

THE ROLE OF THE GENE 9 PRODUCT IN THE ASSEMBLY AND
TRIGGERING OF BACTERIOPHAGE T4

Thesis by
Jeffrey Edward Flatgaard

In Partial Fulfillment of the Requirements
For the Degree of
Doctor of Philosophy

California Institute of Technology
Pasadena, California

1969

(Submitted December 10, 1968)

ACKNOWLEDGMENTS

To Bob Edgar my deepest appreciation for his advice and inspiration, his patience during the last stages of this work, and his concern with my development as a scientist rather than just my scientific work.

To my parents for the many ways in which they encouraged and guided me.

To Bob Huskey, Jon King, and Dick Russell my many thanks for providing a remarkable and competitive atmosphere for scientific research and to Steve Beckendorf, Richard Josslin and Sandy Parkinson who are carrying on the tradition.

To Jean Weigle and Max Delbrück for their interest.

To Ilga Lielausis for her help in performing some of these experiments.

To Lee Simon and Ron Luftig for their aid in the electron microscopic investigations.

To Bertha Jones, Jeannette Navest, and Irene von Hartman who helped provide the essentials for lab work - media and clean glassware.

To Barrie Logan who provided a sounding board for my ideas on the philosophy of science and listened when I felt discouraged.

To Kathie Wheeler and Sally Schneider for their companionship and encouragement.

To all the guys in Prufrock - Alan, Al, Walter, Bill, John and Paul for providing a quality to life that must be unmatched elsewhere.

To Robbi Hunt for her meticulous care in typing this thesis.

And finally to National Institutes of Health Training Grant

5 T1 GM 86 and the National Science Foundation for financial support.

ABSTRACT

Gene 9 of phage T4 specifies a protein needed in the assembly of the virus particle, whose site of action is on the baseplate. Assembly without gene 9 product results in the production of inactive particles which lack tail fibers. These defective particles can be converted to complete active phage in vitro by the sequential action of the gene 9 product and the addition of tail fibers. Gene 9 defective particles are unstable and spontaneously convert to an aberrant "triggered" form that has a contracted sheath, but retains the DNA in the head. Action of the gene 9 product on the particle stabilizes it from converting to the triggered form, provides for DNA release when the sheath contracts during infection, and provides sites on the baseplate for the attachment of tail fibers. For the particle to be subsequently active the gene 9 product must act at least three times on the gene 9 defective particle; there is some experimental evidence to suggest that the gene 9 product may act on the particle stoichiometrically rather than catalytically.

A model is proposed for the action of the gene 9 product on the particle in which six gene 9 products are incorporated into the baseplate. These components join the tail plug to the baseplate vertices such that the change which occurs in baseplate configuration during infection will eliminate the tail plug by pulling it apart. Removal of the tail plug opens the end of the core to allow DNA release. The attachment site for the tail fiber on the baseplate is proposed to be at the junction of the gene 9 product and the baseplate.

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INTRODUCTION

The life cycle of a bacteriophage starts with the attachment of the virus to a bacterial cell and with the injection of the viral DNA; for it is the entry of the phage genome into the host cell which re-directs the cell machinery toward the formation of new progeny phages. The process of infection converts the entering phage particle into a non-infectious state; with phage T2 this loss of infectivity is due to the separation of the DNA from the protein components of the phage. The phage DNA enters the cell while the protein components remain on the cell surface and can be removed without affecting phage growth in the infected cell (Hershey and Chase, 1953). Since after infection the protein components of the phage are not essential for the further steps in the phage life cycle, the genome of the phage must be carried in the DNA.

The electron microscopic observations of Anderson (1953) on the adsorption of T2 phage particles to cells suggested that the phage protein components have more than just the function of protecting the DNA from the environment. These T2 phage particles have both a head and a tail. The head contains the DNA and the tail functions as an adsorption organ since phage particles adsorb by the tips of their tail to the cell wall, suggesting perhaps, the injection of the phage DNA into the cell is a mechanical process performed by the phage tail.

Many experiments on the nature of infection were performed with phage T2 while the work done in this thesis has been performed exclusively

with phage T4. Since both of these phages are members of the T-even group and seem to be phylogenetically closely related (Russell, 1967), the main results from the study of phage T2 structure and infection have been assumed to be true for phage T4 as well.

The use of negative staining with phosphotungstic acid or uranyl acetate provided a means to observe in the electron microscope details of phage structure not seen with metal shadowing procedures and has given a detailed knowledge of the arrangement of protein components in the phage. Observations by several groups of investigