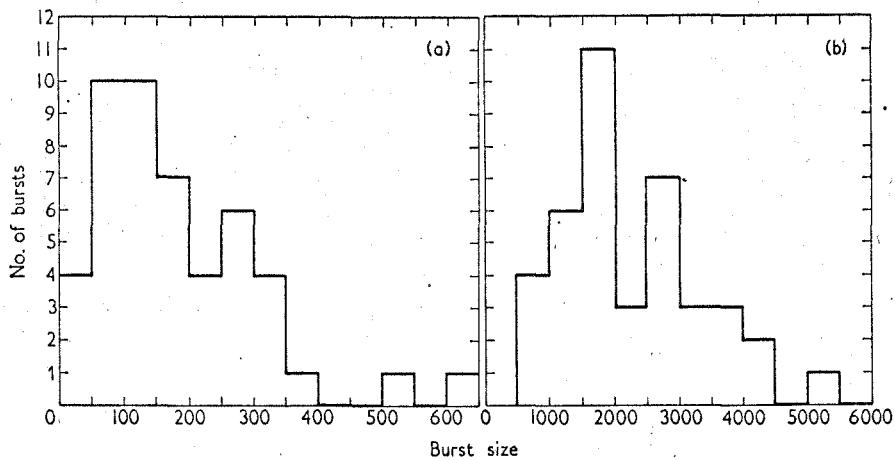


FIG. 3. Distribution of phage among single burst tubes.

(a) ϕX wt bursts (multiplicity of 3). Forty-eight bursts were observed. Thirty-one were found among 200 tubes at one dilution, and 17 among 200 tubes at a higher dilution.

(b) ϕX am3 bursts. After artificial lysis (multiplicity of 5). Forty bursts were observed. Thirty-six were found among 180 tubes at one dilution, and 4 among 60 tubes at a higher dilution.

tube and pouring onto an agar plate. The tubes containing ϕX am3-infected cells were lysed by adding lysozyme and EDTA, followed by freezing and thawing. Then a portion containing one-tenth of the burst was plated in the conventional manner.

The mean burst sizes calculated from the single burst data agree well with values obtained in mass culture. In the experiments shown in Fig. 3, mean burst size (ϕX wt) = 1.7×10^2 , and mean burst size (ϕX am3) = 2.1×10^3 . The distributions are quite broad and are roughly similar in shape.

(d) Observations on ϕX am3-infected cells

As mentioned above (Fig. 1(b)), the turbidity of a culture of C infected with ϕX am3 continues to rise following infection, although infected cells are killed as judged by colony-forming capacity. This growth of infected cells is not due to continued cell division but to enlargement of existing cells.

Figure 4 shows the result of an experiment in which identical cultures of C growing in KC broth at 37°C were infected with ϕX wt and ϕX am3 at a multiplicity of three phage per cell. A third identical culture was uninfected. Changes in turbidity followed the normal time-course (as in Fig. 1(b)). Samples were removed at various times and cell counts were made. The ϕX am3-infected cells show a slight initial increase in number of not more than a factor of two. The cell count of the ϕX wt infected culture falls upon lysis, whereas in the uninfected culture, cell division continues normally.

Although the infected cells do not divide more than once following infection, they elongate to form "snakes". Plate I shows the appearance of uninfected C, and cells infected for two hours at 37°C by ϕX am3. C at a concentration of 1×10^8 /ml. in KC broth was infected with a multiplicity of $2.5 \phi X$ am3 per cell. A control culture was uninfected. The infected cells are variable in length and sometimes irregular in shape; they may be bent or bulge in places. They range in length from a few doublets

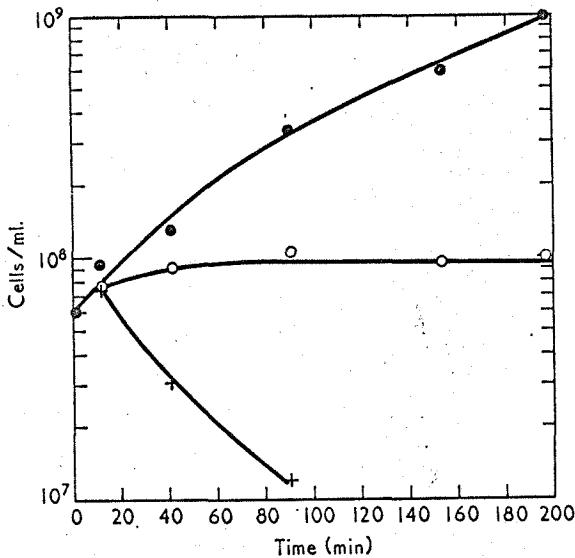


FIG. 4. Cell number as a function of time after infection by ϕX am3. C was infected non-synchronously in KC at 37°C at a multiplicity of 3. Cell concentration was determined by microscopic count. —○—○—, ϕX am3 infected C; —+—+, ϕX wt infected C; —●—●—, uninfected C.

about 2.5μ in length (probably residual uninfected cells) to "snakes" about five times this length. The uninfected cells are predominantly small doublets.

(e) Complementation among ϕX lysis mutants

The lysis function defective in the class III type amber mutants (e.g. am3) can be complemented in a mixed infection with *wt* or *ts* or other *am* (class I and II) mutants which may have either normal or inhibited lysis (Table 1). Under these conditions, mature phage are formed and released.

Seven independent isolates of *am* mutants of class III have been made. These do not complement among each other. Six provide large intracellular phage yields and do not lyse. The other, *am6*, makes only about 5 to 10% of the *wt* phage yield (although this is subnormal, it is larger than the yield of any other ϕX *am* class).

Evidently the lytic function affected in those mutants showing a defect in both phage production and lysis (class II) is distinct from the function affected in the mutants of the *am3* type.

In complementation tests between ϕX *am* mutants of class III and *am* mutants of the other classes, at least 75% of the infected cells (C) form plaques when plated on the permissive host (CR). Thus the yields observed in the complementation cultures do not derive from a few cells with very large bursts.

Many of the successful complementations with *am3* produce larger phage yields than *wt*. This may be a consequence of a slightly delayed lysis allowing a longer period of phage maturation.

Table 1 also includes data on the synthesis by these mutants of the ϕX coat protein responsible for serum blocking power (S. G. Krane, personal communication) and of infective, single-stranded DNA (Dowell & Sinsheimer, 1966).

TABLE 1

Growth of ϕX conditional lethal mutants under restrictive conditions

ϕX	Lysis	Relative intracellular phage yield	Serum-blocking power	Infective DNA	Relative phage release in complementation with ϕX am3	Relative phage release in complementation with self	Class
wt	normal	1.0	+	+	2.0	1.0	
am9	normal	<0.01	—	n.m.	0.35	<0.001	
tsy	normal	<0.01	+	—	1.1	0.02	
ts9	normal	<0.01	+	—	1.1	0.07	I
ts41	normal	<0.01	+	—	0.75	0.05	
ts79	normal	<0.01	—	n.m.	1.8	0.07	
ts4	inhibited	<0.01	+	+	1.8	0.01	
am18	late	~0.01	+	n.m.	1.5	0.01	II
am30	late	<0.01	+	n.m.	1.8	0.009	
am3	none	3.2	+	+	0.01	0.01	
am6	none	0.07	+	(+)	0.02	<0.001	III
am20	none	3.1	+	+	0.003	0.006	
am27	none	4.4	+	+	0.003	0.002	

Lysis of C was measured at 37°C for ϕX am mutants and at 40°C for ϕX ts mutants.

Intracellular phage yields are normalized to the yield of ϕX wt in the same experiment. In the case of the ϕX am mutants this was determined following artificial lysis of C infected for 60 min at 30°C. (This does not represent a maximal phage yield for mutants in class III.) The intracellular phage yields for ϕX ts mutants were determined after 50 min of infection at 40°C. Serum-blocking power was measured under the same conditions as intracellular phage production. Infective DNA synthesis was measured for ϕX tsy, ϕX ts9 and ϕX ts41 by Dowell & Sinsheimer (1966). The results for class III mutants are merely inferred from phage synthesis. The symbol n.m. means not measured. Complementation tests were performed as described in Materials and Methods. The total yield of released phage from a mixed infection with ϕX am3, assayed at 30°C on CR, is normalized to the yield of ϕX wt in a control performed at the same time. Phage synthesis and release upon infection with each mutant alone were also measured at the same time (complementation with self) as a control for leakiness of the mutants.

(f) Temporal exclusion

If *E. coli* C is infected with one strain of ϕX and is then superinfected several minutes later with a distinguishable mutant strain, only the first strain is found among the yield of progeny phage. This effect has been known for some time (D. Pfeifer, personal communication), but trivial explanations could be advanced such as the possibility that the primary infection induced cell lysis before the secondary infection could produce mature progeny. Such a possibility can now be excluded by the use of ϕX am3 as the primary phage.

When ϕX am3 is used as the primary phage, it is found that ϕX wt as the super-infecting phage is excluded, both from genetic contribution to progeny and from lysis function. A culture of *E. coli* C at a concentration of 1×10^8 cells/ml. in KC broth was infected at a multiplicity of 5 with ϕX am3 and then, 25 minutes later,

the culture was superinfected with ϕX *wt* at the same multiplicity. Control experiments, one without the superinfecting *wt* and one without the pre-infecting *am3* were performed at the same time. Samples were removed at various times for determination of intracellular phage titer, and the course of lysis (if any) was followed by turbidity measurements.

The control cultures produced, in each case, normal yields of the expected genotype. In the exlusion experiment: (1) the superinfecting phage was adsorbed and went into eclipse normally; (2) the superinfected cells did not lyse; (3) the superinfected cells produced a normal yield of the primary phage, ϕX *am3*; (4) the superinfected cells produced less than one ϕX *wt* per cell.

If ϕX *am3* and ϕX *wt* are added at equal multiplicities (5) at the same time to an *E. coli* C culture, in an unsynchronized infection, some 60 to 70% of the cells lyse. If the ϕX *am3* is given a five-minute pre-infection period before adding the ϕX *wt*, 30 to 35% of the cells lyse, while if the ϕX *am3* is given a ten-minute pre-infection period, exclusion is complete.

Exclusion does not develop in the presence of 0.003 M-KCN. ϕX *wt* added after a five-minute pre-infection period with ϕX *am3* in KCN induced as much lysis as if the *wt* had been added simultaneously with the *am3*.

To ascertain the fate of the DNA of the superinfecting phage, cells pre-infected for 25 minutes with ϕX *am3* at a multiplicity of 4 were superinfected with ^{32}P ^{15}ND - ϕX *wt* at a multiplicity of 0.5 in KC broth at 37°C. The superinfecting phage was absorbed and went into eclipse. At 15 minutes following superinfection, samples were removed and the cells were washed to remove unadsorbed phage. After the cells were lysed with lysozyme and EDTA, the DNA was prepared from the resultant lysate by treatment with phenol. A control culture, infected only with the labeled *wt* phage, was treated in the same manner.

The DNA samples were centrifuged to equilibrium in a CsCl density-gradient. ϕX γh DNA was added in each case as a density marker. Alternate two-drop fractions were assayed for radioactivity and for infectivity in spheroplasts (Fig. 5).

In the control experiment (Fig. 5(a)), after 15 minutes, more than half of the parental ^{32}P counts are found at the density of hybrid RF,† near the density of the γh DNA marker. The residue of the parental ^{32}P has remained at the density of ^{15}ND -labeled ϕX DNA single strands. Infective *wt* progeny DNA, which has the same density as the γh DNA marker, has been formed to the extent of approximately twenty DNA equivalents per input phage.

In the superinfection experiment (Fig. 5 (b)) none of the ^{32}P of the parental *wt* DNA is found at the density of hybrid RF. All of the ^{32}P counts have remained at the density of heavy single-strand DNA. Almost all of the *wt* infectivity is found at this position, and the amount is equal to the *wt* phage input.

The ϕX *am3* infectivity, which is found at the same density as the γh marker, amounts to about 500 DNA equivalents per infected cell. The small peak of ϕX *wt* infectivity which is found at the density of the γh marker amounts to about 5×10^{-5} of the *am3* infectivity at this density, and almost certainly represents revertants present in the ϕX *am3* DNA and not replication of the superinfecting *wt*.

Evidently pre-infection prevents conversion of the DNA of the superinfecting phage into an RF. The superinfecting DNA remains as an infective, hence undegraded, single-strand.

†Abbreviations used: RF, replicative form; SS, single-stranded DNA.

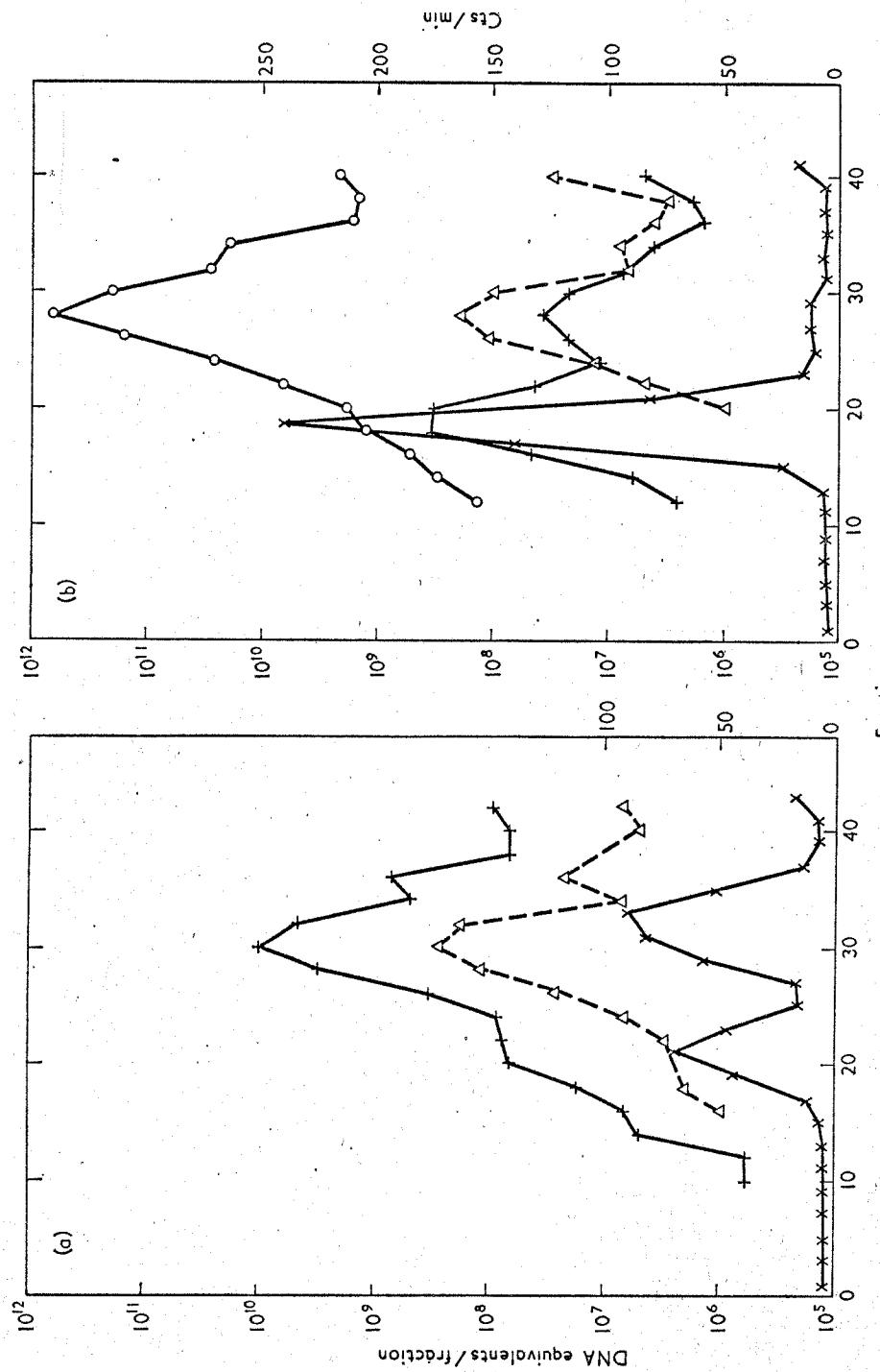


Fig. 5. Temporal exclusion of RF synthesis. *E. coli* C was infected as described in the text, DNA extracted and centrifuged to equilibrium in a CsCl density-gradient ($\bar{p}=1.72$). $\phi X\gamma h$ DNA was added as a density marker. Alternate two-drop fractions were counted to measure ^{32}P and assayed for DNA infectivity to spheroplasts. The progeny phage from the spheroplasts were assayed selectively to determine the genotype.

(a) Cells were infected with ^{32}P 15ND- ϕX *wt*. DNA was extracted after 15 min of growth at 37°C. —○—○—, Cts/min/fraction (^{32}P); —Δ—Δ—, $\phi X\gamma h$ marker infectivity assayed by plating on C at 30°C; —+—+, ϕX *wt* infectivity assayed on C at 40°C.

(b) Cells were pre-infected for 25 min with ^{32}P 14NH- ϕX *wt*, DNA was extracted 15 min later. —×—×—, Cts/min/fraction (^{32}P); —Δ—Δ—, $\phi X\gamma h$ marker infectivity; —+—+, ϕX *wt* infectivity; —○—○—, ϕX *am3* infectivity plus ϕX *wt* infectivity assayed by plating on CR at 40°C (this assay is essentially a titer of ϕX *am3* infectivity since the ϕX *wt* contribution to total infectivity is negligible).

(g) *Effect of chloramphenicol on replication of ϕX am3 DNA*

The increased time span of infection afforded by the use of ϕX am3 has permitted an analysis of the effect of the addition of chloramphenicol at various times after infection upon the subsequent synthesis of RF, single-stranded progeny DNA and mature phage.

A culture of *E. coli* C cells at 2×10^8 /ml. in KC broth was infected at 37°C with am3 at a multiplicity of 3 and immediately divided into six portions. To five of these, chloramphenicol (30 μ g/ml., final concentration) was added at 5, 10, 15, 25 and 50 minutes after infection, respectively. At various times before and after the addition of chloramphenicol, portions were removed from the culture for assay of infective centers and intracellular phage, and for preparation of a DNA extract. The infectivity of the DNA extract was assayed in spheroplasts. The proportions of such infectivity

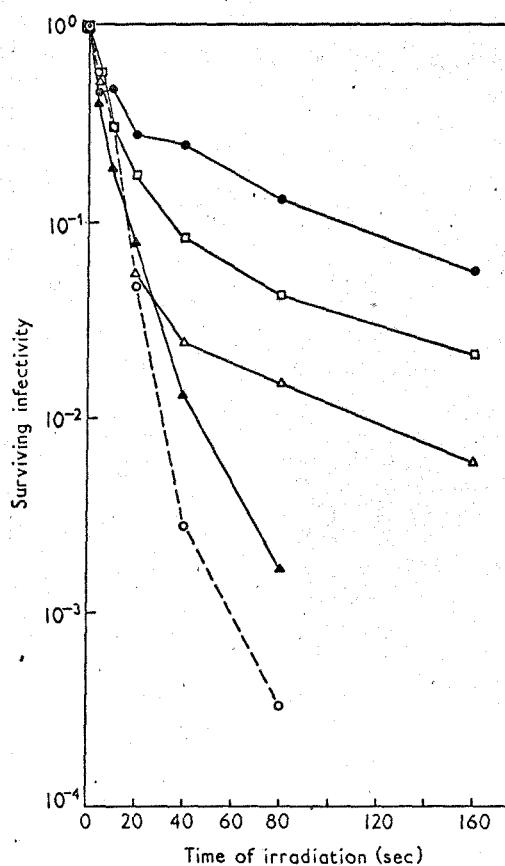


FIG. 6 Ultraviolet inactivation of the infectivity of DNA samples extracted at various times subsequent to the addition of chloramphenicol at 5 min following infection with ϕX am3. The DNA extracts from 10^9 infected cells, dissolved in 0.5 ml. were diluted 10^3 into 0.05 M-tris (pH 8.1). 1.5 ml. of each was irradiated in a 5-cm Petri dish at 30 cm from a pair of 8 w germicidal lamps. Single-stranded ϕX γh DNA was added as an internal standard. 0.1-ml. samples were removed at 0, 5, 10, 20, 40, 80 and 160 sec, diluted 10 times, and assayed for both am3 and γh DNA. Results are normalized to the time zero assay.

—●—●—, extract at 50 min; —□—□—, 25 min; —△—△—, 15 min; —▲—▲—, 5 min; ---○---○---, γh .

attributable to single-stranded DNA and to RF were determined by a measurement of the ultraviolet inactivation curve of the infectivity of each DNA preparation.

The cross-section to ultraviolet inactivation of RF is one-tenth that of SS DNA (Sinsheimer *et al.*, 1962). Therefore the inactivation curve of a preparation containing both SS and RF DNA will break when the infectivity of surviving SS becomes less than that of surviving RF (Fig. 6). Extrapolation of the second (lesser slope) portion of the inactivation curve back to zero dose provides a determination of the fraction of the initial infectivity attributed to RF (and by difference, the fraction attributable to SS). Figure 6 illustrates the inactivation curves obtained with DNA preparations extracted at various times after addition of chloramphenicol five minutes after infection.

In converting infectivity data to molecules, it is assumed that the specific infectivity of SS DNA is 20 times that of RF DNA (Sinsheimer, Lawrence & Nagler, 1965).

The results are presented in Table 2. In normal ϕ X *am3* infection, there is little synthesis of RF after 20 minutes, while the amount of single-stranded DNA, largely present in mature phage, increases 30-fold. The addition of chloramphenicol at five

TABLE 2
Effect of chloramphenicol on synthesis of viral DNA

Time of addition of chloramphenicol	Time of removal of sample (min)	Intracellular phage/cell	SS DNA/cell	RF/cell
No chloramphenicol	10	0.08	2.5	1.6
	20	15	27	5.4
	60	104	375	15.0
	90	670	830	8.3
5 min	5	0.06	—	—
	15	—	1.9	1.8
	25	—	1.7	3.8
	50	0.01	1.5	20
10 min	10	0.09	1.6	2.3
	20	—	2.1	4.6
	30	—	3.0	15
	60	0.15	2.7	54
15 min	15	7.5	17	20
	25	—	58	47
	35	—	150	67
	60	24	88	100
25 min	25	22	104	32
	35	—	156	58
	50	—	210	83
	75	28	210	148
50 min	50	37	166	20
	60	—	290	35
	80	—	335	60
	100	83	290	60

or ten minutes, before the appearance of any progeny SS DNA or progeny phage, prevents the synthesis of SS DNA and permits RF synthesis to rise appreciably above normal levels. The addition of chloramphenicol at later times allows a continued synthesis of SS DNA for 20 to 25 minutes (albeit at a lesser rate than normal) and induces a continued (or renewed) synthesis of RF for 40 to 50 minutes, up to concentrations exceeding 100/cell. In the presence of chloramphenicol the concentrations of SS DNA/cell considerably exceed the concentrations of mature, intracellular phage.

4. Discussion

These results concerning the existence and properties of ϕ X lysis mutants have a bearing on several aspects of the ϕ X infective process.

(a) *Mechanism of ϕ X-induced lysis of the host*

It is clear that mutations in the phage can alter the normal lytic process in the ϕ X-infected cell. This observation implies control of the lytic process by phage genes. Three classes of mutants have been identified. (These classes are defined only with respect to lysis and phage production and do not represent complementation groups.)

Class I

No mature progeny phage are produced; cell lysis is normal. Both temperature-sensitive (*ts*) and amber (*am*) mutants in this class are known. Markert & Zillig (1965) have also reported ϕ X *ts* mutants of this type. The existence of these mutants demonstrates that the presence of mature infective progeny is not required for normal lysis.

It has been found that several of the ϕ X *ts* mutants of this type do not make infective single-stranded DNA at the restrictive temperature (Dowell & Sinsheimer, 1966). Similarly, in studies of ϕ X coat-protein production using a serum blocking power assay, it has been found that some of the ϕ X *ts* and *am* mutants which lyse normally under restrictive conditions fail to show any serum blocking power under these same conditions (S. G. Krane, personal communication).

Thus it appears that neither the mature phage nor any previously described functional component of the phage is required for normal phage-induced lysis. This is consistent with the findings of Markert & Zillig (1965).

Class II

No mature progeny phage are produced; cell lysis is inhibited. Both *ts* and *am* mutants in this class are known. Their existence demonstrates a requirement for ϕ X gene function in the normal lytic process. However, the specific functions controlled by these mutants are not understood at the present time. Reversion rates indicate that they are single mutants, although the mutation affects both lysis and mature phage production. The existence of the mutants suggests that the lytic process may be more complex than the simple interpretation of the results with class III mutants, discussed below, would indicate. The lytic process may require more than one phage gene product, or may be under the influence of some control mechanism which also influences progeny phage production.

Class III

Mature progeny phage are produced; cells do not lyse. Only *am* mutants have so far been found in this class. All of the mutants of this class found so far fall in a single complementation group. They will complement mutants of both classes I and II. The existence of these mutants again demonstrates the requirement for a phage gene product in the lytic process. Accepting current ideas of the nature of *am* mutations (Sarabhai, Stretton, Brenner & Bolle, 1964; Stretton & Brenner, 1965), this gene product is almost certainly a protein. This protein cannot be an essential structural component of the phage particle, since infective progeny phage particles are produced in the restrictive host. These mutations must lie in a gene with the sole function of release of progeny phage from the infected cell. It is interesting that such a small viral genome contains a gene for a function which is not essential for actual replication of the virus.

These experiments do not specify the mechanism of action of this ϕ X lytic protein. It could act directly by enzymic attack upon the cell wall, or indirectly, for example by interference with normal cell-wall synthesis or repair.

It is, at first thought, surprising that similar mutants were not found in Tessman's study of the closely related phage, S13 (Tessman, 1965). This may have been a consequence of differences in the bacterial suppressor used in the selection of the phage mutants (Tessman believes her S13 *su* mutants are "missense" rather than "nonsense" mutants). Alternatively, it may be that there simply are no sites where such a mutation could occur in S13, even if a gene homologous to the ϕ X lysis gene is present. (Although conditional lethal mutants are not specific for a particular function, they probably do require a very specific type of sequence change in the DNA; hence the number of possible sites for such mutations per gene may be quite low. This same circumstance could explain our failure to find *ts* mutants of this type.)

(b) *Mechanism of ϕ X replication*

Since, in a culture of *E. coli* C infected by ϕ X *am3* the cells do not lyse, it is possible to study the process of phage synthesis for extended periods of time. Denhardt & Sinsheimer (1965a) observed that ϕ X *wt* is matured linearly, not exponentially. When lysis is blocked by use of ϕ X *am3*, this result is confirmed and the maturation can be observed for a period of almost two hours at 37°C.

There appears to be no specific control mechanism that halts ϕ X maturation at the end of the normal latent period, since maturation continues if lysis is blocked. In this respect ϕ X *am3* behaves differently from mutants in the lysozyme gene (*e* gene) of phage T4. In these mutants, phage maturation stops at the end of the normal latent period even though lysis does not occur (F. Mukai, G. Streisinger & B. Miller, personal communication).

(c) *Effect of ϕ X infection on the host*

Although lysis of the host does not occur in ϕ X *am3* infection, the infected cells are still killed. They will not multiply to form colonies, nor divide more than once. Instead, they elongate to form "snakes". This non-lytic lethal effect distinguishes ϕ X from the rod-shaped single-stranded DNA phages of the fd and M13 type. These are able to exist in a carrier state in which the infected cells continue to grow and divide while also releasing progeny phage particles (Hoffmann-Berling, Durwald & Beulke, 1963; Hofschneider & Preuss, 1963).

(d) *Temporal exclusion in ϕX infection*

In addition to their intrinsic interest, lysis mutants of the ϕX *am3* type are technically useful in a number of ways. They are obviously useful in the production of large quantities of virus, since the phage yield in the restrictive host is about ten times the ϕX *wt* yield. They also are useful in any type of experiment concerning the ϕX -infected cell in which lysis may produce complications in experimental procedure or in interpretation of results.

An example of such a problem is provided by the phenomenon of temporal exclusion in ϕX infection. The use of ϕX *am3* as the primary infecting phage excludes the possibility that exclusion is due to lysis induced by the primary phage before the secondary phage has had time to produce mature progeny. This experiment also shows that the secondary phage is excluded from performance of the lytic function as well as contribution to progeny.

The finding that the excluded phage is unable to form the parental RF molecule is sufficient to account for its inability to produce progeny or to function. This mechanism of exclusion is different from that found in T2 (Lesley, French, Graham & van Rooyen, 1951), since the excluded ϕX single strands remain infective and therefore, presumably, intact. How the primary phage infection prevents RF formation by the superinfecting phage is not understood. Although adsorption and eclipse of the superinfecting phage are normal, it is possible that the superinfecting phage DNA never actually enters the cell. However, the fact that exclusion is set up rather rapidly (about half the cells exclude after five minutes of pre-infection) makes it seem unlikely that exclusion is a consequence of a change in the cell wall. An alternative possibility is that the superinfecting DNA actually enters the cell and immediately becomes complexed with newly synthesized phage-coat protein. Phage-coat protein begins to be synthesized at about five minutes after infection (S. G. Krane, personal communication). The phenomenon of temporal exclusion in ϕX infection has a different basis from the superficially similar phenomenon of limited participation (M. Yarus & R. Sinsheimer, paper in preparation). In the latter circumstance, the non-participating parental DNA is transformed into an RF molecule, while the DNA of temporally excluded phages is not.

(e) *Effect of chloramphenicol on ϕX DNA synthesis*

The experiments with addition of chloramphenicol confirm earlier observations (Sinsheimer *et al.*, 1962) that if protein synthesis is blocked at an early stage of infection, no progeny single-strands appear. They also indicate that, in normal infection, the initiation of the synthesis of single-stranded DNA appears to preclude further replication of RF. Once initiated, single-strand DNA synthesis can continue for at least 20 to 25 minutes in the absence of protein synthesis. These single strands are not in mature virus particles but may be in incomplete particles, formed as the pool of viral coat subunits is depleted.

If chloramphenicol is added before initiation of SS DNA synthesis, the synthesis of RF continues well beyond the normal time and level of turn-off. If chloramphenicol is added after initiation of SS DNA synthesis, the synthesis of RF can be resumed and reach levels many times normal. It seems likely that the synthesis of RF under these conditions may not involve the semi-conservative replication previously demonstrated (Denhardt & Sinsheimer, 1965b), but rather the conversion of progeny

SS DNA, unencumbered with coat-protein subunits, into RF by the same process responsible for conversion of the parental SS DNA to RF.

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ADDENDUM

The preceding paper does not identify by name the complementation groups which show aberrant lysis. This addendum will rectify this situation, and will present additional information concerning the mutants which exhibit delayed or incomplete lysis.

Class I

No mature progeny phage are produced; cell lysis is normal. This class includes mutants in cistrons III, IV, and V.

Class II

No mature progeny phage are produced; cell lysis is inhibited.

Mutants in cistron II exhibit incomplete lysis. An example is ts 4, as presented in Fig. 1(a) of the preceding paper. The turbidity of the ts 4 infected culture drops somewhat at the normal time of lysis, then increases in the same manner as the uninfected culture. This behavior is consistent with the idea that a fraction of the infected cells lyse, and that the remainder grow at the same rate as uninfected cells. This interpretation has been supported by more recent microscopic observations of cultures infected with ts 4. During the later phase of the experiment the remaining cells appear quite normal morphologically, and are dividing at the same rate as uninfected cells. It has been observed (F. Funk, personal communication) that the presence of the ts 4 mutation, in the infecting phage, causes a marked increase in the fraction of cells which survive infection and are able to form colonies, at 30°C. In view of these facts it seems reasonable to hypothesize that the ts 4 defect

results in a lowered efficiency of initiation of the infection which is more pronounced at 40°C. For example, even though phage were adsorbed to cells at 30°C (using the starvation-block procedure), they may have failed to inject their DNA when transferred to complete medium at 40°C. Since cistron II codes for a structural component of the phage coat (see Part 3.3) it is quite reasonable that a mutation could affect both the maturation process and the efficiency of initiating infections. It is possible to test this hypothesis experimentally.

Cultures infected with mutants defective in cistron VI lyse at a later time than ØX wt infected cells. These include am mutants in VI and a mutant in IV which, as a result of a polar effect, is deficient in VI function (am 30 and am 18 - see Fig.1(c)). The kinetics of the turbidity changes observed, following infection with these mutants, suggest that some fraction of the cells lyses, starting 30 to 40 minutes after infection at 37°C (approximately twice the time required for the onset of wt-induced lysis in the same experiment). The turbidity then remains approximately constant until lysis is reinitiated 2 to 3 hours after infection. The reason for this complex behavior is not clear. It seems possible that the very late phase of lysis is produced by the replication of revertants of the am mutant. It seems very unlikely that the earlier lysis could be produced by revertants (one in 10⁵ of the infecting phage were revertant). It should be remembered also, that am mutants in cistron VI are able to provide the lysis function in complementation with mutants in cistron I, even though the cistron VI mutant is not itself efficiently rescued. B.H. Lindqvist (personal

communication) has observed that the time of lysis of cells infected with am mutants in cistron VI is dependent upon the multiplicity of infection; the time required for lysis decreases and approaches that of the wt with increasing multiplicity. Since these mutants lack the ability to replicate RF under restrictive conditions (see Part 3.4), it is tempting to hypothesize that the rate of production of lytic protein (cistron I product) is proportional to the number of RF molecules in the infected cell. It would be of interest to study this multiplicity effect under conditions of limited participation, in order to clarify the nature of the bacterial site requirement in transcription.

Class III

Mature progeny phage are produced; cells do not lyse. This class is equivalent to cistron I.

3.3 PHAGE COAT GENES

The genes which code for protein components of the mature phage particle offer special advantages for studies of gene function, simply because their products may be readily isolated (in the phage particle) from the thousands of species of host cell proteins. These proteins also provide an excellent experimental system for investigating a biological machine, composed of several different kinds of protein molecules, which must perform several rather complex, non-enzymatic, functions - packaging of the viral + strand, specific attachment, and injection of DNA into a suitable host cell.

This section will begin with some non-genetic evidence for the structural complexity of the ϕ X capsid. Next, evidence which implicates cistrons II, III, IV, and VII as genes for phage coat proteins will be presented. Evidence which identifies cistrons with particular components of the phage, and results related to the assembly and function of the phage particle will also be presented.

Most of the experimental results, as well as ideas concerning their interpretation, are the result of collaboration with Marshall H. Edgell.

3.3.1 Structure of the ϕ X virion.

An electron micrograph of a purified preparation of ϕ X am 3, negatively stained with uranyl acetate, is shown in Fig. 1. The projections or "spikes" which protrude from the 12 vertices of the icosahedral particle can be clearly seen. The use of uranyl acetate (as opposed to phosphotungstate staining), and the selection of fields which are lightly

stained, greatly enhance the visual impression that the spikes are structures appended to a regular shell or capsid. Internal structure can sometimes be seen in the spikes. They generally appear to be somewhat broader at the tips than at the base where they are attached to the shell, and sometimes what appears to be subunit structure may be discerned. The icosahedral virions may be oriented at a variety of angles with respect to the grid surface, and Fig. 2 shows views which are parallel (or nearly so) to the various rotational symmetry axes of the phage particle. It is the comparison of these various views of the phage particle with models which leads to the conclusion that the spikes are directed as from the 12 vertices of an icosahedron. It should be pointed out, however, that all 12 vertices are never visible on a single particle, and that one therefore can not exclude possibilities such as, for example, that one of the vertices has no spike, or has a spike with a significantly different structure from the others.

The impression that the spikes are appendages to an inner shell has been strengthened by the development of a procedure which removes the spikes but leaves the shell intact. An electron micrograph of ϕX am 3 treated with 4 M urea is shown in Fig. 3. The spikes have been essentially completely removed, leaving a shell which now stains internally. One can see what appears to be DNA extruded from some of the particles. Removal of the spikes allows visualization of subunit structure in the virus shell. Fig. 3 shows one particle in which a ring of 10 subunits can be clearly distinguished.

After treatment with 4 M urea the spikeless capsids can be separated from the released spike material by sedimentation in a sucrose gradient (39,110). The shells with trailing DNA sediment in a broad peak at about 50 S. About 20% of the phage protein and nucleic acid remains at the top of the gradient. This top fraction presumably contains the released spike protein.

If purified $\emptyset X$ is disrupted in 10 M urea containing mercaptoethanol, then electrophoresis in acrylamide gel containing urea can resolve 4 bands (39,110). One of these is present in small but variable amount, and moves at the electrophoretic front at either pH 3.8 or pH 9.5. It is present in 10 M urea disrupted preparations of purified shells and spike material separated by sucrose gradient centrifugation. Only one of the three other major bands is found in purified shells. The other two bands are found specifically in the slow sedimenting spike fraction. This result indicates that the spikes have a distinct protein composition, as well as a different morphology, which distinguishes them from the shell of the phage particle. It is worth noting, however, that no evidence exists which proves that the proteins released by 4 M urea treatment are all parts of the spike, as visualized in the electron micrographs. It is possible, for example, that the spike plus some protein which is not visible at all in micrographs of untreated phage, is released from the particle by 4 M urea treatment.

Our working hypothesis, based on these results, is that the $\emptyset X$ particle consists of a shell composed of a single molecular species of protein molecules, containing the viral DNA, and that 12 structurally com-

plex spikes composed of at least two different proteins are appended to this shell.

3.3.2 Identification of mutants in coat genes.

Four methods have been used for the identification of mutants affecting the structure of the ϕX coat proteins: 1) Studies of the thermal stability of mutant phage particles, 2) Measurements of the serum-blocking protein (SBP) synthesized by mutants under restrictive conditions, 3) Measurements of the electrophoretic mobilities of mutant phage particles, and 4) Electrophoretic analysis of the proteins obtained by disruption of mutant phage particles (and defective phage particles produced under restrictive conditions).

Thermal stability of mutant phage. The plaque forming ability of ϕX is inactivated by heating under conditions which should not cause damage to the DNA of the phage. The kinetics of inactivation of ϕX wt and a host range mutant, ϕX h1-3, in 0.025 M Tris buffer, pH 8.1, at 60°C, are shown in Fig. 4. This experiment was performed by heating a mixture of the two phage types, and then scoring the two types among surviving phage by plating on a mixed indicator (wt gives turbid plaques and h1-3 gives clear plaques on a mixture of 5 parts C plating bacteria plus 1 part C₁). It is seen that the two phage strains have identical thermal stabilities under these conditions.

Electron micrographs of heat inactivated phage have been published (9), showing that the inactivated particles extrude DNA. These particles sediment with a mean sedimentation constant of about 47 S (5), presumably

due to an increased frictional coefficient resulting from trailing DNA. These particles are infective to spheroplasts, indicating that the DNA is not damaged (5). It appears, therefore, that the inactivating event results from breakage of the phage coat, which then allows the DNA to escape from the phage particle. One would expect that any point mutations which alter the rate of heat inactivation of the phage could do so only by alteration of the structure of the phage coat.

A number of mutants, representing all seven complementation groups, have been tested for altered thermal stability. In these experiments a sample of the mutant to be tested (from a stock grown under permissive conditions) is mixed with an equal number of the host range mutant h1-3. This mixture is then heated under the conditions of the experiment described in Fig. 4, sampled after 10 and 30 min. of heating, and the survivors of the two genotypes scored. This procedure allows small differences in heat stability from the wt to be reliably detected. At the temperature used (60°C) the actual rate of inactivation is itself quite temperature dependent, so that inactivation rates obtained without the use of an internal standard may be quite variable due to slight variations in the conditions of the experiment. The results of these experiments are expressed as the ratio of the mutant inactivation rate to that of the wt (which is identical to h1-3). This parameter is greater than 1 for mutant particles which are more sensitive to heat inactivation than the wt, and less than 1 for mutants which are more resistant.

Results of such experiments are presented in Table 1. Values for the majority of mutants studied are not significantly different from wt.

Six am mutants in cistron I (which is a lysis gene, and so might be expected not to code for a structural component of the phage) all show values close to 1. On the other hand, several mutants in cistrons III and IV, and the mutant ts 41D in cistron VII show significant alterations in thermal stability. None of the mutants studied which lie in cistrons I, II, V, or VI show altered stability.

These results are interpreted to indicate that cistrons III, IV, and probably VII, code for proteins which determine the structure of the phage coat. The simplest interpretation is that these cistrons code for three of the proteins which are components of the phage particle. The result with cistron VII is not clear, since the only mutant tested (ts 41D) appears to be a double mutant. Experiments with op mutants in this cistron should clarify this point.

SBP synthesis by $\emptyset X$ mutants. A serological assay has been developed to measure the phage antigen which reacts with antibodies, resulting in inactivation of the plaque forming ability of the phage (46). This "serum-blocking power" (SBP) assay can detect antigen when incorporated into mature phage particles, 70 S particles, or slow sedimenting precursors of the phage particle (46, 111). It has been shown that most ts mutants of $\emptyset X$ produce SBP at the high temperature, even though phage is not manufactured under these conditions (46).

A number of $\emptyset X$ mutants have been screened for the ability to manufacture SBP under restrictive conditions of growth, in an attempt to determine which cistrons(s) codes for the SBP antigen of the phage. These include both ts mutants, which were tested in collaboration with

S.G. Krane, and am mutants. It was expected that the majority of ts mutations, since they are missense mutations would result in a protein immunologically similar to the wt protein, but that am nonsense mutations would essentially always result in the production of a fragment which would be undetectable in the SBP assay.

E. coli C was infected with a multiplicity of 5 to 10 of a mutant phage, using the starvation-block synchronization procedure. Development was initiated by the addition of KC broth at 40°C in the case of the ts mutants, and at 30°C in the case of am mutants. A sample was taken at time zero for the determination of background SBP, due to the infecting phage particles. A sample for the assay of newly synthesized SBP was taken after 50 min. of incubation in the 40°C experiment and after 80 min. at 30°C. These samples were chilled and then treated with lysozyme and versene to ensure lysis. The results of SBP assays of these mutant lysates are presented in Table 2.

Three mutants have been found which do not appear to synthesize any SBP under restrictive growth conditions; ts 79, am 9, and am 23. All three of these mutants have been assigned to cistron III. A number of other ts mutants in III were tested, giving positive results (or in some cases ambiguous results due to high background), however the am mutants were the only ones in cistron III which have been tested. These results also show that am mutants in cistrons I, IV, (including polar mutants am 18, and 35), V, and VI are able to synthesize SBP in the restrictive host. These results suggest that the cistron III product is the SBP antigen. It is possible, however, that the products of cistron

III, or VII are also essential parts of the antigen since nonsense type mutants in these cistrons have not been tested.

S.G. Krane (46) and L.L. Greenlee (111) have studied the physical state of the SBP antigen produced by $\emptyset X$ ts mutants at the restrictive temperature. Mutants in cistrons III and IV produce SBP which sediments slowly in a sucrose gradient (estimated at 6 S, compared to 114 S for phage particles). The cistron II mutant ts 4, on the other hand, incorporates SBP into particles which nearly co-sediment with infective phage. Evidence concerning the nature of the defect present in these particles will be presented.

Electrophoretic mutants. We have developed a method for performing acrylamide gel electrophoresis of infective $\emptyset X$ particles (a published account of this work is included in part 3.3.3 of this thesis). This technique has identified several mutants with mobilities different than that of the $\emptyset X$ wt. These include several independently isolated host range mutants, and the cistron III mutant ts 79. This result provides further confirmation for the fact that cistron III codes for a protein component of the phage coat.

Electrophoresis of mutant proteins. When a mutation results in an infective particle with an altered electrophoretic mobility, it is expected that the mobility of one protein component will also exhibit an altered charge following dissociation of the phage. Highly purified phage in sufficient quantity for protein analysis has been prepared from the mutant strains am 3 $H_a H_b$ and am 3 ts 79 (III). The defective phage particles produced at 40°C by am 3 ts 4 (II) were also purified. The

protein composition of all three of these preparations can be distinguished electrophoretically from that of ØX wt.

The electrophoretic patterns obtained following disruption of these preparations are shown in Fig. 5, which also presents patterns obtained from am 3 for comparison. The H_aH_b and ts 79 mutations cause changes in the patterns at pH 9.5. One of the spike proteins which is removed by mild urea treatment has a lower mobility resulting from the H_aH_b mutation. One of the spike proteins from the ts 79 particle has an increased mobility. Both of these changes are in the same direction as the change in charge of the infective phage particle, as would be expected. The patterns observed with ts 79 were surprising since some protein still migrates at the wt position of the altered band. The phage preparation from which this material was obtained was genetically pure, and electrophoretically homogeneous when infective particles were electrophoresed. This makes it seem unlikely that the preparation contained two types of particles with different protein compositions. Two possibilities are : 1) the ts 79 mutation results in an ambiguous codon which causes insertion of two amino acids, with different charges, in a fixed proportion (so that the resulting phage population is homogeneous), or 2) there are two different protein components of the phage coat which have nearly identical mobilities in the wt particle, but which are resolved after one (the cistron III product) is altered by the ts 79 mutation. The latter possibility seems more likely in view of the fact that four cistrons (II, III, IV, and VII) have been implicated in various ways as structural components of the phage.

The defective particles produced at 40°C by am 3 ts 4 appear to be missing one of the spike proteins. Fig. 5 compares the protein patterns obtained by electrophoresis at pH 3.5 from a purified preparation of defective particles and a control preparation, purified in the same manner, of infective am 3 ts 4 produced at 30°C. Electron microscopy of the defective particles shows spikes which we have been unable to distinguish from wt spikes (see Fig 6). These results suggest that the cistron II product is a minor component of the ϕ X spike, and that assembly can proceed without incorporation of this protein, resulting in a particle which is physically quite like the normal phage, but is not infective.

Summary of coat protein mutants. 1) Some mutants in cistrons III, IV, and VII produce phage particles under permissive conditions which exhibit altered thermal stability. 2) am mutations and some ts mutations in cistron III result in the inability to synthesize SBP under restrictive growth conditions. 3) Host range mutants and cistron III mutants may produce phage particles with altered electrophoretic mobility. 4) A host range mutant and a cistron III mutant contained spike protein components with altered mobility. Two different bands were altered by the two mutations. The electrophoretic pattern observed with the cistron III mutant suggests that the wt cistron III product co-electrophoreses with another spike protein (at pH 9.5). 5) The defective phage particle produced by a ts mutant in cistron II is missing one spike protein band (at pH 3.5).

We interpret these results to mean that cistrons II, III, IV, and VII code for protein components of the phage coat. The host range mutants are in a coat protein (probably one of the cistrons listed). The cistron III product and the host range protein are different spike proteins. The cistron II protein is also a spike component, presumably either the host range protein or the protein which co-electrophoreses with the cistron III product at pH 9.5. Since cistron VII appears to correspond to cistron I of phage S13, and since this S13 cistron contains host range mutations (107), it seems likely that the $\emptyset X$ host range mutants are in cistron VII.

3.3.3 Assembly and function of the $\emptyset X$ coat.

The publication reproduced on the following pages describes the methods used for the identification of electrophoretic mutants of $\emptyset X$, and the use of such mutants in phenotypic mixing experiments. This method allows quantitative determination of hybrid phenotypes, thereby giving some new insight into the in vivo assembly of the $\emptyset X$ capsid, and the structure of the phage adsorption site.

In reading this paper please keep in mind that ts 79 (III) and $H_{a}H_{b}$ (probably VII) are in different cistrons which code for two protein components of the phage spike. This was not known at the time the paper was written.

The Process of Infection with Bacteriophage ϕ X174XII†. Phenotypic Mixing between Electrophoretic Mutants of ϕ X174

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A technique for the acrylamide gel disc electrophoresis of ϕ X174 particles has been developed. Using this technique, mixtures of several phage mutants, altered in genes which affect the structure of the viral capsid, can be physically separated. This technique allows also the separation of incomplete phage particles produced in ϕ X174 infection from infective phage.

The progeny phage particles from cells mixedly infected with two electrophoretically distinguishable phage strains (ϕ X174 wild type and ϕ X174 H_aH_b) have been examined. Phenotypic mixing occurs, with the result that progeny DNA molecules of either genotype may be contained in hybrid capsids. Such hybrid capsids contain the wild-type and mutant protein subunits in varying ratios. Electrophoretically homogeneous preparations of phage with hybrid capsids can be isolated. The existence of these hybrid capsids supports the idea that the ϕ X174 coat is assembled by withdrawing subunits from a pool.

Since ϕ X174 H_aH_b is an extended host range mutant (it can form plaques on *Escherichia coli* C₁ which does not adsorb wild type ϕ X174), it is possible to compare the biological phenotype of a hybrid capsid with the molecular composition of the capsid, as estimated from the electrophoretic mobility. We find that a phage particle must have an almost pure H_aH_b capsid in order to infect C₁ bacteria. Provided that certain structural assumptions are made, the minimum size of the adsorption site on the phage particle can be estimated from these results: this site must contain 4 to 6 subunits of the type altered by the H_aH_b mutations.

1. Introduction

By means of sucrose density-gradient electrophoresis, Aach (1963) was able to detect a change in the mobility of infective particles of bacteriophage ϕ X174‡ accompanying a host range mutation. This finding has led us to develop a technique for electrophoresis of ϕ X particles in polyacrylamide gel.

This technique is useful for identification of mutations in those genes which specify structural components of the virus particle. We have also used this electrophoretic analysis to study the process of assembly of ϕ X particles from a pool of protein subunits, by examination of the progeny from cells mixedly infected with two electrophoretically "labeled" phage types.

Phage particles with hybrid capsids, produced in such a mixed infection, allow study of the relationship between structure and function of the ϕ X capsid.

† Part XI of this series precedes this paper.

‡ Abbreviation used: ϕ X174 is referred to as ϕ X throughout this paper.

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2. Materials and Methods

(a) Biological

The biological materials and methods used here are those described by Hutchison & Sinsheimer (1966) with a few additions described below.

$\phi X H_a H_b$ is a two-step extended host range mutant isolated by Pfeiffer (1961) and kindly supplied by him. $\phi X H_a H_b$ will form plaques on *Escherichia coli* C, the normal host for ϕX wild-type (wt), and on strains Cab and C₁, two independently isolated mutants of C that are resistant to ϕX wt.

ϕX hr is a restricted host range mutant of ϕX . It forms plaques on C but not on the mutant strain CR, whereas ϕX wt forms plaques on both strains. The plaques on C are small and ragged.

Mixed indicator is made by mixing 5 vol. of *E. coli* C plating bacteria with 1 vol. of C₁ plating bacteria. If mixed indicator is used as plating bacteria and the plates are incubated 2 to 3 hr at 30°C and then an additional 2 to 3 hr at 40°C, then it is possible to distinguish plaques formed by ϕX wt, $\phi X H_a H_b$, ϕX ts mutants, and a double mutant that is ts and also has an extended host range (ϕX yh). The ϕX wt forms large turbid plaques; $\phi X H_a H_b$ forms large clear plaques; ϕX ts mutants form small turbid plaques with crisp edges; ϕX yh forms small clear plaques with crisp edges.

Eclipse measurement. Cells were grown to a turbidity of $A_{650\text{m}\mu} = 0.20$ in KC broth (about 1×10^8 cells/ml.). KCN was then added to a concentration of 0.003 M. Phage were diluted to a concentration of 1×10^7 plaque-forming units/ml. in KC broth. 0.1 ml. of phage was added to tubes containing 0.9 ml. of cells. The tubes were incubated 20 min at 30°C and then chilled to 0°C. Each tube was then diluted tenfold into KC broth saturated with chloroform and shaken at room temperature. Surviving phage were then assayed. The chloroform treatment kills infected cells, so they do not form plaques. Therefore the phage measured after chloroform treatment should represent those that have not made an irreversible attachment during the 20-min adsorption period. This fraction was calculated by comparison with a control in which phage were subjected to the same procedure except that cells were absent.

[¹⁴C]Leucine-labeled ϕX am3 was prepared as follows. A culture of *E. coli* C grown in TPG medium (minus FeCl₃ (Sinsheimer, Starman, Nagler & Guthrie, 1962)) at 37°C to a concentration of 2×10^8 cells/ml. was infected with 10 ϕX am3 per cell. At the time of infection 2.5 μ C/ml. of [¹⁴C]leucine were added. Aeration was continued for 2 hr at 37°C. The infected cells were collected by centrifugation and lysed with lysozyme, EDTA, and 3 cycles of freezing and thawing. Cellular debris was removed by low-speed centrifugation (about 10,000 rev./min). The phage and the associated 70 s material were separated from each other and from other material in the lysate by sucrose density-gradient centrifugation.

(b) Gel electrophoresis

We have used essentially the same method described by Ornstein & Davis (1962) except for a more dilute gel. Materials were obtained from Canal Industrial Corporation, Rockville, Md. The compositions of the stock solutions used are listed for reference.

(A) 24 ml. of 1 N-HCl, 18.2 g of Tris, and 11.5 ml. of Temed (*N,N,N',N'*-tetramethyl-ethylene-diamine) are dissolved in water to make 100 ml. of solution. The pH of the solution should be 8.8 to 9.0.

(B) 5.98 g of Tris, 0.46 ml. of Temed, and about 48 ml. of 1 N-HCl are dissolved in water to make 100 ml. of solution with a pH of 6.6 to 6.8.

(C) 22.0 g of acrylamide monomer and 3.9 g of Bis (*N,N'*-methylbisacrylamide) are dissolved in water to make 100 ml. of solution.

(D) 20 g of acrylamide and 5.0 g of Bis are dissolved in water to make 100 ml. of solution.

(E) 4.0 mg of riboflavin is dissolved in water to make 100 ml. of solution.

(F) 40.0 g of sucrose is dissolved in water to make 100 ml. of solution.

(G) 0.14 g of ammonium persulfate is dissolved in water to make 100 ml. of solution.

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(H) 3.0 g of Tris and 14.4 g of glycine are dissolved in water to make 1 liter of solution.

Two different gel recipes for pH 9.5 gels are used in the experiments described here. One system uses light-induced polymerization of the gel with riboflavin as a catalyst. In these experiments a stacking gel was used. The other gels employ no stacking gel and use a persulfate catalyst.

Riboflavin gels. The separating gel is made by mixing 1 part (by volume) (A), 1 part (D), 1 part (E), and 4 parts (F). The stacking gel is made by mixing 1 part (B), 1 part (D), 1 part (E), and 4 parts (F). These polymerize upon exposure to bright fluorescent light for about 20 min. These gels have a final acrylamide concentration of 3.7%.

Persulfate gels. The gel is made by mixing 0.5 part (A), 0.2 part (C), 0.3 part water, and 1 part (G). These gels polymerize on standing at room temperature for about 20 min. These gels have a final acrylamide concentration of 2.6%.

The gels are polymerized in glass columns with an inside diameter of 5 mm. The total length of a column is approximately 5 cm. When a stacking gel was used, it was about 0.75 cm in length.

The buffer used in the electrode compartments is solution (H).

Samples, either in 0.05 M-Tris buffer, pH 8.1, or in broth, are mixed with a small amount of 10% sucrose to facilitate layering of the sample onto the column. 50 μ l. of sample is mixed with 20 μ l. of 10% sucrose containing enough bromphenol blue to give a visible color to the solution and then layered onto the column. The upper (sample) end of the column is connected to the negative terminal of the power supply.

During electrophoresis the current flow is maintained at about 4 mA per column. This requires from 150 to 300 v potential across the columns. In order to compensate for variation between columns, the time required for the tracking dye (bromphenol blue) to traverse the column is recorded for each column. This time is referred to as 1 time unit and is usually about 40 min. Using this time unit it is possible to estimate the R_F (the ratio of distance moved by the sample to the distance moved by the tracking dye) of samples after long runs in which the tracking dye has left the column.

After electrophoresis, the gels are removed from the glass columns by rimming under water with a small cannula. The dilute gels must be rimmed for their entire length before removal from the tubes to prevent fragmentation. The gels are then placed on a glass slide which is placed on top of a block of aluminum cooled by a dry-ice-acetone bath. After the gels are frozen, they are fractionated by slicing with razor blades. In some cases the gels are sliced by hand into approximately 60 slices. In other cases we use a device consisting of 50 parallel razor blades, bolted together through the central holes, but separated by washers, in order to slice the whole gel at once.

The individual slices of gel are placed in tubes containing 1 or 2 ml. of Tris buffer (0.05 M, pH 8.1) or sodium tetraborate solution (0.05 M) and the phage are generally allowed to elute overnight before assay.

Slices of gel containing [14 C]leucine-labeled particles are eluted in 1 ml. of 0.05 M-sodium tetraborate. After elution, 0.5 ml. is added to 10 ml. of Bray's solution and this sample then counted in a liquid-scintillation spectrometer.

The results obtained with the riboflavin- and persulfate-catalyzed gels have been essentially identical. Presence or absence of the stacking gel has made no detectable difference.

(c) *Sucrose gradients*

Sucrose was dissolved in the same buffer used in the acrylamide gel columns (a seven-fold dilution of solution (A)). Equal volumes of 5% and 20% (w/v) sucrose were used to form a linear gradient in cellulose nitrate centrifuge tubes.

Samples of 0.2 ml. each, in 0.05 M-Tris buffer, pH 8.1, were layered on top of the gradients which were then centrifuged for approximately 1 hr at 37,000 rev./min in the SW50 rotor of a Spinco model L2 ultracentrifuge. After centrifugation, 50 to 60 fractions (2 drops each) were collected into 1 ml. of 0.05 M-Tris buffer, pH 8.1, through a hole punctured in the bottom of the centrifuge tube.

Sedimentation coefficients ($S_{20,w}$) were calculated by the method described by Martin & Ames (1961).

3. Results

(a) Electrophoresis of infective ϕX particles and the separation of capsid mutants

In the first attempts at acrylamide gel electrophoresis of ϕX we used a mixture of ϕX wt and $\phi X H_a H_b$. These strains had previously been separated by sucrose density-gradient electrophoresis (Aach, 1963). Although $\phi X H_a H_b$ is a double mutant from the wild type, only the H_b mutation affects the charge of the phage particle, since Aach found the mobilities of ϕX wt and $\phi X H_a$ to be identical.

A sample containing 5.0×10^4 infective $\phi X H_a H_b$ particles and 7.7×10^4 infective ϕX wt particles was applied to a 2.6% acrylamide gel column. After electrophoresis for 3.5 time units (140 minutes at a current of 4 mA) the column was fractionated and the eluted phage were assayed on mixed indicator (Fig. 1(a)).

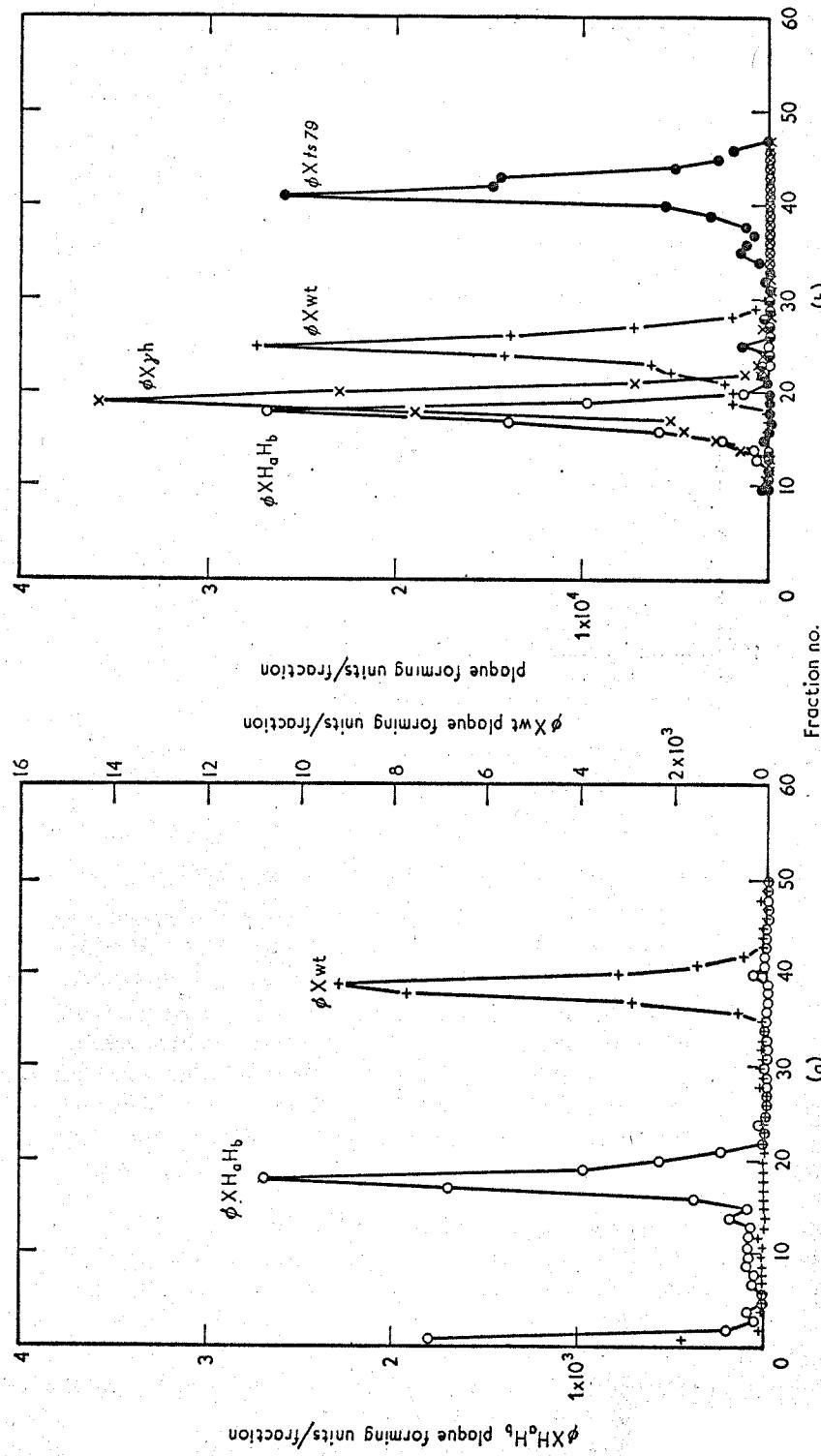
The two types of phage particles are clearly separated by electrophoresis in this system. The peaks are quite sharp and very well resolved from the background. In each case only the two peak fractions exceed one-half of the peak value, and the background is less than 10^{-2} of the peak value. In this experiment 20% of the $\phi X H_a H_b$ and 39% of the ϕX wt infectivity were recovered from the column. A fraction of the phage is trapped at the top of the gel in this experiment. Such trapping does not usually occur.

Although the band width and recovery of infective phage from the gels varies slightly in different experiments, we have not observed any correlation of either of these parameters with the number of phage particles for samples containing 10^4 to 10^{11} infective phages.

A mixture containing four strains of ϕX (each having a different mobility) was run for 2.5 time units in a 2.6% gel (Fig. 1(b)). The mixture is resolved into the four components. Although the difference in mobility between the two host range mutants, $\phi X H_a H_b$ and $\phi X \gamma h$, is quite small, these mutants can be cleanly separated using a longer column. As in the experiments of Fig. 1(a), the resolution between $\phi X H_a H_b$ and ϕX wt is very good, although this is a somewhat shorter run. The mutant $\phi X ts79$ is even further separated from the ϕX wt than are the host range mutants.

The effect of acrylamide concentration on the rate of phage migration in these gels is illustrated in Fig. 2. In this experiment three gels, containing 2.6, 3.6 and 5.3% acrylamide, were run simultaneously. The phage sample contained $\phi X \gamma h$, ϕX wt and $\phi X ts79$. Electrophoresis was allowed to proceed for three time units. After fractionation of the column and assay of the eluted phage on mixed indicator, the R_F of the peak fraction was calculated in each case. Over the range studied, the logarithm of the R_F decreases approximately linearly with increasing acrylamide concentration for all three ϕX strains. Since we are operating at an acrylamide concentration where R_F is dependent on acrylamide concentration, it is conceivable that some of the mutant separations observed are based on differences in size or shape of the phage particles rather than simple charge differences.

Mobility data on several ϕX mutants are summarized in Table 1. In addition to the mutants with mobilities different than wild type, a number of mutants have mobilities indistinguishable from ϕX wt by this method. The mobilities of such mutants were directly compared to ϕX wt by running a mixture of wt and the mutant in the same gel and assaying the fractionated gel for both phage types. The known or suspected function affected by each mutation is also listed in Table 1.



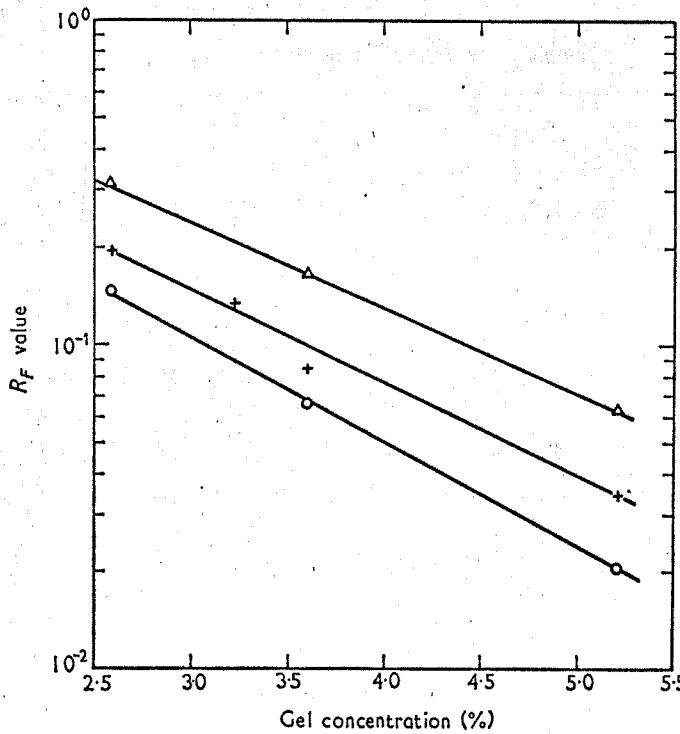


FIG. 2. Dependence of phage mobility on gel concentration at pH 9.5.

—Δ—Δ—, ϕX ts79; —+—+, ϕX wt; —○—○—, ϕX yh. The term R_f is explained in the text.

(b) *Electrophoresis of radioactively labeled 114 s and 70 s ϕX particles*

In the experiments described above, only infective phage particles are assayed. Since ϕX stocks always contain some inactive particles, it was of interest to analyze the distribution of ϕX particles during electrophoresis by some method other than infectivity assay. ϕX am3 labeled with [14 C]leucine was used for this purpose. The 114 s and 70 s components were purified by sucrose gradient centrifugation. The distribution of radioactivity and infectivity of the 114 s material is shown in Fig. 3(a). The two curves are essentially identical in shape. In this experiment about 90% of the input radioactivity was recovered (after correction for quenching). We conclude that the inactive 114 s particles in the stock have very nearly the same mobility as the infective phage.

The mobility of particles such as viruses in general depends only on charges at the surface of the particle. The mobility of the ϕX -associated 70 s particle was studied in order to see if the surface charge of such particles is identical to that of the infective particle. In addition to a small amount of infective 114 s material contaminating the sample, unlabeled ϕX ts9 was added to mark the phage position. (In some runs ϕX ts9 has appeared to separate from ϕX wt by one fraction.) The distribution of radioactivity and infectivity after electrophoresis is shown in Fig. 3(b). The 70 s material moved as a sharp peak about 15% more slowly than the infective virus. In this run about 65% of the radioactivity was recovered from the gel.

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TABLE I
Electrophoretic mobilities of ϕ X strains

ϕ X	R_f (2.6% gel)	Function affected	Reference
<i>ts</i> 79	0.31 \pm 0.05	Capsid protein (serum blocking protein)	<i>a</i>
<i>am3ts</i> 79	0.31 \pm 0.05	Lysis and capsid protein (a double mutant)	<i>a, b, d</i>
wt	0.20 \pm 0.05	None	
<i>am</i> 3	0.20 \pm 0.05	Lysis	<i>b</i>
<i>am</i> 9	0.20 \pm 0.05	Capsid protein (serum blocking protein)	<i>a, b</i>
<i>ts</i> γ	0.20 \pm 0.05	Capsid protein (serum blocking protein)	<i>a, b, c</i>
<i>ts</i> 4	0.20 \pm 0.05	[Capsid structure or assembly and lysis]	<i>a, b</i>
<i>ts</i> 9	0.20 \pm 0.05	Capsid component (different than serum blocking protein)	<i>a, b, c</i>
<i>am3ts</i> 4	0.20 \pm 0.05	[Capsid structure or assembly and lysis (a double mutant)]	<i>a, b, c, d</i>
<i>ts</i> γ h	0.14 \pm 0.03	Adsorption or injection, and capsid protein (a double mutant)	<i>b, c</i>
H_4H_6	0.13 \pm 0.03	Adsorption or injection	<i>e</i>
hr	< 0.02	Adsorption or injection	<i>d</i>

References

- a* — Sinsheimer, Hutchison & Lindqvist (1967).
- b* — Hutchison & Sinsheimer (1966).
- c* — Dowell & Sinsheimer (1966).
- d* — Unpublished results.
- e* — Pfeiffer (1961).

Functions in square brackets are only tentatively assigned. The errors listed are an estimate of the error in determination of the absolute R_f values. The difference between mobilities listed as equal must be much smaller than this error since such equalities are established by failure to separate from a marker phage.

(c) *Electrophoretic analysis of the progeny from mixed infections*

Since it is possible to identify by these means the protein coats produced by various mutant strains of ϕ X, it was of interest to examine the phage coats produced in a cell mixedly infected with two electrophoretically distinguishable strains. It was known from previous work that when broth-grown cells of *E. coli* C are mixedly infected with two ϕ X strains, both genotypes may be represented in the progeny of a single cell (Yarus, 1966); hence both types of single-stranded DNA are synthesized within a single cell. Since mutants defective in the production of the ϕ X serum blocking antigen may be rescued by complementation (Sinsheimer, Hutchison & Lindqvist, 1967), a single cell can probably produce two types of phage coat protein. If mature virus particles are assembled by random withdrawal of coat subunits from a pool containing two types of molecules, one might expect the formation of hybrid phage capsids.

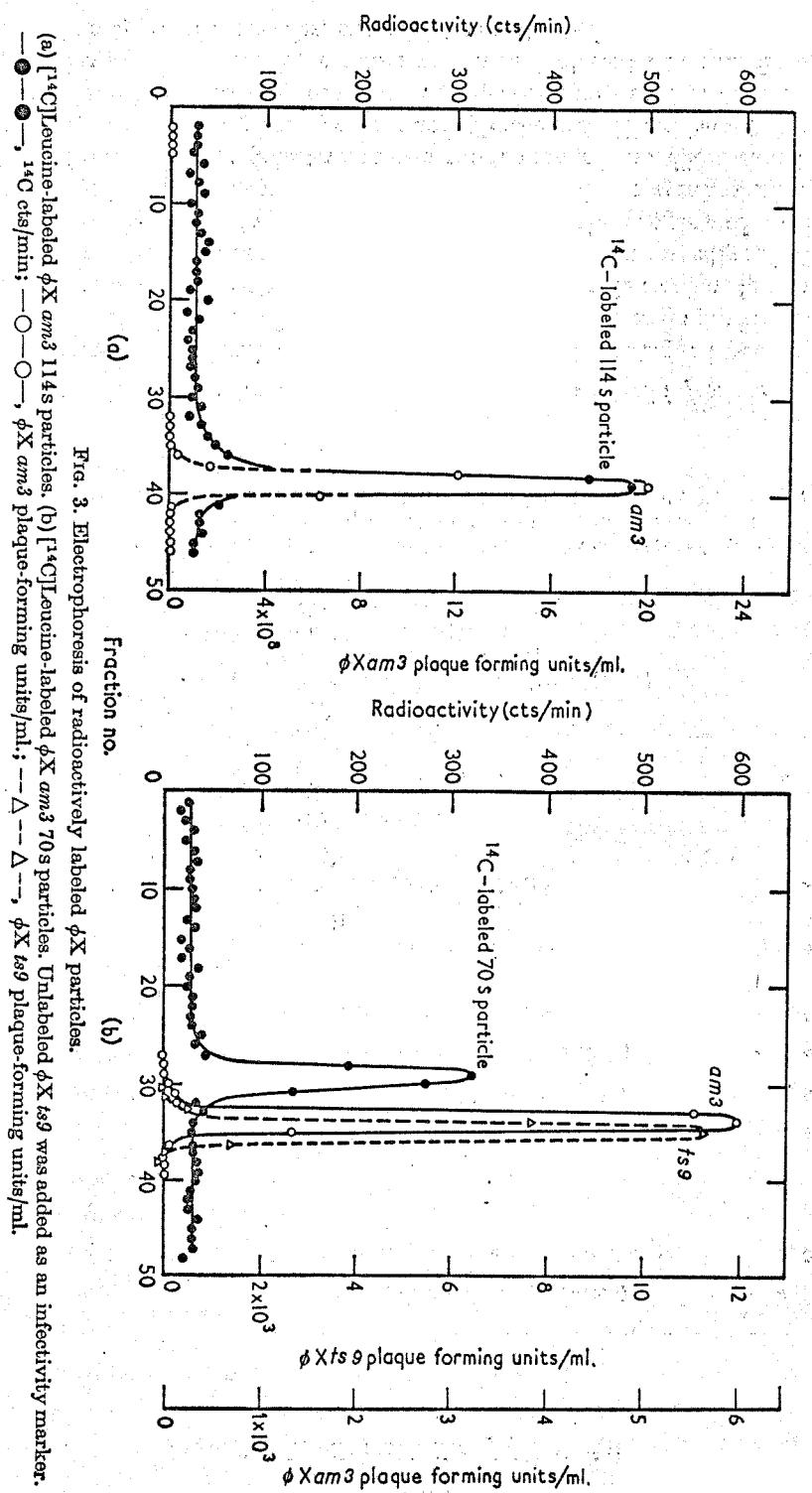


Fig. 3. Electrophoresis of radioactively labeled ϕX particles.
 (a) ^{14}C Leucine-labeled ϕX *am3* 114s particles. (b) ^{14}C Leucine-labeled ϕX *am3* 70s particles. Unlabeled ϕX *ts9* was added as an infectivity marker.
 —●—●—, ^{14}C cts/min.; —○—○—, ϕX *am3* plaque-forming units/ml.; —△—△—, ϕX *ts9* plaque-forming units/ml.

A culture of *E. coli* C grown in KC broth, with aeration, at 37°C, to a concentration of 1×10^8 cells per ml., was infected with a multiplicity of 9 ϕ X H_aH_b and 7 ϕ X wt. Thirty minutes later, after visible lysis had occurred, the cellular debris was sedimented by centrifugation at 10,000 rev./min. Under these growth conditions most of the phage released are adsorbed to cellular debris. Therefore the supernatant solution was discarded and the phage eluted from the pellet by resuspension in 1/20 the initial volume of 0.05 M-sodium tetraborate solution. This sample was diluted tenfold into 0.05 M-Tris buffer, pH 8.1, containing ϕ X ts9 (to serve as a marker for the ϕ X wt position in the electrophoresis experiment). As a control, a synthetic mixture was prepared which contained ϕ X H_aH_b and ϕ X wt (not derived from a mixed infection) and also the ϕ X ts9 marker.

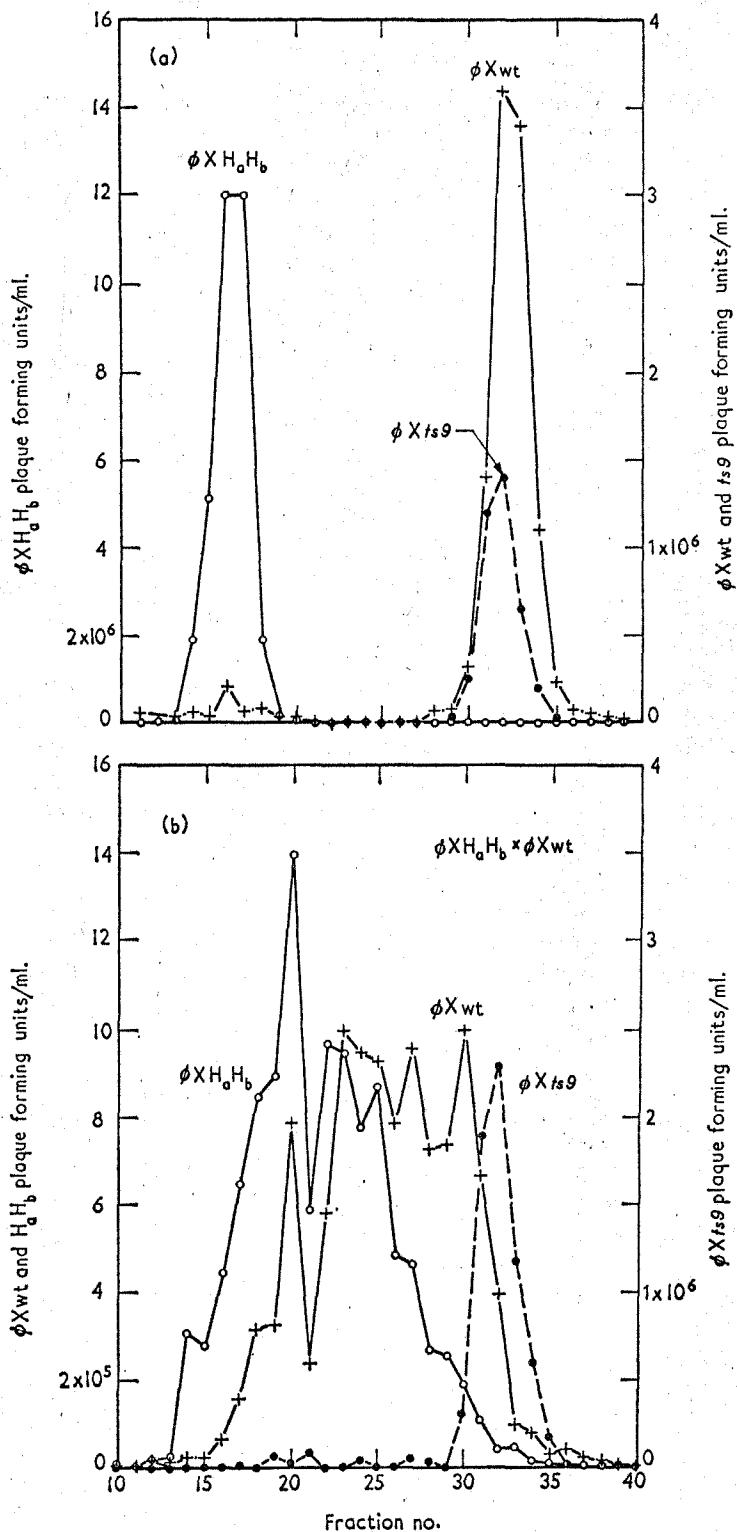
Upon electrophoresis of the control sample, the ϕ X H_aH_b and ϕ X wt were cleanly separated (Fig. 4(a)) as has been shown previously. The ϕ X ts9 migrated at the same R_f as the ϕ X wt. It is therefore a suitable marker for the ϕ X wt mobility.

Electrophoresis of the phage progeny from the mixed infection resulted in a much different pattern (Fig. 4(b)). Both the ϕ X H_aH_b and ϕ X wt peaks are much broader, and the two peaks overlap extensively. This broadening cannot be a peculiarity of this particular gel since the ϕ X ts9 marker peak is quite sharp.

We interpret this alteration of the mobility profile, produced by mixed infection, as resulting from the production of hybrid phage particles containing capsid subunits contributed by both parents (ϕ X (H_aH_b, wt) hybrids). In this connection it should be pointed out that the plaque type formed by such a hybrid phage, when assayed on mixed indicator, should be determined solely by the genotype (DNA molecule) of the particle. All the phage particles can adsorb to the C cells in the mixed indicator to produce a burst of progeny. The adsorption properties of these progeny then determine whether or not the C₁ in the mixed indicator will be lysed, to produce a clear plaque. It will be shown later that the progeny of the hybrid particles, after a cycle of unmixed infection, revert to the parental mobility.

In order to prove that the broadening of the electrophoretic pattern produced by mixed infection is actually due to particles with various mobilities, fractions 20, 24 and 28 from the experiment of Fig. 4(b) were re-run (Fig. 5). In each case ϕ X ts9 and ϕ X γ h were added as markers for the positions of unmixed ϕ X wt and—very closely— ϕ X H_aH_b. The positions of the marker peaks and the relative positions (with respect to the markers) of the original fractions in the experiment of Fig. 4(b) are indicated by arrows. All three re-run samples formed relatively sharp peaks (although not quite as sharp as completely pure ϕ X wt or H_aH_b), which moved with essentially their original mobilities with respect to the marker phage. In each case the two genotypes moved with the same mobility. It thus appears that phage of either genotype released from this mixed infection may have any electrophoretic mobility in the range between the parental mobilities.

To exclude the possibility that these intermediate mobilities are produced by aggregation of ϕ X wt and ϕ X H_aH_b particles in various ratios, some sedimentation experiments were performed. Fractions 20, 24 and 28 from the experiment of Fig. 4(b) were sedimented in sucrose gradients. In each case ϕ X ts9 and ϕ X γ h were added as markers. The results for fractions 20 and 24 are shown in Fig. 6(a) and (b). The phage from the mixed infection sediment at the same rate as the added marker phage. The result for fraction 28 (not shown) was the same. The calculated sedimentation coefficients ($S_{20,w}$) for the three samples are 115 s, 119 s and 115 s. These agree

FIG. 4. Phenotypic mixing between $\phi X H_aH_b$ and ϕX wt.

(a) Control. Electrophoresis of $\phi X H_aH_b$, ϕX wt and ϕX ts9 (not derived from mixed infection).
 —○—○—, $\phi X H_aH_b$; —+—+, ϕX wt; —●—●—, ϕX ts9.

(b) Mixed infection. Electrophoresis of $\phi X H_aH_b$ and ϕX wt from a mixed infection, plus ϕX ts9 added as a marker. Symbols as for (a).

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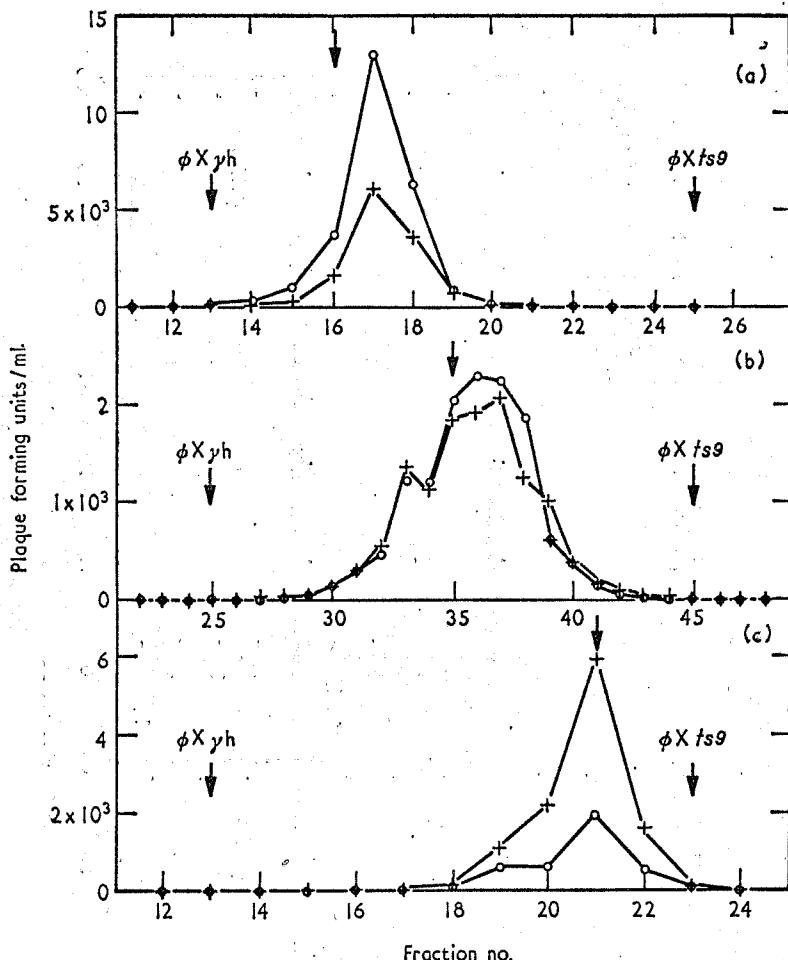


FIG. 5. Re-electrophoresis of hybrid phage particles.

Fractions from the experiment shown in Fig. 4(b) were re-run with added ϕ X yh and ϕ X ts9 markers. The positions of the marker peaks are indicated by arrows. Another arrow indicates the expected position of the re-run fraction with respect to the markers (estimated from the position in Fig. 4(b)).

—○—○—, ϕ X H_sH_b; —+—+, ϕ X wt.

(a) Fraction 20 of Fig. 4(b). (b) Fraction 24 of Fig. 4(b). (c) Fraction 28 of Fig. 4(b).

within the experimental accuracy of the determinations with the previously published sedimentation coefficient of 114 s for non-aggregated particles of ϕ X (Sinsheimer, 1959).

In order to demonstrate that the phage particles resulting from the mixed infection are only phenotypically hybrid, such hybrid phage were used to infect cells at low multiplicity and the progeny were examined by electrophoresis. Phage from fraction 24 of the experiment presented in Fig. 4(b) were used to infect C at a concentration of 1×10^7 cells/ml. in KC broth at 37°C. The multiplicity of infection was approximately 0.1. After allowing ten minutes for adsorption the culture was diluted 100-fold into fresh KC broth. After aeration for an additional 30 minutes the culture was assayed

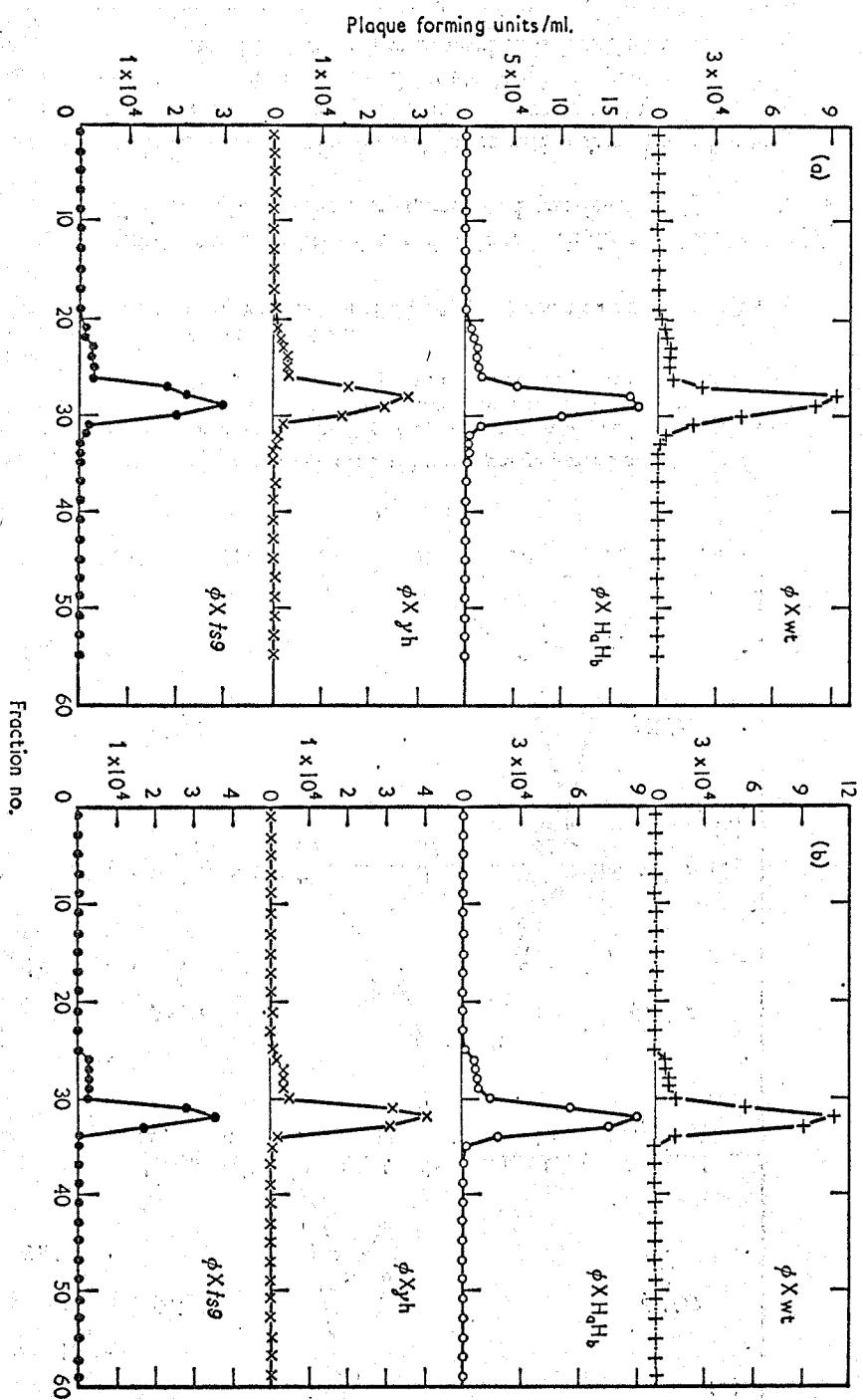


FIG. 6. Sucrose gradients of hybrid phage particles.

Fractions from the experiment shown in Fig. 4(b) were sedimented with added $\phi X yh$ and $\phi X ts9$ markers.
 (a) Fraction 20 of Fig. 4(b). (b) Fraction 24 of Fig. 4(b).

for progeny phage. The infection yielded 200 to 300 progeny phage for each infecting particle.

ϕ X *yh* and ϕ X *ts9* were added to these progeny to serve as markers and the mixture subjected to electrophoresis. The two genotypes ϕ X H_aH_b and ϕ X *wt* were cleanly separated and migrated with about the same R_f as the markers (Fig. 7). This is in sharp contrast to the pattern obtained before the low multiplicity infection (Fig. 5(b)).

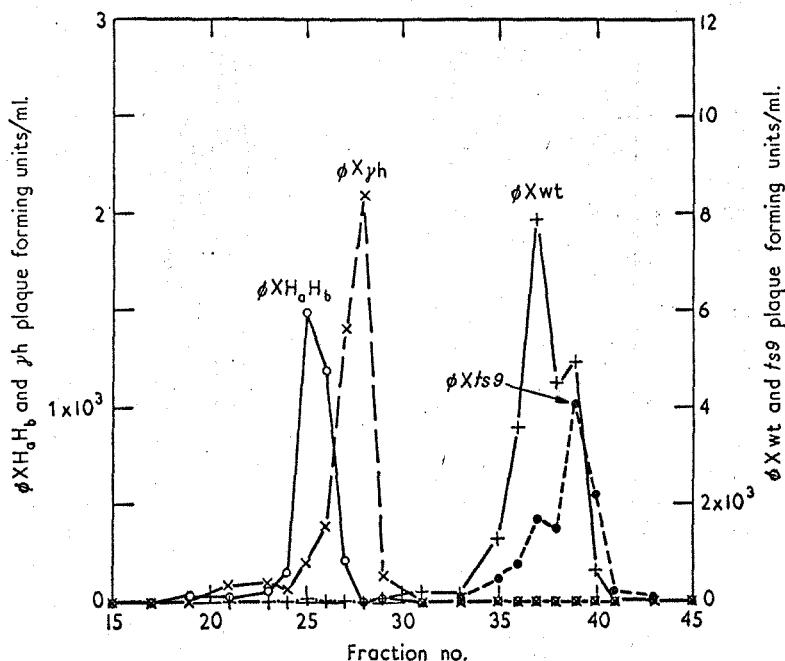


FIG. 7. Electrophoresis of the progeny of hybrid phage particles after a single cycle of infection at low multiplicity.

ϕ X *yh* and ϕ X *ts9* markers were added to the progeny from fraction 24 of Fig. 4(b).
 —○—○—, ϕ X H_aH_b ; ---×---, ϕ X *yh*; ---+---, ϕ X *wt*; —●—●—, ϕ X *ts9*.

We conclude that in a mixed infection between ϕ X *wt* and ϕ X H_aH_b , particles with hybrid capsids are produced. Hybrid capsids have also been observed in experiments with other pairs of mutants, for example ϕ X *am3* and ϕ X *ts79*; also ϕ X *ts79* and ϕ X H_aH_b . The mobility distribution of progeny from mixed infection with ϕ X H_aH_b and ϕ X *ts79* (at a multiplicity of 5 of each parent) is shown in Fig. 8, along with a control showing the distribution of the pure parental phage. The shape of this mobility distribution is somewhat different from that for the ϕ X (H_aH_b , *wt*) hybrids shown previously (Fig. 4(b)). The ϕ X (H_aH_b , *ts79*) hybrids show definite peaks at the positions of the pure ϕ X H_aH_b and ϕ X *ts79* phenotypes, in addition to the broad spectrum of hybrids at intermediate mobilities, whereas this was not apparent in the case of the ϕ X (H_aH_b , *wt*) hybrids. The different multiplicities of infection in the two experiments may be responsible, since another experiment in which cells were infected with only four each of ϕ X H_aH_b and ϕ X *wt* showed a

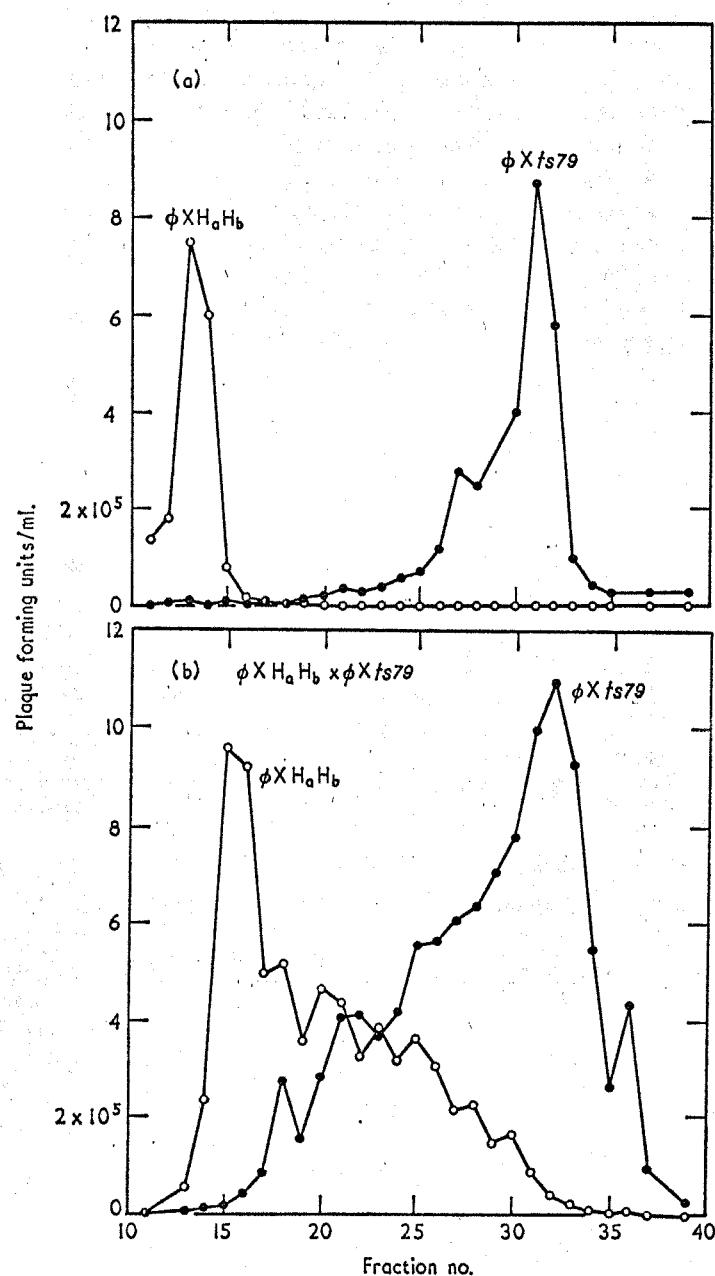


FIG. 8. Phenotypic mixing between $\phi X H_4H_5$ and $\phi X ts79$.

(a) Control. Electrophoresis of $\phi X H_4H_5$ and $\phi X ts79$ (not derived from mixed infection).

—○—, $\phi X H_4H_5$; —●—, $\phi X ts79$.

(b) Mixed infection. Electrophoresis of $\phi X H_4H_5$ and $\phi X ts79$ from a mixed infection. Symbols as in (a).

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distribution more similar in shape to the ϕ X ($H_aH_b, ts79$) distribution shown in Fig. 8(b).

(d) *The phenotype of phage particles with hybrid capsids*

The H_aH_b mutations confer the ability to infect certain strains of *E. coli* that are resistant to infection by ϕ X wt. Two such strains are Cab and C_1 . The ϕ X wt is unable to initiate infection of these cells because it fails either to adsorb or to eclipse. The eclipse and plating properties of ϕ X wt and ϕ X H_aH_b on bacterial strains C, CR and C_1 are shown in Table 2. C_1 clearly is altered in its ability to eclipse either the ϕ X wt or H_aH_b , when the infection is carried out in liquid culture. The ϕ X H_aH_b eclipses more efficiently than ϕ X wt in the strains C and CR. The failure of ϕ X H_aH_b to eclipse with C_1 was surprising. Since ϕ X H_aH_b forms plaques on C_1 , it must be able to eclipse fairly efficiently under the conditions of the plaque assay. These results suggest that the extended host range of ϕ X H_aH_b particles is due to an altered capsid which will adsorb to and eclipse in a cell with an altered ϕ X receptor. For this reason we were interested in determining the phenotype of phage particles with hybrid capsids composed of wt and H_aH_b subunits.

TABLE 2
Effect of the H_aH_b mutations on ϕ X eclipse and plaque formation

Phage	Fraction uneclipsed			Relative e.o.p.		
	C	CR	C_1	C	CR	C_1
ϕ X H_aH_b	0.003	0.005	0.97	1.0	1.0	0.7 ± 0.3
ϕ X wt	0.035	0.12	1.00	1.0	0.6	$< 10^{-6}$

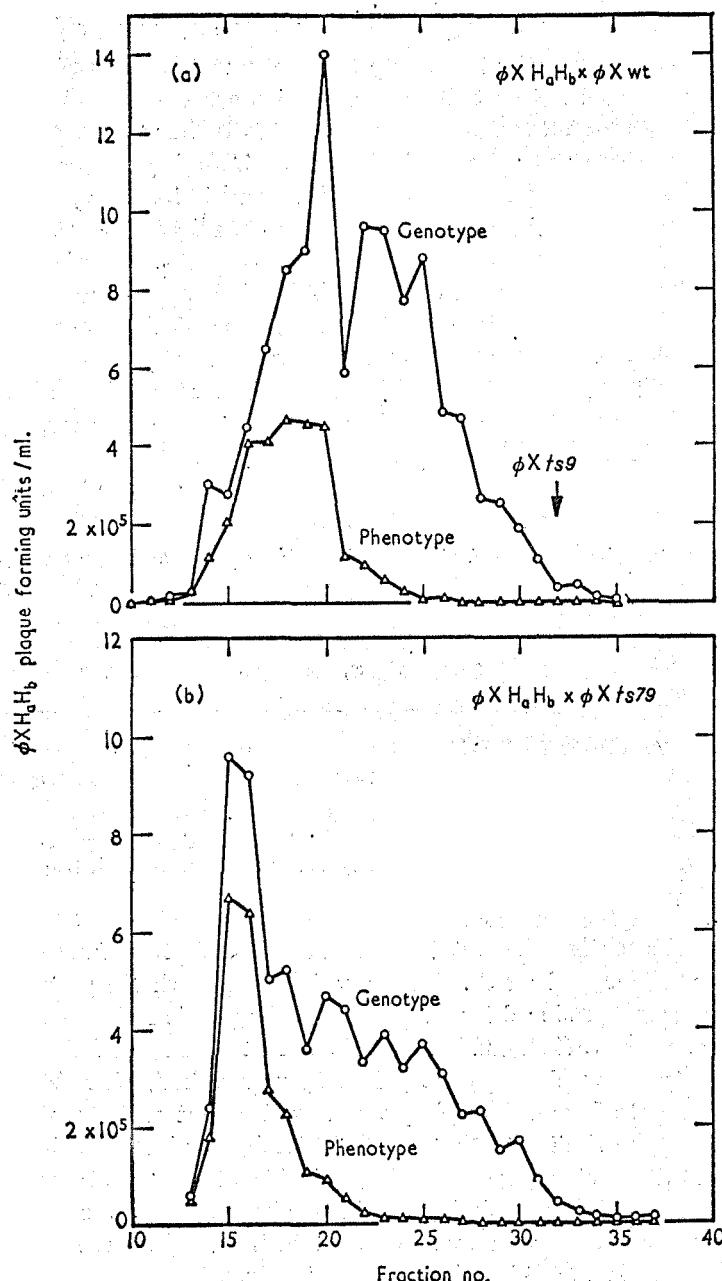
Relative e.o.p. is the ratio of phage titer to that obtained with C plating bacteria. Although the efficiency of plating of ϕ X H_aH_b varies slightly in different experiments, it is quite reproducible within an experiment.

Figure 9(a) shows the result obtained when the hybrid phage from the experiment of Fig. 4(b) were assayed on C_1 . The assay for phage with the genotype H_aH_b on mixed indicator is reproduced for reference, and the position of the ϕ X *ts9* marker is indicated by an arrow. It should be pointed out that in order to form a plaque on C_1 , both the genotype and phenotype of ϕ X H_aH_b are required, whereas only the genotype is necessary to form a clear plaque on mixed indicator. Phage with an electro-phoretic phenotype similar to that of stock ϕ X H_aH_b plate as efficiently as pure ϕ X H_aH_b on C_1 . Hybrid phage containing a significant fraction of wt protein show a greatly reduced efficiency of plating on C_1 . Similar results were obtained when the phenotype measurements were made on another host, Cab. The result of a similar analysis of ϕ X ($H_aH_b, ts79$) hybrids is shown in Fig. 9(b).

4. Discussion

(a) *Electrophoresis of ϕ X particles*

Infective particles of ϕ X move as a single discrete component in the gel electrophoresis system described. The patterns compare favorably with those obtained by Aach (1963), and the system is easy to use and requires only very simple equipment.

FIG. 9. Assay of hybrid phage particles on C_1 .

(a) $\phi X (H_a H_b, wt)$ hybrids. —Δ—Δ—, Phage titer on C_1 ; —○—○—, titer of total $\phi X H_a H_b$ genotype, reproduced from Fig. 4(b).

(b) $\phi X (H_a H_b, ts79)$ hybrids. —Δ—Δ—, Phage titer on C_1 ; —○—○—, titer of total $\phi X H_a H_b$ genotype, reproduced from Fig. 8(b).

It is convenient to run several samples simultaneously. Rough calculations of the mobility of ϕ X wt (in 2.6% gel) assuming constant field strength along the column (probably not valid), agree within a factor of two with Aach's measurement.

The radioactivity of a labeled virus preparation displays the same mobility distribution as does the infectivity of the preparation. This suggests that the non-infective particles present in the stock have a normal protein capsid.

The 70 s particles produced in ϕ X infections are separable from infective virus by electrophoresis. These particles are composed of an apparently intact protein capsid and contain small fragments of viral DNA (Eigner, Stouthamer, Van der Sluys & Cohen, 1963). The 70 s particles are less negatively charged than the intact 114 s particles. Some possible explanations are: (1) the protein capsid of the 70 s particle is altered, or (2) the negative charge on the DNA component of the 114 s particle contributes to the viral mobility (which should depend only on the surface charge of the virus), or (3) the 70 s particle has an increased frictional coefficient, possibly due to a trailing piece of DNA.

(b) *Electrophoretic separation of ϕ X capsid mutants*

We have confirmed Aach's finding of a sizable difference between the mobilities of ϕ X wt and ϕ X H_aH_b . Mobility shifts produced by some other mutations have also been identified. An independently isolated extended host range mutation, the h mutation in the double mutant ϕ X $ts\gamma h$, produces a mobility shift similar to that produced by the H_b mutation. The mobilities of ϕ X H_aH_b and ϕ X $ts\gamma h$ differ by an amount small compared to the separation between these mutants and ϕ X wt. Another ϕ X mutant with altered mobility is the temperature-sensitive mutant ϕ X $ts79$. This mutant cannot replicate at 40°C because it fails to synthesize a protein with serum blocking capacity (Krane, 1966). This mutation produces a mobility change larger in magnitude and opposite in sign to the change produced by the extended host range mutants. ϕ X $ts79$ is more negatively charged than the ϕ X wt, whereas both host range mutants are less negatively charged. It is reasonable to assume that single amino acid changes in the protein subunits of the capsid could produce mobility shifts of the type observed.

Those mutants which exhibit altered electrophoretic mobilities must have some mutational alteration in the structure of the phage particle. This is almost certainly a change in the structure of the protein capsid of the virus. Other types of data on these mutants also implicate an alteration in the viral capsid protein. In the case of the host range mutants the adsorption properties of the phage are altered. In the case of ϕ X $ts79$, mutant-infected cells fail to synthesize serum blocking power at the restrictive temperature, in contrast to the result obtained with the ϕ X wt or with other ts mutants.

Since the host range mutants we have used are not conditional lethals, it has not been possible to use a complementation test in order to determine whether all the electrophoretic mutants fall in a single cistron.

As would be expected, the majority of ϕ X mutants studied have the same electrophoretic mobility as the wild type. These include some mutants for which the available evidence indicates that the mutation affects the structure of the capsid protein, as well as others (such as lysis mutants) in which no alteration in the capsid structure was expected. It is not surprising that certain mutations affecting coat structure have no detectable effect on the mobility.

(c) *The formation of phage with hybrid capsids in mixedly infected cells*

Analysis of the progeny from cells mixedly infected with ϕ X wt and ϕ X H_aH_b (or with other pairs of mutants) demonstrates that phage particles with hybrid capsids are formed. These are detected as infective particles which move with mobilities intermediate between the mobilities of the parental phage. Phage of intermediate mobility isolated from the gel are fairly homogeneous with respect to mobility (and presumably capsid composition) upon re-electrophoresis in a similar gel. A possible alternative interpretation of these results would be that stable aggregates composed of pure particles of ϕ X wt and ϕ X H_aH_b are formed, and that these may contain varying ratios of the two phage types. It would be necessary to assume that these aggregates are formed during infection, not during the electrophoresis run, since control experiments using phage derived from unmixed infections did not show any phage of intermediate mobility. In order to rule out the possibility that the phage of intermediate mobilities are actually such aggregates, several fractions containing hybrid phage were sedimented in sucrose gradients. Samples with mobilities 1/4, 1/2 and 3/4 of the distance between the mobilities of pure ϕ X wt and ϕ X H_aH_b were used for this purpose. In order to account for these mobilities in terms of the aggregate model suggested above, the aggregates would have to contain a minimum of 4, 2 and 4 particles, respectively. All three samples sedimented at the same rate as an added phage marker (presumably unaggregated). Calculated sedimentation constants of 115 s, 119 s and 115 s agree within the errors of the method with the previously published value of 114 s for the unaggregated particle (Sinsheimer, 1959). The change in sedimentation rate which would be produced even by dimerization would certainly be detected in such experiments. An aggregate which is probably a tetramer with a sedimentation coefficient of 155 s was observed by Sinsheimer (1959). This aggregate only formed at slightly acid or neutral pH in very high concentrations of virus (1 mg/ml. or greater), and dissociated on dilution. The sample containing the hybrid phage was at alkaline pH and only contained approximately 10⁹ phage per ml. In view of these facts it seems impossible that the phage observed at intermediate mobilities are in aggregates. We conclude that such phage possess hybrid capsids containing protein subunits contributed by both parents.

The term "phenotypic mixing" has been used to describe the formation of particles containing the genotype of one parent and a phenotype of the other parent (or a hybrid phenotype) in mixedly infected cells (see Stent (1963) for a general discussion). Several investigations of this phenomenon were performed using large phages. The phenotypes observed involved either the host-range or the serum-inactivation properties of the phage, so that the mixing observed concerned only the components of the phage tail (see Streisinger, 1956). In the case of the small spherical RNA phage, f2, Valentine & Zinder (1964) have observed encapsulation of the RNA of a serum-sensitive mutant in the protein coat of a serum-resistant mutant. This phenotypic mixing experiment was not set up in such a way that it would be possible to detect the formation of particles with hybrid capsids. Ledinko & Hirst (1961) have observed the formation of hybrid particles of poliovirus which exhibited serological properties contributed by both parents, and concluded that mixed protein coats were responsible. We find that phenotypic mixing results in the formation of such hybrid particles in cells multiply infected with a pair of ϕ X mutants.

(d) *Estimation of the molecular composition of phage with hybrid capsids*

Under the assumption that the total charge on the virus particle is the simple sum of the charge contributions of the individual subunits, the molecular composition of a hybrid capsid may be inferred directly from its electrophoretic mobility. This seems to be a valid assumption for smaller structures composed of two types of subunits, such as isozymes, which give a series of approximately equally spaced bands. (For example, see the electrophoretic patterns of the lactate dehydrogenase isozymes in the review by Wilkinson (1965).) Under this assumption, the fractional amount of H_aH_b protein in hybrid capsids which contain wt protein as well should vary linearly with position in the gel, ranging from 100% H_aH_b capsids at the position of unmixed ϕ X H_aH_b to 0% (100% wt) at the ϕ X wt position. The apparently continuous distribution of mobilities indicates that the viral capsid contains a large number of identical protein subunits of the type altered by these mutations.

(e) *Phage maturation from the intracellular protein pool*

The existence of phage with hybrid capsids supports the idea that ϕ X particles are assembled by withdrawing subunits from a pool, and that a single pool may be composed of subunits coded by more than one parental phage.

If there is no preferential selection of a particular phenotype of capsid subunit during the actual assembly process, then the capsid composition of phage of a particular mobility should be a reflection of the proportion of the two subunits in the pool from which the phage are derived. (With a large number of subunits per phage the statistical fluctuations will be very small.) We would expect, therefore, that the broad distribution of mobilities observed in the phage derived from mixed infections results from a similar distribution in the compositions of intracellular subunit pools.

Such a distribution in pool composition might arise from heterogeneity among the cells with respect to the relative numbers of infecting phage capable of contributing to the subunit pool. If the proportions of the two subunit types within a cell reflect the number of phage-synthesizing sites within that cell, we might expect that the proportions of the two DNA genotypes derived from that cell would also reflect the relative number of synthesizing sites and thus that there will be a simple correlation between the mobility of the progeny and the proportions of the two genotypes at that mobility.

The fraction of ϕ X wt in the ϕ X (H_aH_b , wt) hybrid phage is plotted as a function of position in the gel in Fig. 10(a). (This is calculated from the data in Fig. 4(b).) The correlation is striking, with the fraction of ϕ X wt increasing linearly throughout the mobility range from the position of pure ϕ X H_aH_b to that of pure ϕ X wt. This same correlation has been observed in other experiments which resulted in a different actual distribution of progeny mobilities (also shown in Fig. 6). This result suggests that the rates of progeny DNA synthesis and capsid protein synthesis carried out under the direction of a particular infecting phage are proportional. This inference is probably valid even if the distribution in pool composition actually is due to causes other than the particular possibility outlined above. If, for example, geographical variation in pool composition due to partial mixing of phenotypically homogeneous pools were responsible, we should have to assume that the ratio of protein to DNA was the same in the various pools in order to account for the result of Fig. 10.

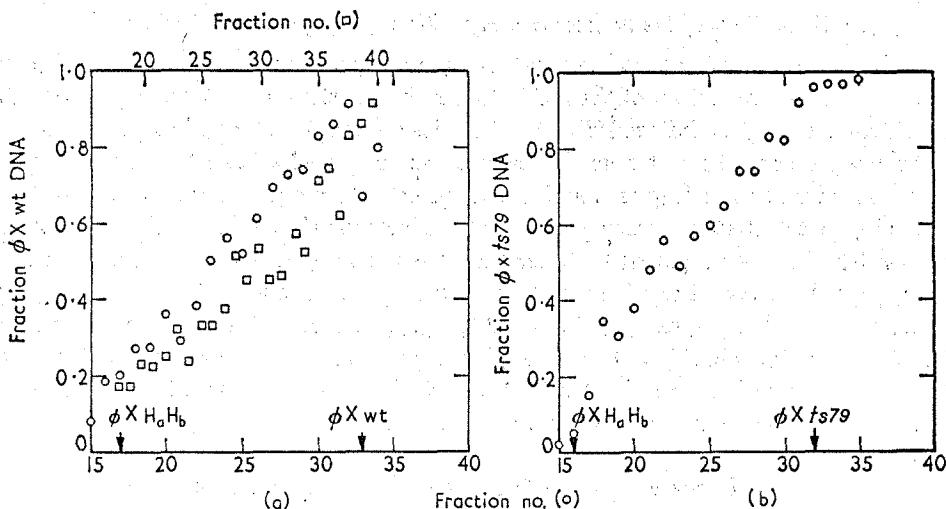


FIG. 10. Proportions of the two genotypes in hybrid phage as a function of mobility.

(a) ϕX (H_aH_b , wt) hybrids. The fraction of ϕX wt genotypes (the ratio of ϕX wt plaques to the sum of ϕX wt plus $\phi X H_aH_b$ plaques) is plotted *versus* fraction number. (○), Calculated from the data presented in Fig. 4(b); (□), data from another mixed infection with the same strains.

(b) ϕX (H_aH_b , ts79) hybrids. The fraction of ϕX ts79 genotypes (the ratio of ϕX ts79 plaques to the sum of $\phi X H_aH_b$ plus ϕX ts79 plaques) is plotted *versus* fraction number. The points are calculated from the data presented in Fig. 8(b).

(f) *The phenotype of phage with hybrid capsids and the nature of the ϕX adsorption process*

It is possible to calculate the fraction of hybrid phage showing the extended host range phenotype of $\phi X H_aH_b$ as a function of the molecular composition of the hybrid particles. We have done this using the selective plating data of Fig. 9, and the result is presented in Fig. 11. The fraction of phage showing the host range phenotype is calculated as the ratio of phage titer on C_1 to the titer of total $\phi X H_aH_b$ genotype on mixed indicator. Since the efficiency of plating of pure $\phi X H_aH_b$ on C_1 is usually less than 1.0, this ratio (C_1 titer/mixed indicator titer) is normalized to 1.0 for the phage with the mobility of pure $\phi X H_aH_b$. The molecular composition of the phage capsids is estimated as described in (d) of this discussion. We estimated the mobilities of the pure parental phenotypes from the position of the marker phage and the control experiment in the case of the ϕX (H_aH_b , wt) hybrids and from the positions of the peaks of pure parental phenotype in the ϕX (H_aH_b , ts79) experiment.

In order to express the extended host range phenotype of $\phi X H_aH_b$, a phage particle with a hybrid capsid must be predominantly composed of H_aH_b subunits. For example, although many particles containing approximately equal numbers of wt and H_aH_b subunits are synthesized in a mixed infection, these plate with only a few per cent efficiency on C_1 (Fig. 11). Several types of models of the ϕX adsorption mechanism might be able to account for this result.

In one type of model the infection specificity could be thought to be a property of the entire phage capsid. The critical property for infection of C_1 might be the total charge on the particle, with the requirement that the charge not exceed some critical

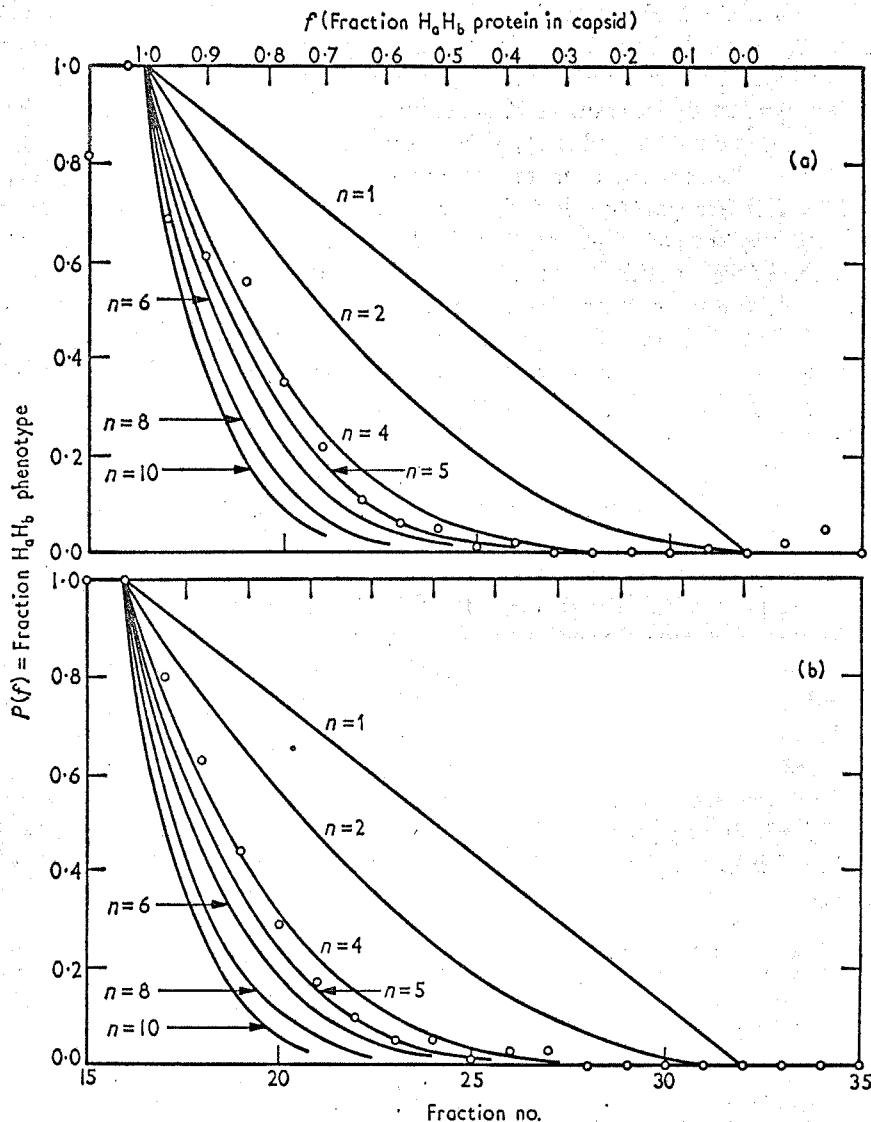


FIG. 11. Variation of phenotype with capsid composition.

Calculated as described in the text. Points represent the experimental data. The curves are plots of the function $P(f) = f^n$ for various values of n (see text).

(a) From the data of Fig. 9(a). (b) From the data of Fig. 9(b).

value in order for adsorption to occur. Or the critical property might be a steric configurational change in the structure of the whole capsid, produced by the H_aH_b mutations.

Total charge on the phage particle cannot be the factor which determines the ability to infect C_1 , since the fraction of hybrid phage showing the extended host range phenotype is the same function of the fraction of H_aH_b subunits in the capsid for the two different sets of hybrids, $\phi X (H_aH_b, wt)$ and $\phi X (H_aH_b, ts79)$. The mobili-

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ties of ϕX wt and ϕX ts79 are quite different, so that a plot of fractional phenotype versus mobility would be different for the two types of hybrids. However, we have no way to exclude other models of adsorption which involve the entire capsid.

Another type of model assumes that adsorption occurs at a particular site on the phage surface, and that the phenotype is determined only by the molecular composition of this site. (There may be more than one such site on the particle if it is assumed that one site is irreversibly selected, at random.) Additional assumptions must be made concerning the phenotype of the particle as a function of the composition of this critical site. We will consider a particular model of this type since it is possible to make some quantitative predictions. Assuming that every molecule in the adsorption site must be H_aH_b in order for the H_aH_b phenotype to be expressed, and assuming that the adsorption site is a random sample of the protein molecules of the capsid, the probability $P(f)$ that a phage with a fraction f of H_aH_b subunits will have the H_aH_b phenotype is

$$P(f) = f^n,$$

where n is the number of molecules in the adsorption site. Figure 11 compares the observed data for both the ϕX (H_aH_b , wt) and ϕX (H_aH_b , ts79) hybrids with the values of $P(f)$ calculated for several values of n . The data are consistent within experimental error with the curve generated by this function, with a value of $n = 5$ (± 1). The value of $n = 5$ would fit nicely with the idea that ϕX adsorption occurs at one of the 12 vertices of the virus (which has icosahedral symmetry), since a five-fold rotational axis passes through each vertex, so that we might expect a cluster of five molecules at this point.

Regardless of the validity of the particular model considered above, the value $n = 5$ is a reasonable lower limit on the number of capsid protein molecules involved in the ϕX adsorption process. The requirement that every molecule in the site be H_aH_b , in order for the phenotype to be expressed results in a minimum estimate of the site size.

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TABLE 1
THERMAL STABILITIES OF $\emptyset X$ MUTANTS

Heat inactivation was measured in 0.025 M Tris, pH 8.1 at 60^oC, as described in Materials and Methods. Each mutant was mixed with a host range mutant with a heat stability which is identical to $\emptyset X$ wt under these conditions, and the ratio of inactivation rates was determined after 10 and 30 min of heating (rate for mutant/rate for wt). The actual ratio of the surviving fractions after 30 min of heating is also tabulated (surviving fraction for mutant/surviving fraction for wt). Mutants which are judged significantly more resistant to heat inactivation than $\emptyset X$ wt are indicated by R, those which are more sensitive by S, and mutants which do not differ significantly from $\emptyset X$ wt by W.

TABLE 1

THERMAL STABILITIES OF $\emptyset X$ MUTANTS

Cistron	$\emptyset X$	t=0 to 10	t=0 to 30	Ratio of surviving fractions at t = 30	Stability
I	<u>am</u> 3	0.87	0.90	2	W
I	<u>am</u> 6	1.10	1.03	0.9	W
I	<u>am</u> 20	0.82	0.86	2	W
I	<u>am</u> 26	1.13	1.00	1.0	W
I	<u>am</u> 27	0.93	0.92	1.5	W
I	<u>am</u> 34	1.70	0.97	1.2	W
<hr/>					
III	<u>ts</u> 41	0.61	0.71	20	R
III	<u>am</u> 9	0.62	0.62	20	R
III	<u>am</u> 23	1.35	1.25	0.2	S
<hr/>					
IV	<u>ts</u> 9	0.73	0.51	100	R
IV	<u>am</u> 14	2.29	1.60	0.02	S
IV	<u>am</u> 16	0.92	0.77	4	R?
<hr/>					
VII	<u>ts</u> 41D	0.79	0.74	10	R

TABLE 2

SBP SYNTHESIS BY ϕ X MUTANTS UNDER RESTRICTIVE CONDITIONS

The experimental procedure is described in the text. Mutants which show a significant increase (at least a factor of 5) in SBP between the early and late samples are indicated by "4". Mutants which did not show a significant increase in SBP because of a high background of SBP at time zero are classified "?". A "--" indicates that no significant amount of SBP was synthesized, above a normal background level. Mutants in parentheses were not included in this experiment, but were studied by Krane (46).

TABLE 2

SBP SYNTHESIS BY ϕ X MUTANTS UNDER RESTRICTIVE CONDITIONS

Cistron	Mutants	Is SBP Synthesized?
I	<u>am</u> 3, 6, 20, 26, 27, 34	+
	<u>am</u> 24	?
II	<u>ts</u> 4, 28	+
	<u>ts</u> 34	?
III	<u>ts</u> 10, 41, 53, <u>74</u> , 81, (8)	+
	<u>ts</u> 106	?
	<u>ts</u> 79	-
	<u>am</u> 9, 23	-
IV	<u>ts</u> (9)	+
	<u>ts</u> 70, <u>95</u>	?
	<u>am</u> 14, 16, 18, 35	+
V	<u>am</u> 10	+
VI	<u>am</u> 8, 30, 33	+

Figure 1. Electron micrograph of $\emptyset X$ am 3.

Purified $\emptyset X$ am 3 was negatively stained with uranyl acetate. The micrograph is printed at a total magnification of 1.8×10^5 X.

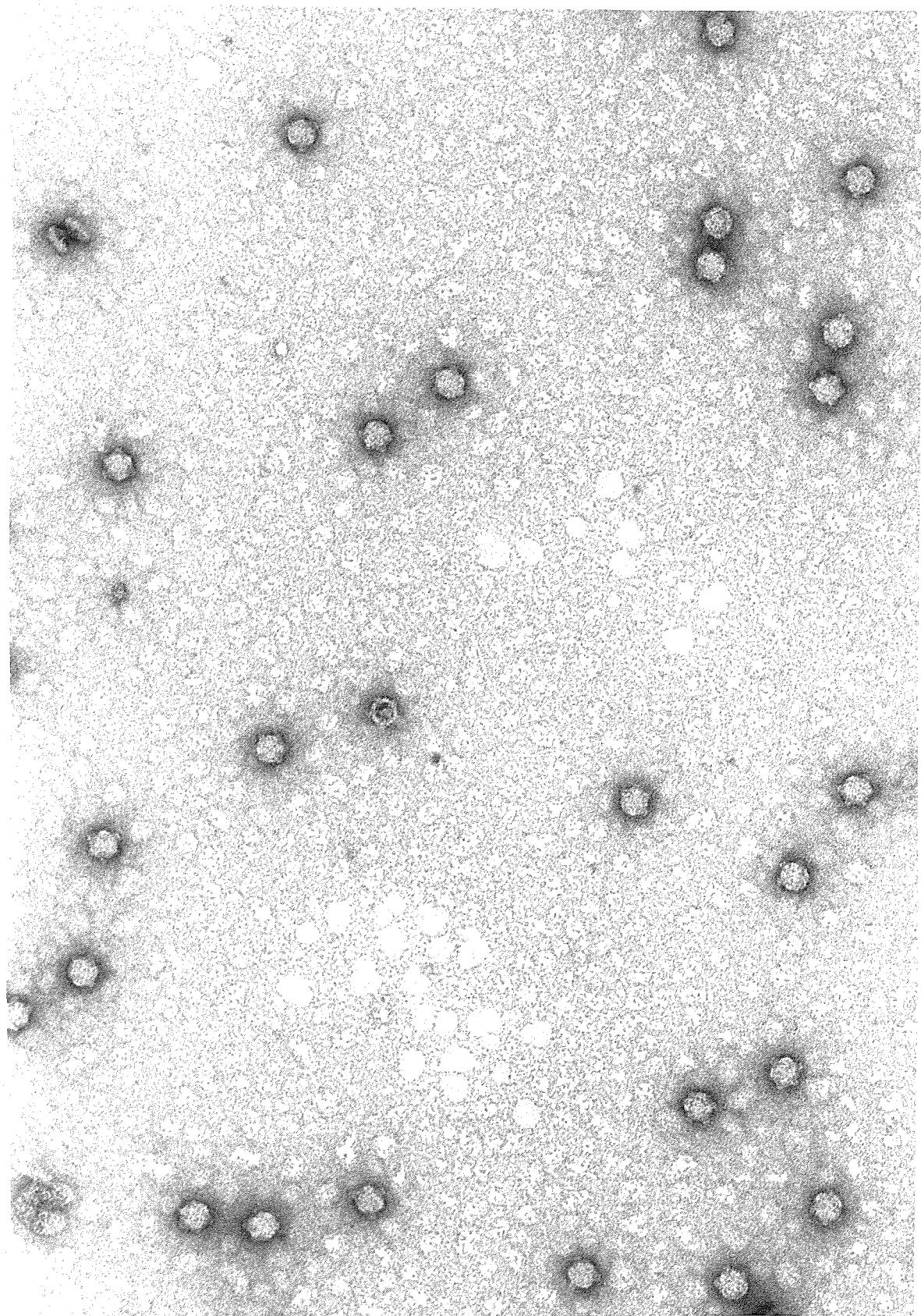


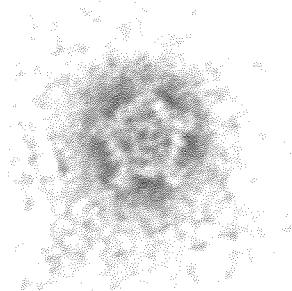
Figure 2. Symmetry of the ϕ X capsid.

This figure shows individual particles of ϕ X am 3 viewed parallel to the 5, 3, and 2-fold rotational symmetry axes of the particle.

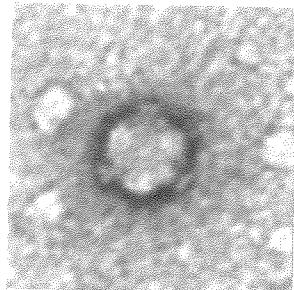
Total magnification is approximately 5×10^5 X.

- a) parallel to the 5-fold axis
- b) parallel to the 3-fold axis
- c) parallel to the 2-fold axis

(a)



(b)



(c)

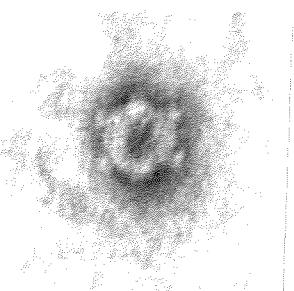


Figure 3. Electron micrograph of \emptyset X am 3 shells, following spike removal

Purified \emptyset X am 3 was treated with 4 M urea (see Materials and Methods), then immediately applied to a grid and negatively stained with uranyl acetate. The micrograph is printed at a total magnification of 3.7×10^5 X.

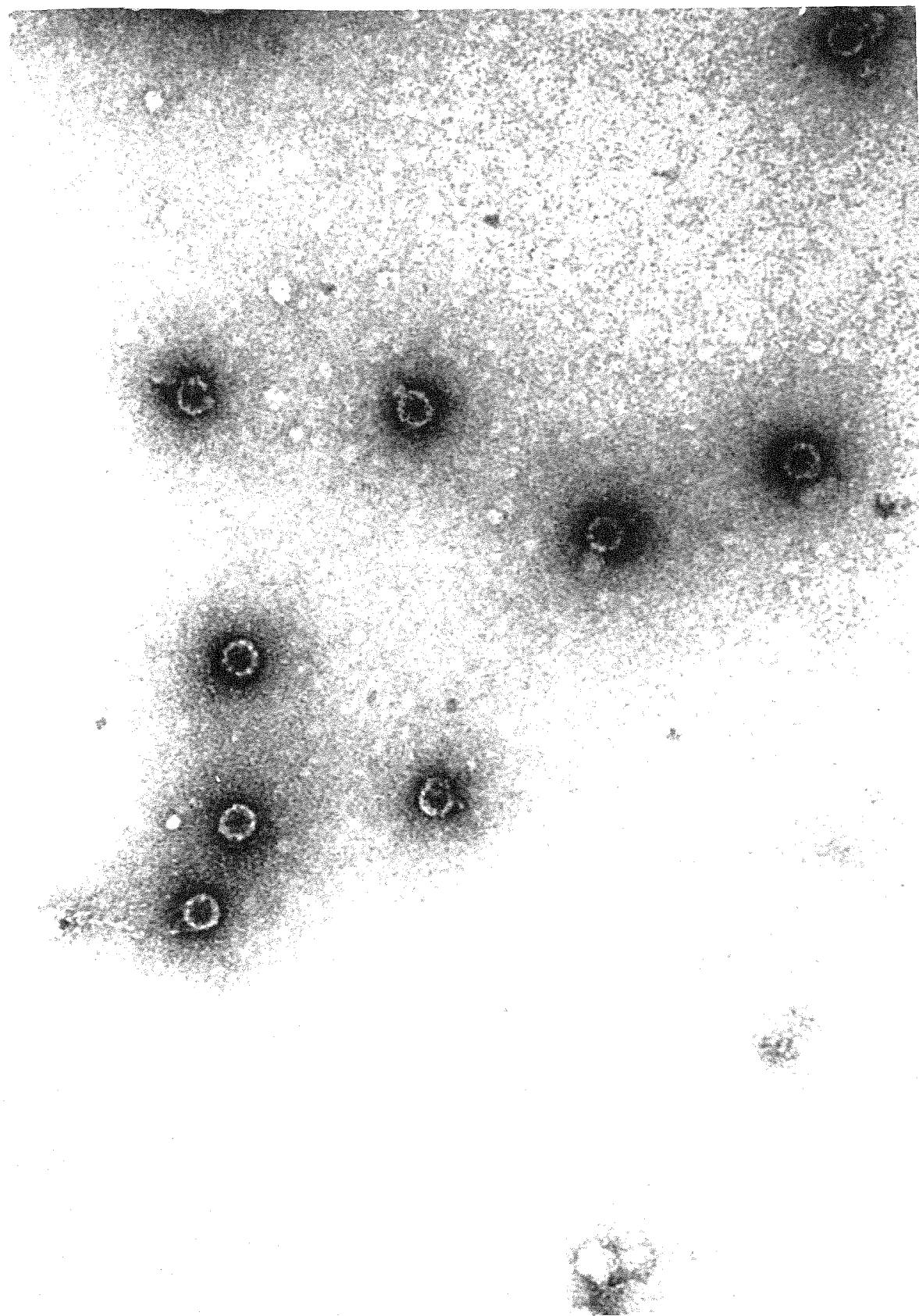


Figure 4. Thermal inactivation of ϕ X wt and ϕ X h1-3.

A mixture of ϕ X wt and ϕ X h1-3, each at a concentration of approximately 1×10^6 pfu/ml, was heated at 60°C in 0.025 M Tris buffer, pH 8.1. Samples were withdrawn at various times and selectively assayed for the two genotypes by plating on mixed indicator bacteria (see Materials and Methods).

• ϕ X wt

0 ϕ X h1-3

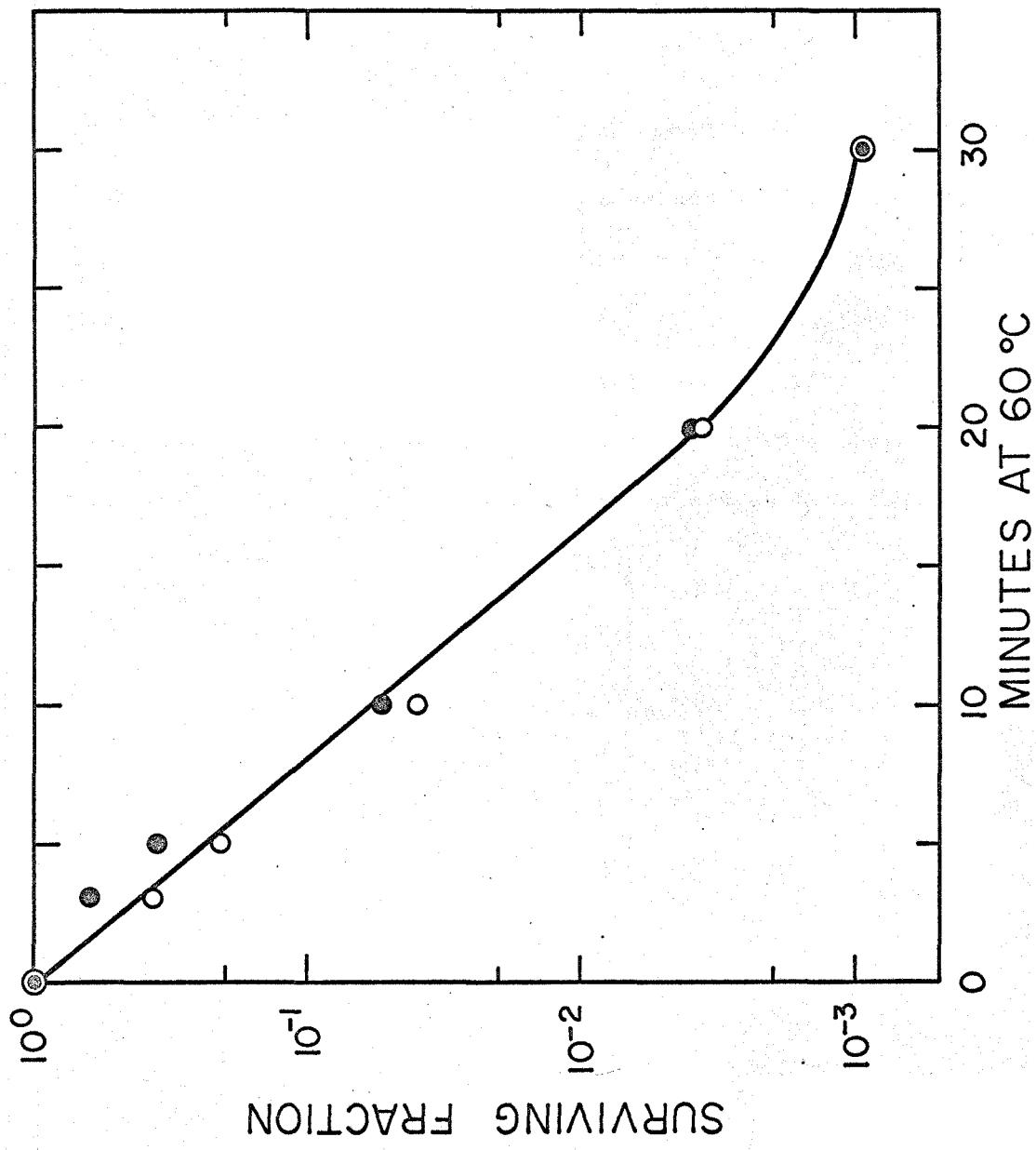


Figure 5. Electrophoresis of $\emptyset X$ coat proteins from disrupted particles.

Particles were disrupted in 9 M urea containing mercaptoethanol, and electrophoresed, as described in Materials and Methods. Abbreviations used: P = proteins from whole phage, C = proteins from 50 S capsid material after 4 M urea treatment, S = proteins from slow sedimenting spike proteins, DP = proteins from defective phage particles. Gels marked "*" were electrophoresed for a longer time than the other gels (approximately 1.5 times as long).

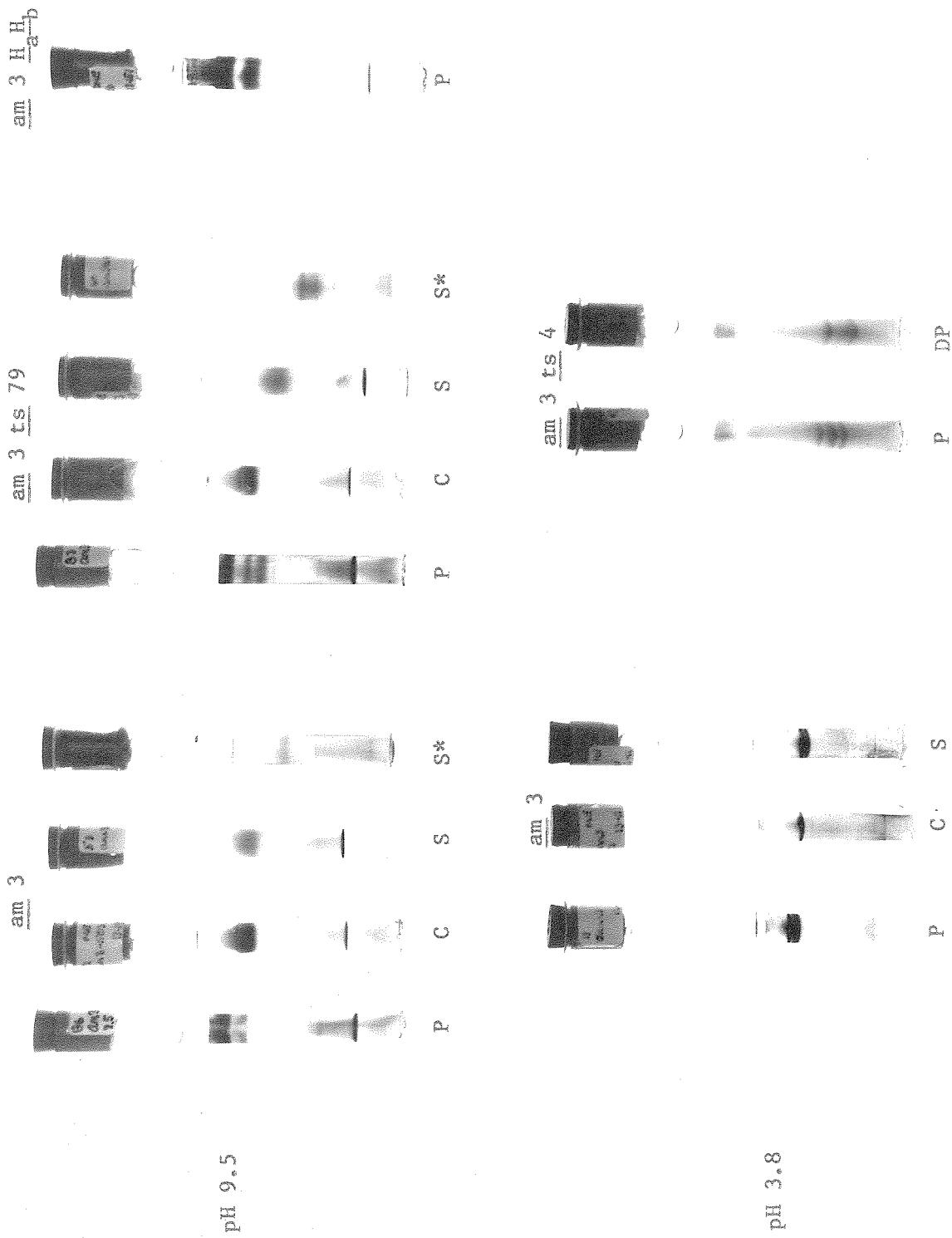
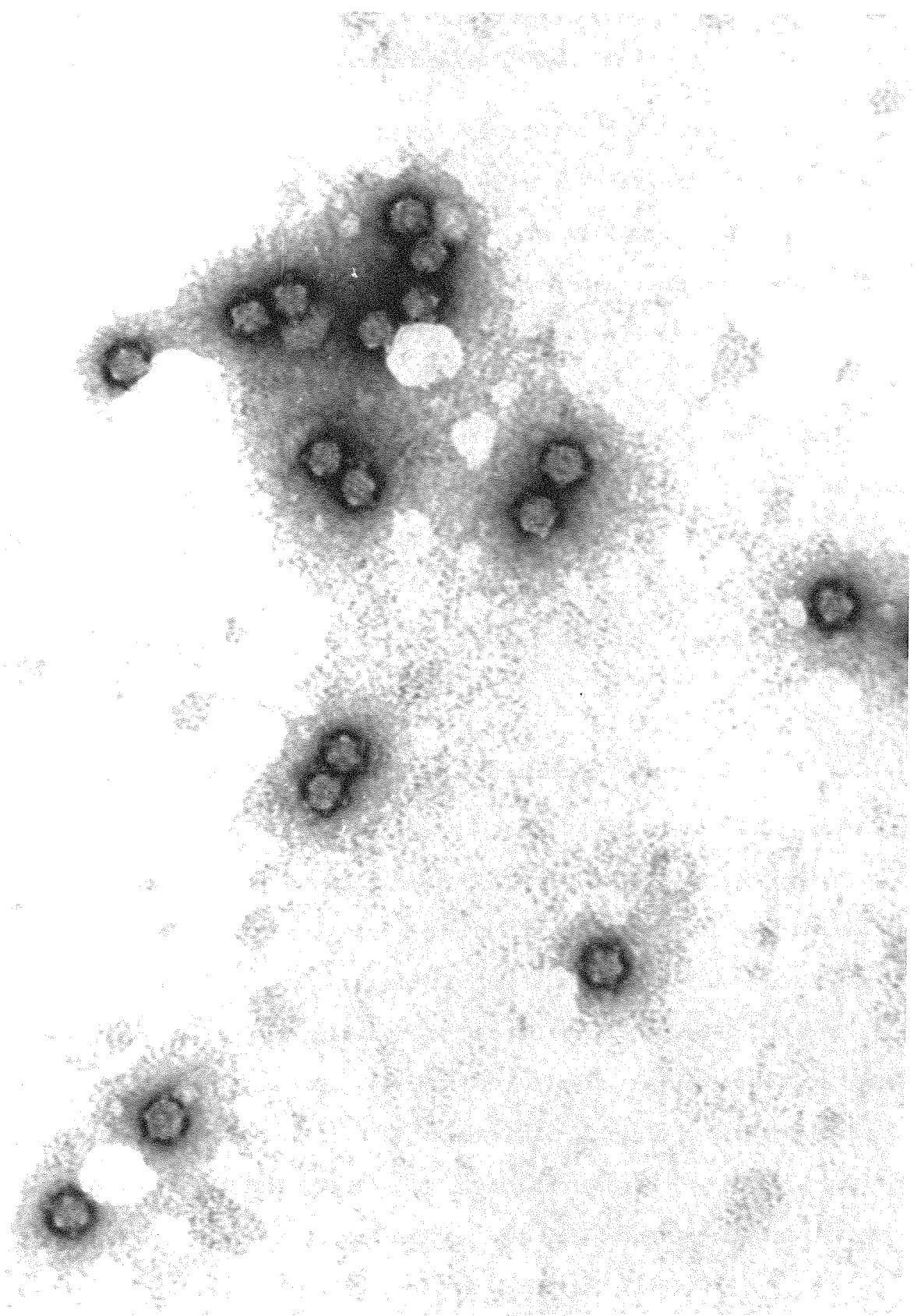


Figure 6. Electron micrograph of am 3 ts 4 defective particles.

Purified am 3 ts 4 defective particles were negatively stained with uranyl acetate. Total magnification is 3.1×10^5 X.



3.4 DNA SYNTHESIS GENES

Mutants of $\emptyset X$ have been found which alter the normal pattern of DNA synthesis following infection in several ways. The product of at least one cistron (VI) is essential for the replication of RF-DNA. Several additional cistrons, including some coat structural genes (III, IV, and probably VII) and one gene with a poorly defined function (V), are necessary for the formation of SS-DNA.

The extended latent period which is made available for study following infection with lysis-less mutants of $\emptyset X$ (cistron I), has made it possible to detect two new characteristics of DNA synthesis in $\emptyset X$ -infected cells. These are: 1) $\emptyset X$ -induced cessation of host DNA synthesis (67), and 2) the exclusion of superinfecting phage from the synthesis of parental RF-DNA (68).

3.4.1 Genes essential for RF replication.

$\emptyset X$ mutants in cistron VI are similar in several respects to mutants in one cistron of the closely related phage S13 (S13 cistron IV). Both sets of mutants are very poorly rescued by complementation, and exhibit aberrant lysis kinetics, suggesting that similar functions are defective. Tessman (51) has shown that the S13 suppressible mutants of this type are able to form parental RF following infection of the restrictive host, but that this RF is not able to replicate (as measured by infectivity). For this reason it was decided to investigate DNA synthesis in $\emptyset X$ amVI-infected cells. The following experiment was performed in collaboration with Bjorn H. Lindqvist (109).

If ϕ X-sensitive host cells, defective in their ability to repair UV induced lesions (HCR⁻), are treated with the drug Mitomycin C, bacterial DNA synthesis is halted while ϕ X is able to reproduce normally within the treated cells (109). It then becomes possible to label the newly synthesized ϕ X-DNA selectively by the incorporation of ^{3}H -thymidine. After extraction of DNA from the infected cells, the various forms of ϕ X-DNA may be resolved by preparative band centrifugation. These procedures are described in Materials and Methods (Part 3.1).

The results of such an experiment, in which cells infected with an am mutant in cistron VI (am 33) were labelled for 12 min. from the time of infection at 40°C, is shown in Fig. 7. At the same time an identical experiment was performed with an am mutant in cistron V (am 10), as a control. In the am 10 infection no single-strand DNA was made, however a normal amount of RF was synthesized (about 20 molecules per cell). Infection with am 33 resulted in a greatly reduced synthesis of RF; again no single-strands were formed. The small amount of radioactivity incorporated into RF in the am 33 infection is approximately enough to account for the conversion of input parental single-strands to RF. It appears from this result that am 33 is able to form parental RF, but not to replicate RF under restrictive conditions. The interpretation has been strengthened by further experimental work of B.H. Lindqvist (109) and A.J. Levine (personal communication).

It is of interest to attempt an interpretation of the other effects of mutation in cistron VI, in terms of the observation that the product of this gene is essential for duplication of ϕ X RF. The phenomenon of

asymmetric complementation observed with cistron VI mutants can be explained by assuming that the protein product of this gene somehow remains associated with the RF molecule which supplied the information for its synthesis, and can only function in the replication of this molecule. This is perhaps most readily visualized in terms of an RF-mRNA-ribosome-nascent-protein complex, attached to a bacterial site. It is possible to interpret the delayed lysis observed with am VI mutants either simply as a gene dosage effect on the rate of synthesis of the cistron I product, or as a result of some necessary connection between the rate of protein synthesis from an RF template and replication of that RF (53).

A.J. Levine (personal communication) has obtained evidence that the mutant ts 41D (cistron VII) is temperature-sensitive in its ability to synthesize RF. The mutant appears somewhat leaky, however, and the results are less clearcut than with cistron VI mutants. It seems possible that these results are a result of the complex nature of the ts 41D mutation(s). Experiments with the newly discovered op mutants in cistron VII should clarify this situation.

3.4.2 Genes essential for single-strand synthesis.

It might be hoped that the screening of $\emptyset X$ mutants for the ability to produce viral single-strands under restrictive conditions of infection would lead to the identification of a specific gene involved in the synthesis of this novel form of DNA. Surprisingly, however, such experiments have shown the failure to produce single-strands to be the rule rather than the exception.

Mutation in cistron II, IV, V, VI, or VII results in a block to the synthesis of viral single-strands (45, 109, A.J. Levine, personal communication). Three of these cistrons (III, IV, and VII) appear to code for coat structural proteins (Part 3.3). One of these cistrons (cistron VI) is essential for RF duplication. In view of the evidence that viral single-strands are derived from progeny RF molecules (64) it seems reasonable to assume that the failure of cistron VI mutants to synthesize single-strands is a direct consequence of the block to RF replication. The nature of the role played by the cistron V product in the synthesis of single-strands is not clear. In view of the large number of functions necessary for single-strand synthesis it would be premature to assume that the cistron V product is "directly" involved.

The failure of a variety of coat protein mutants to make single-strands suggests that incorporation of a single-strand into a phage particle is necessary for its synthesis. It should be noted that the only two cistrons in which mutation does not block single-strand production do synthesize either phage (cistron I) or phage-like particles (cistron II) under restrictive conditions of infection. In this connection it is important to demonstrate that the single-strands produced in infection by the cistron II mutant ts 4 (Part 3.3) are actually contained in the defective phage particles which are synthesized. An experiment to prove this point has been performed in collaboration with S.G. Krane (46, 116). Strain C was infected at 40°C with a multiplicity of 2 ts 4 plaque forming particles per bacterial cell. 25 min. later a

sample of the infected culture was treated with lysozyme and versene to insure lysis of the infected cells, and analysed by sedimentation in a sucrose gradient. The fractions collected from the sucrose gradient were assayed for SBP, for infective phage, and for infectivity to spheroplasts both before and after phenol extraction. It should be noted that under these conditions essentially all the SBP and infective DNA present after 25 min. of infection are newly synthesized during the infection. In this experiment the total amount of SBP in the 25 min. sample was approximately 50 times that present in a 5 min. sample. The small amount of SBP present in the 5 min. sample was not above the background level after sedimentation in a sucrose gradient. The results of sedimentation analysis of the material formed during 25 min. of infection by ts 4 at 40°C are shown in Fig. 8. The majority of the SBP sediments at about 71 S. The defective phage particle produced by ts 4 sedimented just slightly more slowly than residual infective phage (114 S) which was present in the preparation, at 107 S. In addition, SBP components sedimenting at approximately 43 S and 21 S were observed.

The majority (approximately 80 to 85%) of the infective single-strands, recovered from the gradient fractions by phenol extraction, sediment in the defective particle (107 S) peak, indicating that the single-strands synthesized at 40°C by ts 4 are actually contained in the defective particles. Less than 1% of this infective DNA can be accounted for by the intact phage particles (produced due to leakiness of ts 4) which sediment at 114 S. The remaining 15 to 20% of the in-

fective DNA present in the gradient fractions sediments in the trailing edge of the 71 S peak (about 64 S) and at approximately 21 S, again coincident with SBP components.

It seems reasonable to suppose that the particles containing infective DNA, which sediment at 64 S, trail the peak at 71 S because of a larger frictional coefficient resulting from a greater length of extruded phage DNA. (The majority of 70 S particles, at least in wt infection, contain only a non-infective fragment of a DNA molecule.) The infective material at 21 S is particularly interesting. It appears that this DNA is associated with a SBP component which renders the DNA non-infective to spheroplasts until it is removed by phenol extraction. It is not known whether this material represents an intermediate in the assembly of the defective phage particle, a breakdown product, or simply a non-functional byproduct of the infection.

The result of this experiment confirms the idea that single-strands are produced only in situations where they can be incorporated into a phage-like particle. It is tempting to hypothesize that the act of particle synthesis is actually essential for single-strand synthesis. Perhaps the nascent single-strand is pulled from an RF molecule by the process of coat assembly.

3.4.3 Superinfection exclusion - a ϕX gene function?

ϕX -infected cells acquire the ability, as the infection proceeds, to exclude superinfecting ϕX particles from reproducing themselves. The physiological basis of this exclusion has been described (68).

this paper is reproduced in Part 3.2 of this thesis). We have used the non-lysing mutant am 3(I) as the primary infecting phage, and then superinfected with $^{32}\text{P}^{15}\text{ND-wt}$. A centrifugal analysis of the intracellular DNA showed that the superinfecting phage was unable to form parental RF molecules under conditions of exclusion. The superinfecting DNA remains as an infective, and therefore presumably intact, single-strand. Superinfecting phage do attach, and go into eclipse as measured by plating chloroform treated infective centers. Cistron I function cannot be performed by excluded phage (as would be expected since no - strand is formed to serve as a template for mRNA synthesis). Exclusion normally takes 5 to 10 min. at 37°C to be fully established, but is not established or is much delayed in the presence of KCN.

Additional experiments have been performed in order to determine whether protein synthesis is necessary for the establishment of superinfection exclusion. Strain C was pretreated with chloramphenicol (CAM) at 30°C afor 10 min. in KC broth. Six cultures were treated, two with 100 $\mu\text{g}/\text{ml}$, two with 30 $\mu\text{g}/\text{ml}$, and two controls with no CAM. One culture at each concentration of CAM was then infected with a multiplicity of 15 plaque formers of unlabeled $\emptyset\text{X}$ am 3 ts h (a triple mutant containing an am mutation in I, a ts mutation in III, and an extended host range mutation). After 30 min incubation at 30°C , all six cultures were infected with a multiplicity of 0.5 pfu of $^{32}\text{P}^{15}\text{ND } \emptyset\text{X}$ am 3. The cultures were incubated for an additional 40 min, chilled, the cells collected by centrifugation and lysed by treatment with lysozyme and versene, and the lysate phenol extracted. Each resulting nucleic acid

preparation was mixed with a small amount of a viral single-strand preparation derived from unlabelled $\emptyset X$ ts \checkmark , brought to a density of 1.72 by the addition of CsCl and centrifuged to equilibrium at 30K rpm in an SW 39 rotor. The contents of the centrifuge tubes were fractionated by drop collection, and alternate fractions were dried on planchets for the determination of ^{32}P or assayed for infectivity to spheroplasts. Progeny phage from the spheroplast infections were assayed selectively for the various genotypes involved (am 3 ts h was plated on CR/ $\emptyset X$ at $30^{\circ}C$; am 3 on HF 4714 at $40^{\circ}C$; ts \checkmark on C at $30^{\circ}C$). The results of these analyses are presented in Fig. 9.

The distribution of parental ^{32}P provides an assay for exclusion. In each gradient, a fraction of the radioactivity bands as free heavy labelled single-strands (peak at fraction 20 to 30). Hybrid density RF has a density nearly the same as light single-strands (indicated by the ts \checkmark marker at about fraction 50). In the absence of preinfection the majority of parental ^{32}P is found as hybrid RF, either in the absence of CAM, or in the presence of 30 or 100 micrograms CAM per ml. The preinfected control culture without CAM exhibits exclusion. Less than 10% of the ^{32}P bands at the position of hybrid RF. However, the presence of CAM at either 30 or 100 $\mu g/ml$ appears to prevent the establishment of exclusion. In the presence of CAM the majority of parental ^{32}P is converted to hybrid density RF by the preinfected cells.

The infectivity assays give information concerning replication of the viral DNA. In the absence of CAM progeny single-stranded DNA is formed. Some light am 3 single-strands are apparently formed in the

preinfected culture, presumably by the small fraction of parental strands which were not excluded. The amount of am 3 infectivity found at the light single-strand position is reduced by a factor of approximately 10^2 by preinfection. In the presence of 30 $\mu\text{g}/\text{ml}$ of CAM the am 3 RF is able to replicate both in singly infected and in preinfected cells, resulting in a band of fully light RF infectivity. The quantity of replicated RF appears to be smaller in the case of the preinfected culture, presumably because am 3 RF represents a small fraction of the replicating pool in this case. The quantities of infectivity at the position of the light single-strand marker suggest that a very small number of single-strands may be made in the cultures containing 30 $\mu\text{g}/\text{ml}$ of CAM, particularly on the culture without preinfection. (RF is about 20 times less infective, per molecule, than single-stranded DNA.) Detectable replication of RF is not observed in this experiment at 100 $\mu\text{g}/\text{ml}$ of CAM.

This experiment demonstrates that CAM treatment can block the establishment of the ability to exclude superinfecting phage from synthesis of parental RF. This is true both at levels of CAM which (almost completely) prevent single-strand synthesis, but allow RF replication, as well as at CAM concentrations high enough to prevent RF replication. When exclusion is blocked by low levels of CAM, the RF formed by the superinfecting phage is able to replicate. These results suggest that some protein must be formed following infection in order for exclusion to be established, and that the synthesis of this protein is prevented by 30 $\mu\text{g}/\text{ml}$ of CAM. This result does not tell us whether

the protein is a host or a virus gene product. We can probably eliminate the cistron VI product as the causative agent of exclusion, since its synthesis is CAM resistant.

A similar type of experiment can be performed in order to ask whether continued protein synthesis is necessary for the maintenance of superinfection exclusion, once it has been established. C was pre-infected with am 3 ts ✓ h at 30°C. After 30 min of incubation (which is sufficient for exclusion to be established), CAM was added to the culture to give a concentration of 30 μ g/ml. After an additional 30 min the culture was superinfected with $^{32}P^{15}ND$ am 3. The culture was chilled 40 min. later and DNA prepared and banded as in the previous experiment. Two control experiments were performed at the same time. One sample was simultaneously infected with both phage types at time zero (multiplicities of both phages are the same as in the previous experiment). This sample should not show exclusion of the am 3 labelled phage. Another sample was superinfected 30 min after the primary infection in order to demonstrate that exclusion actually had been established by the time CAM was added to the experimental culture. Both of these cultures were harvested 40 min. after infection with the labelled am 3. Half of the second control culture was treated with 0.05 M sodium tetraborate and 0.2% versene in order to see whether excluded phage DNA can be eluted by this procedure. The cells were collected by centrifugation, resuspended, the supernatant solution and the resuspended cells were counted to determine the distribution of ^{32}P , and DNA was prepared from the cells. The resulting distributions of radioactivity

following equilibrium centrifugation in CsCl are shown in Fig. 10. The results suggest that CAM can at least partially reverse superinfection exclusion, since the amount of ^{32}P at the position of hybrid RF in the CAM treated sample is intermediate between the two types of control samples. The reason for the broadness and splitting of the ^{32}P band at the hybrid RF position is not known. It may be some kind of artifact due to viscosity of cellular DNA which bands near this position; however, the ts \vee single-strand marker peaks were symmetrical. A fraction (about 25 to 30%) of the excluded phage DNA was removed by the borate-versene treatment, indicating that at least part of the parental DNA remains extracellular.

It appears, on the basis of these results, that continued protein synthesis is necessary in order to maintain complete exclusion. The nature of the protein involved is not known. One might suppose, for example, that the incoming viral single-strand is blocked from the formation of RF by forming a complex with free phage coat protein. In CAM the pool of coat protein could be depleted, allowing superinfecting phage to now form RF. In order to test this idea, and the more general idea that the product of one of the known $\emptyset\text{X}$ cistrons is necessary for exclusion, mutants in all seven cistrons should be tested to see whether they can establish exclusion.

Figure 7. Sedimentation of am 33 (VI) and am 10 (V) DNA.

Strain HF4714, grown in T3A medium at 37°C to a concentration of 8×10^7 cells/ml, was treated with Mitomycin C (109). The cells were collected by centrifugation and resuspended in fresh T3A medium containing 10 micrograms of thymidine/ml. The culture was split into two portions which were infected at 40°C with the mutants am 33 and am 10, each at a multiplicity of approximately 7. Tritiated thymidine was added at the time of infection to give a concentration of $20 \mu\text{c}/\text{ml}$ of culture (a specific activity of approximately $2 \mu\text{c}/\text{mg}$ of thymidine). After 12 min of infection the cells were collected, and DNA was extracted and analysed by sedimentation in a preformed CsCl gradient at neutral pH (2 hr at 37 K rpm in a SW39 rotor). Single-stranded DNA from the mutant ts ∇ h was added as a marker. Fractions were collected in 0.05 M Tris (pH 8.1). Aliquots were removed for infectivity measurements and radioactivity was determined by TCA precipitation, collection on glass filters, and counting in Toluene-Liquiflor in a liquid scintillation counter.

—●— radioactivity (^3H labeled DNA)

—○— infectivity (ts ∇ h SS marker)

The arrows at 0.415 and 0.36 of the total length of the gradient mark the expected positions of RF I and RF II, respectively.

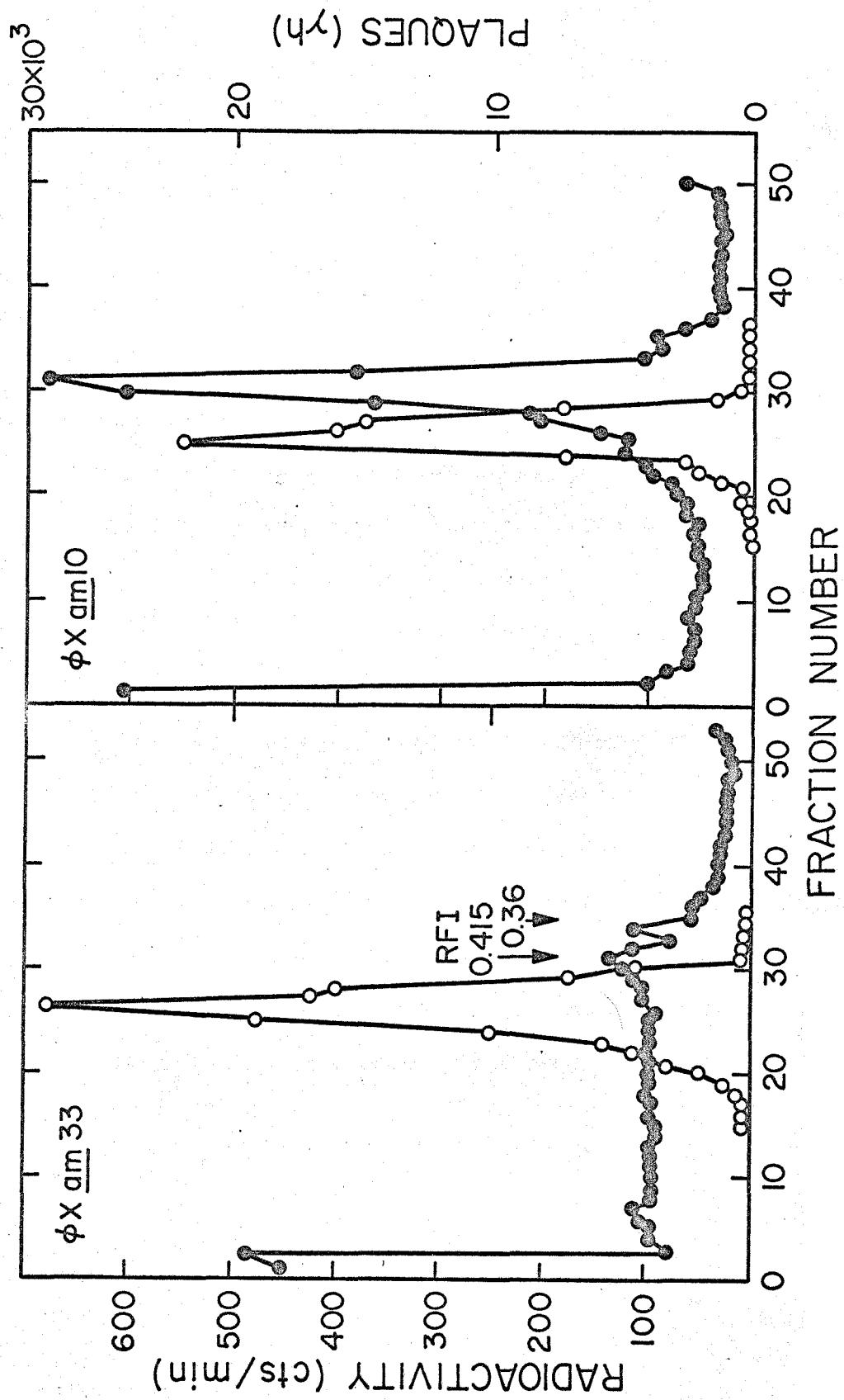


Figure 8. Sedimentation analysis of SBP and infective DNA produced by ts 4 at 40° C.

A lysate was prepared from cells infected with ts 4 at 40° C as described in the text. 1 ml of lysate was layered on a 25 ml, 5 to 20% sucrose gradient and centrifuged in a SW 25 rotor (46). The gradients were fractionated following centrifugation, and assayed for SBP and infective phage. (A few infective phage are produced due to leakiness of the ts mutation.) The position of the infective phage peak is indicated by the arrow marked 114 S. S values indicated for other peaks are simply the ratio of distance sedimented to the distance traveled by infective phage, multiplied by 114 (not true sedimentation constants). Each fraction was assayed for infectivity to spheroplasts, before and after phenol extraction. The infectivity values before phenol extraction are corrected for infective phage trailing from the peak at 114 S, by subtraction. The only significant infectivity before phenol extraction, above the background from phage, is in the 21 S peak. It should be noted, however, that small amounts of infectivity at higher S values could have been obscured by the phage background.

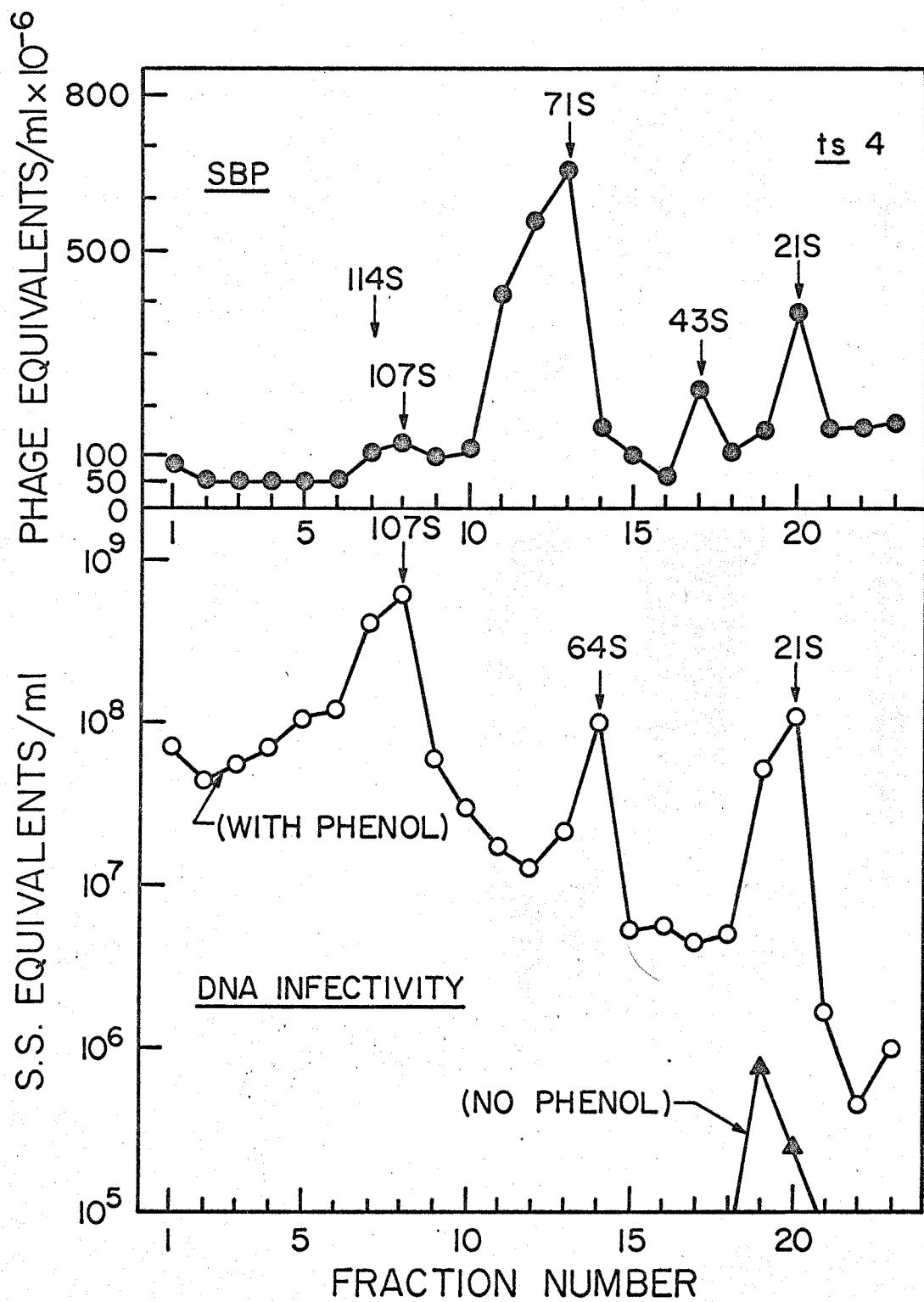


Figure 9. Inhibition of superinfection exclusion by chloramphenicol.

Chloramphenicol (CAM) treatment, pre-infection, and superinfection were performed as described in the text. The distribution of parental ^{32}P (from superinfecting $^{32}\text{P}^{15}\text{ND} \emptyset \text{X am 3}$), and the infectivity of am 3 ts h (primary infection), am 3 (superinfection), and ts (light SS marker DNA peak indicated by arrow) are shown following equilibrium CsCl density gradient centrifugation.

- a) no CAM, no preinfection
- b) 30 micrograms per ml CAM, no preinfection
- c) 100 micrograms per ml CAM, no preinfection
- d) no CAM, preinfection
- e) 30 micrograms per ml CAM, preinfection
- f) 100 micrograms per ml CAM, preinfection

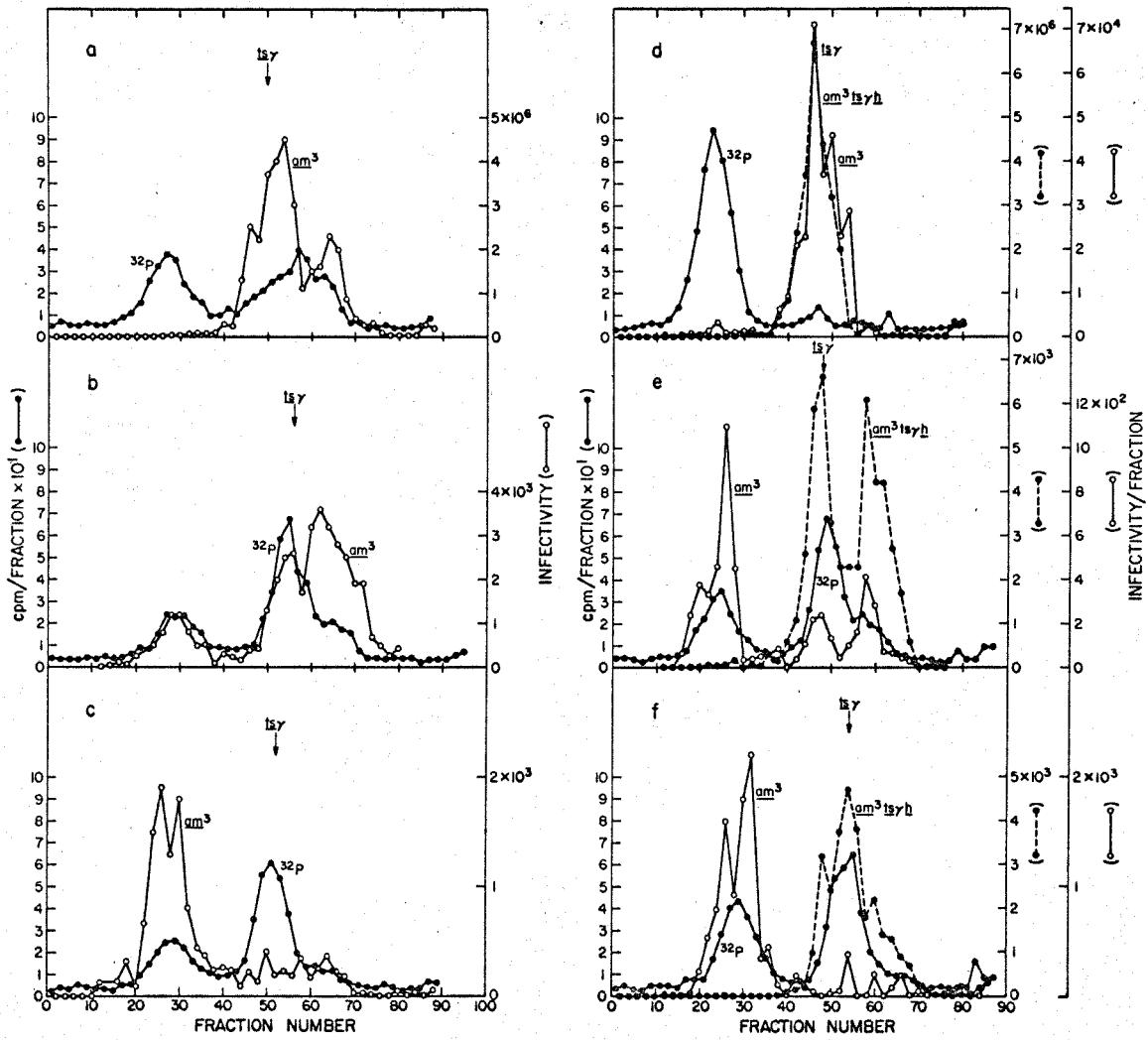
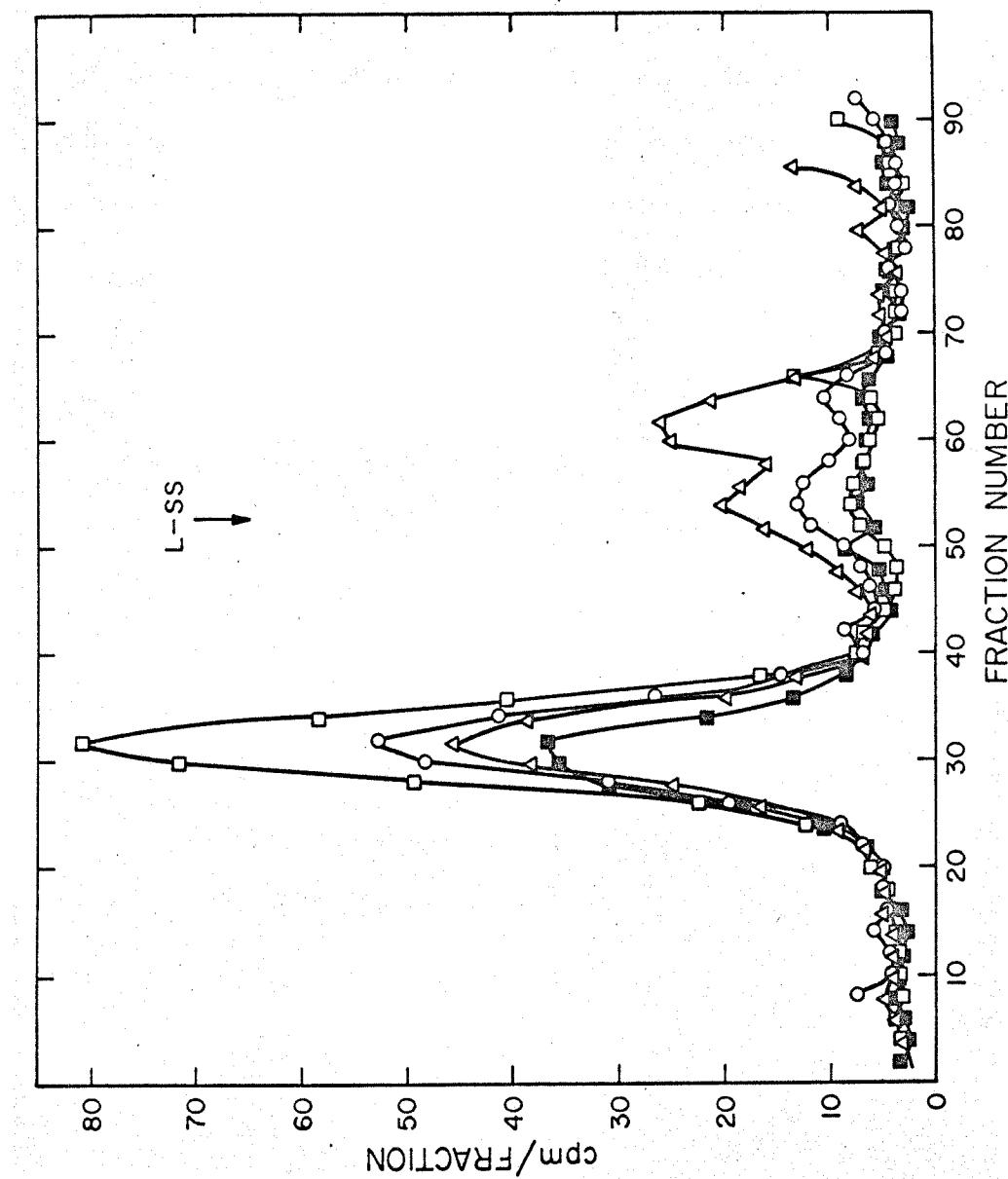


Figure 10. Reversal of superinfection exclusion by chloramphenicol.

The infections were performed, and DNA was extracted and banded, as described in the text. Distributions of parental ^{32}P following equilibrium CsCl density gradient centrifugation are plotted.

- Δ — Δ simultaneous infection (no exclusion control)
- \square — \square superinfection experiment without CAM (exclusion control)
- \blacksquare — \blacksquare superinfection experiment without CAM, eluted with borate-versene
- 0 — 0 superinfection after CAM treatment (experimental sample)

The patterns have been shifted horizontally so that the ts ∇ infective single-stranded DNA markers coincide (indicated by arrow L-SS).



3.5 OTHER GENES - CISTRON V

There is at present no definitive evidence concerning the function of the cistron V product. am mutants in this cistron initiate infections in the restrictive host, replicate a normal amount of RF-DNA, but make no SS-DNA, produce SBP, and lyse the host. In all these respects they behave identically to mutants in cistrons III and IV which code for protein components of the phage coat. However, no alterations in the physical properties of the phage particle have been found to be associated with mutations in cistron V. In addition, the number of coat structural genes already identified appears to be sufficient to account for the proteins observed by electrophoresis following disruption of the phage.

In view of the available information concerning the defect in cistron V mutants, and the known physiological steps in the ϕ X infection, it seems reasonable to suggest that cistron V is either required in some specific way for the synthesis of SS-DNA, or in the (apparently simultaneous) process of phage maturation.

4. CONCLUSIONS AND GENERAL DISCUSSION

4.1 THE CISTRONS OF ϕ X AND THEIR FUNCTIONS - A SUMMARY

4.1.1 Mutants

Several types of conditional lethal mutants of ϕ X have been characterized. These include missense mutants of the ts (temperature-sensitive) type, and nonsense mutants of the am (amber) and op (opal) types. These am and op mutations are believed to be mutations to the nonsense triplets UAG and UGA, respectively. Preliminary results with ss (solvent-sensitive) missense mutants have also been obtained. These mutants are more sensitive than the wt (wild-type) to the presence of denaturing solvents such as urea, dimethylsulfoxide, and ethylene glycol, during plaque formation.

Mutants of ϕ X which exhibit altered host range have been selected. These include both extended (h) and restricted (hr) host range mutants. Some mutants have been isolated which appear to have acquired both the ts and the h phenotype as the result of a single mutation.

A number of multiple mutants have been constructed. Multiples of the type am-ts have been constructed by recombination, and ts-h and am-h multiples have been made by mutation of the ts or am strains as well as by recombination. It is also possible to recover triple mutants of the composition am-ts-h as recombinants from the cross am x ts-h.

4.1.2 Cistrons

Complementation studies of the ts and am mutants (as well as a few op mutants) have resulted in the identification of seven complementation

groups or cistrons in ϕ X. These cistrons are referred to by the numerals I through VII. We believe that these represent seven genes of the phage which code for seven different polypeptide chains which are essential for the normal reproductive cycle of the virus.

4.1.3 Cistron functions

Physiological experiments have been performed with representative mutants from the seven complementation groups in an attempt to elucidate the roles of the various gene products during the course of the normal infection. These studies have included an analysis of the abortive infections resulting from growth of conditional lethal mutants under restrictive conditions and also determinations of the properties of mutant phage particles produced under permissive conditions. Results of these studies are summarized in Table 1.

Cistron I. The protein product of this gene is necessary for lysis of the infected cell. am mutants in this gene are able to replicate normally in the restrictive host except that the newly synthesized phage are not released. Phage synthesis continues for an extended period under these conditions so that burst sizes of several thousand phage per cell are produced (some ten times the wt burst size).

The role of the cistron I product in the lytic process is not known. No one has been able, so far, to detect a lytic enzyme associated with ϕ X infection (42, 73, 71). It seems possible that this protein somehow interferes with the normal process of cell wall repair, thereby causing lysis without the need for a lytic enzyme of the type associated with

larger phages such as T4 or λ (126,127). It should be noted that the lytic process of the large phages is quite complex and requires more than the simple production of a lysozyme. Mutants of both T4 and λ have recently been discovered which are defective in lysis and which mimic the properties of $\emptyset X$ cistron I mutants by continuing phage maturation for periods much longer than the wild-types (128,129). These mutations do not map in the structural genes for the phage lysozymes. It is not known whether the resemblance between mutants of these apparently regulatory genes of the large phages, and mutants of $\emptyset X$ cistron I, is more than superficial.

Cistron II. The available evidence strongly indicates that this cistron codes for a structural component of the spikes projecting from the phage coat. One representative of this cistron, ts 4, has been carefully studied. This mutant produces a phage-like defective particle which is not infective to intact bacterial cells, but which contains viral DNA infective to spheroplasts. The particle appears normal in the electron microscope, but was shown to be missing one of the protein components of the phage spikes by electrophoretic analysis of the proteins obtained by disruption of the particles. It is concluded that cistron II is the structural gene for the missing spike protein.

Cistron III. This cistron codes for another component of the spikes which carries the serum-blocking antigen. Mutations in this cistron may result in alterations of the stability of mutant phage particles to thermal inactivation, and in alterations of the electrophoretic mobility of the phage particle. Both of these results indicate that the product of this gene is a structural component of the phage particle. Electrophoretic

analysis of the protein components of an electrophoretic mutant in cistron III shows that one of the spike proteins exhibits an altered mobility. am mutants and one ts mutant in this cistron fail to synthesize SBP under restrictive conditions of growth. Another ts mutant has been shown to be unable to assemble its SBP protein into phage size particles.

Mutants in this cistron (as well as the other coat cistrons IV and VII) do not synthesize single stranded DNA. It thus appears that coat proteins are somehow involved in the process of synthesis of viral single strands.

Cistron IV. This cistron codes for another protein component of the phage particle which we feel is probably (by a process of elimination) the main structural protein of the phage capsid. Several mutants in this cistron show alterations in the thermal stability of mature phage produced under permissive conditions. We have not yet detected any changes in electrophoretic mobility of the phage particles resulting from mutation in this cistron which suggests, albeit weakly, that the protein product of this cistron may be electrically shielded from the external field (as we might expect the capsid of the phage to be shielded by the spikes). Both am and ts mutants in this cistron produce SBP under restrictive conditions. One ts mutant which has been studied appears unable to assemble this SBP protein into phage size particles. Cold-sensitive (cs) mutants of $\emptyset X$ (45) which have also been characterized as coat protein mutants, appear to be in cistron IV (C.E. Dowell, personal communication). Since the available evidence indicates that cistrons II, III, and VII are structural genes for components of the phage spikes, this leaves cistron

IV as the only likely candidate for the main capsid protein.

Again, mutants in this coat gene cannot make single-stranded DNA under restrictive growth conditions.

Cistron V. We do not yet know the function of the product of this cistron. The only function necessary for normal reproduction which we know is not performed in the presence of a defect in this cistron, is the synthesis of single-stranded DNA. However, since single-strand synthesis is clearly an intricate process, involving unexpectedly at least three protein components of the phage coat, we can only speculate concerning the molecular function of the cistron V product. It seems possible that this protein could be a nuclease or polymerase which is specifically needed for the process of single-strand synthesis. It also seems possible that this protein could be required for single-strand synthesis by virtue of some role which it plays in the structure or assembly of the virus particle.

Cistron VI. The product of this cistron is a protein which is necessary for the replication of RF. Why this protein is necessary for RF replication is not known, although some pertinent information is available. The fact that mutants in this cistron are rescued only very poorly, if at all, by complementation suggests that the cistron VI protein is not freely diffusible within the cell, but rather remains associated with the RF template from which it was translated (or with the particular bacterial site to which this RF is attached). It appears that the protein is not needed for attachment of the RF to the bacterial membrane, since attachment occurs in the absence of synthesis of the protein (A.J. Levine,

personal communication). The possibility of obtaining purified preparations of this cistron product (65) should be extremely helpful in determining its function. Obvious possibilities such as a polymerase or nuclease activity of this material should be carefully investigated.

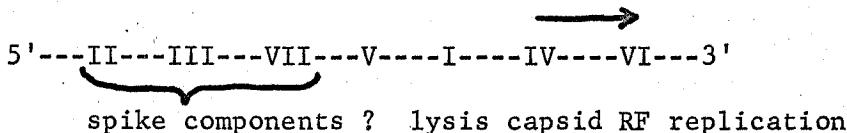
Cistron VII. This cistron appears to code for a third protein component of the phage spike, and is probably the cistron in which extended host range mutations occur. A mutant in this cistron (ts 41D) is very closely linked by recombination to an extended host range mutant. This same cistron VII mutant is abnormally resistant to thermal inactivation, further suggesting that it codes for a structural component of the phage particle. This latter result is obscured by the fact that ts 41D appears to contain, in addition to a ts mutation in VII, another mutation (probably a very leaky ts) at some other site. Experiments with recently discovered op mutants in this cistron, and an am mutant isolated by Hayashi which falls in this cistron should provide additional evidence. It should be noted here that $\emptyset X$ cistron VII appears to be homologous to cistron I of the closely related phage S13 (cistron VII is homologous to cistron E of Hayashi, which corresponds to S13-I (134)), and that this S13 cistron has also been implicated as a structural protein of the phage particle in which mutational changes can produce altered host range (107). Electrophoretic analysis of the dissociated proteins has shown that one of the spike proteins is altered by a host range mutation. Electrophoresis of infective phage particles produced by cells mixedly infected with a host range mutant and an am mutant in III or IV or a ts mutant in II show that mutants in all three of these cistrons can contribute pro-

tein of the type which is electrophoretically altered by the host range mutation (130). This argues strongly that cistrons III and IV do not code for the host range protein, and less strongly (since a ts mutant was used) that cistron II is not the host range gene. This finding strengthens, by the process of elimination, the arguments that cistron VII codes for the host range protein. Analogous experiments with cistron VII mutants will be performed.

Cistron VII mutants appear to be unable, as are mutants in cistrons III and IV, to synthesize single-stranded DNA. Initial findings which implicated cistron VII in the process of RF replication (22) are probably related to a secondary mutation present in the cistron VII mutant studied (ts 41D). Recent results with an am mutant in this cistron, isolated by Hayashi, indicate that RF synthesis is essentially normal in this mutant (A.J. Levine, personal communication).

4.1.4 Organization of the ϕ X genome

By combining the map of ϕ X presented in part 2.4 of this thesis, with the available information relating to cistron functions, we obtain:



where the ends should be joined to form a circular map. The direction of translation inferred from the polar mutant is indicated by an arrow and the corresponding direction of the viral DNA strand is indicated. It is interesting that the three cistrons which code for protein components of

the spikes are adjacent, as is generally the case for functionally related genes. No other organization of the genome is obvious.

TABLE 1

PROPERTIES OF ØX MUTANTS

This table summarizes the results of physiological studies of ØX mutants performed by a number of workers (see text). A few representative mutants in each cistron are listed. Symbols: wt indicates that the property measured does not differ significantly from ØX wt (wild-type); S = more sensitive than wt; R = more resistant than wt; SBP = serum blocking power; SS = single-stranded DNA; RF = replicative form DNA; * indicates asymmetric complementation; -- indicates that the appropriate experiment has not been performed.

TABLE 1

PROPERTIES OF ϕX MUTANTS

Cistron Function	Mutants	Phage Particle Properties				Products of Abortive Infection			
		Thermal Stability	Electrophoretic Mobility	SBP	SS	RF	Mature Phage	Lysis	
I	Lysis	<u>am</u> 3, 30	<u>wt</u>	<u>wt</u>	<u>wt</u>	<u>wt</u>	<u>wt</u>	<u>wt</u>	0
II	Coat (spike)	<u>ts</u> 4, 28	<u>wt</u>	<u>wt</u>	<u>wt</u>	<u>wt</u>	<u>wt</u>	<u>wt</u>	<u>wt</u>
III	Coat (spike-SBP)	<u>ts</u> 41, 79 <u>am</u> 9, 23	<u>wt, R, S</u>	<u>wt, >wt</u>	<u>0, wt</u> (low S)	<u>0</u>	<u>wt</u>	<u>0</u>	<u>wt</u>
IV	Coat (shell ?)	<u>ts</u> 6, 9 <u>am</u> 14, 16	<u>wt, R, S</u>	<u>wt</u>	<u>wt</u> (low S)	<u>0</u>	<u>wt</u>	<u>0</u>	<u>wt</u>
V	?	<u>am</u> 10, 42	<u>wt</u>	<u>wt</u>	<u>wt</u>	<u>0</u>	<u>wt</u>	<u>0</u>	<u>wt</u>
VI*	RF Replication	<u>am</u> 8, 30, 33	<u>wt</u>	<u>wt</u>	<u>wt</u>	<u>wt</u>	<u>0</u>	parental only	late
VII	Coat (spike-host range?)	<u>ts</u> 41D, <u>op</u> 6, 9 probably $\underline{H_aH_b}, \underline{h}$	<u>R</u>	--	<u><wt</u>	--	<u>0</u>	<u>(wt)</u>	0

$(\underline{H_aH_b})$ is not a conditional lethal

4.2 IS THE $\emptyset X$ GENE SET NOW COMPLETE?

The number of cistrons identified by these studies of $\emptyset X$ conditional lethal mutants -- seven -- is consistent with estimates based on the information content of the $\emptyset X$ DNA molecule (see the General Introduction to this thesis). It is not clear, however, that existing methods could prove that all of the $\emptyset X$ genes had been identified, assuming for the sake of argument that they had. Detailed mapping of a large number of sites within each cistron might show the absence of gaps between the known cistrons. Analysis of the proteins produced within the $\emptyset X$ -infected cell might reveal a one to one correspondence between phage induced proteins and the known cistrons. But it is doubtful whether either of these methods could exclude the existence of a $\emptyset X$ -coded peptide of small size which is produced in small quantity. In the near future the analysis of $\emptyset X$ reproduction in a cell free system may allow a much better characterization of all the molecular components necessary. Eventually the base sequence of the $\emptyset X$ DNA molecule, coupled with our - by then - more perfect knowledge of the genetic code and its punctuation, will permit a quite reliable determination of the number, and amino acid sequences, of the $\emptyset X$ -coded proteins.

At present the best we can do is try to determine whether any evidence exists which suggests that $\emptyset X$ codes for proteins that we have been unable, so far, to alter mutationally. This question can be approached by considering whether genes of closely related phages all appear to be homologous to one of the $\emptyset X$ genes, and also by considering whether the

known $\emptyset X$ gene products are sufficient to account for all known features of the infective process.

4.2.1 The genes of related phages

Seven cistrons have been identified in the phage S13, which is very closely related to $\emptyset X$ (114). Six cistrons of the phage M13 have been detected by the conditional lethal technique (115). This phage is similar to $\emptyset X$ in the nature and replication of its DNA - it contains a single-stranded ring DNA of nearly the same size as $\emptyset X$ which replicates via a double-stranded RF - but appears to differ in its strategy for entering and leaving the bacterial host. M13 incorporates its DNA into rod shaped phage particles which specifically infect male strains of *E. coli*, presumably by parasitizing the nucleic acid transfer mechanism of the host, and are able to exit without lytic disruption of the host cell. Although the similarity in the number of cistrons identified is suggestive that some sort of limit is being approached, $\emptyset X$ and M13 are different enough so that there is no reason to expect a completely homologous set of cistrons. No other phages closely related to $\emptyset X$ have received detailed genetic study.

$\emptyset X$ and S13. A comparison of the findings concerning functions of the $\emptyset X$ cistrons and the homologous S13 cistrons is presented in Table 2. The conclusions of these two studies are in excellent agreement, although some of the results differ in detail. Some differences which have not been discussed previously are:

1) Dowell and Sinsheimer (131) found "normal or nearly normal" synthesis of infective single-strands by ts 4 (II), while Tessman (51) found that S13 su mutants in the homologous cistron IIIb "show either no decrease or a small increase" in infective single-strands under conditions where most mutants in the other cistrons tested (I, II, IIIa, and IV) "show some decrease". $\emptyset X$ nonsense mutants in cistron II should be studied to see whether they are able to synthesize normal amounts of single-stranded DNA under restrictive conditions.

2) Only one of the S13 mutants studied synthesized SBP under restrictive growth conditions (107). This is quite different than the situation with $\emptyset X$, where the only mutants so far identified which fail to synthesize SBP are in cistron III. Since the one S13 mutant which did synthesize SBP is the only representative of cistron IIIb (which corresponds to $\emptyset X$ cistron II) tested, it seems likely that this SBP is incorporated into a defective phage particle similar to the one produced by ts 4(II). The results of the two systems can then be made compatible by assuming that the SBP assay used in the S13 experiments was incapable of detecting SBP in subunit form, before it was incorporated into phage-size particles.

3) No lysis has been observed with S13 cistron IV mutants, while $\emptyset X$ mutants in the homologous cistron VI do lyse but at a later time than the wt.

We feel that all these differences are more likely to reflect variations in experimental procedure than any significant disparity in function between homologous $\emptyset X$ and S13 cistrons.

The main point which needs to be determined is whether mutants in ϕ X cistron V and S13 cistron VI will complement. Complementation between these cistrons would strongly indicate that both phages have at least eight cistrons, while a negative result would lead to the conclusion that the two sets of seven cistrons are completely homologous, thereby tending to support the idea that all cistrons capable of mutating to ts and am have been identified.

It should be pointed out that there is no good reason to believe that all genes can be tagged, in a single mutational step, by am or ts mutants. Although such mutations are not function specific they are quite site specific. am mutants, for example, can occur only by mutation to the triplet UAG. Of the nine triplets which can mutate by a single base change to UAG, one is a nonsense codon (UAA) which would not be found within a cistron, and five others code for amino acids which could also be coded by triplets which could not mutate to UAG (in one step). This leaves three triplets, two of which code for tyrosine and one for tryptophan. It would therefore be possible that a gene for any protein which does not contain tryptophan or tyrosine might not be able to mutate to am. It seems possible that specific types of changes in protein structure may be necessary to produce a ts phenotype, so that certain genes might not be able to mutate to a temperature-sensitive form. The best approach therefore seems to be the examination of as many types of conditional lethal mutants as possible.

4.2.2 Can known cistron functions explain the infective process?

ϕ X DNA synthesis. The replication of ϕ X DNA probably requires, according to current ideas, a polymerase, a ligase, and a nuclease. An initiator protein (132) may also be required. The product of one ϕ X cistron (VI) is known to be involved, in an unknown manner, in RF replication. Three proteins of the phage coat (III, IV, and VII) as well as cistron V, are necessary for the production of single-stranded DNA. It seems very unlikely that the cistron VI product can by itself perform all the functions necessary for RF replication. It seems quite plausible, on the other hand, to assume that host cell enzymes could perform the majority of these functions and there is no reason to require any additional phage-coded proteins. Cistrons III, IV, VII, and V seem ample (perhaps excessive) to account for the change from RF replication to single-strand synthesis, with the concomitant incorporation of the single-strands into phage particles.

Phage maturation. The number of cistrons implicated as structural genes for phage coat components (4 - II, III, IV, and VII) is sufficient to account for the major proteins obtained by disruption of phage particles. The minor band of highly charged material which migrates near the electrophoretic front (part 3.3) could represent either a contaminant, a host coded component of the coat, or the product of an unidentified cistron.

Lysis. One ϕ X cistron (I) is necessary for lysis of the host. Since it is very unclear how ϕ X induces lysis it is difficult to guess whether other phage genes are likely to be involved.

Other functions. In addition to the functions mentioned above, all of which are obviously essential parts of the normal ϕX infective cycle, several other properties of the ϕX infected cell are known which have so far not been identified with the function of any ϕX cistron. These are: 1) shutoff of host DNA synthesis, 2) cessation of net RF synthesis, and 3) superinfection exclusion. It seems possible that one or more of these functions may require a ϕX -coded protein not yet identified.

4.2.3 Conclusion.

Seven ϕX cistrons have been identified. This number is consistent with predictions based on the information content of ϕX DNA. The same number of cistrons have been identified in the closely related phage S13, and at least six of the ϕX cistrons appear to be homologous to S13 cistrons (the last pair has not been tested). These results support the idea that the majority, if not all, of the ϕX cistrons have been identified.

There is no strong evidence that any properties of the ϕX infection result from products of unidentified ϕX cistrons. Several unexplained features of the infection are known, however, which might involve products of additional cistrons.

TABLE 2

COMPARISON OF FUNCTIONS OF HOMOLOGOUS ϕ X AND S13 CISTRONS

Phage ϕ X		Phage S13	
Cistron	Function	Cistron	Function
I	Lysis	V	Lysis
II	Spike Component	IIIb	Phage Coat
III	Spike Component	IIIa	Phage Coat
IV	Capsid Protein	II	?
V	?	?	-----
VI	RF Replication	IV	DNA Synthesis
VII	Spike Component (Host Range)	I	Phage Coat (Host Range)
?	-----	VI	?

The homologous cistrons are deduced from complementation studies (see Part 2.3 of this thesis). The functions of the S13 cistrons have been studied in the Tessmans' laboratory (107,51,133). The words used to describe the functions are taken from Baker and Tessman (119) except that the fact that host range mutants fall in S13 cistron I (107) has been added.

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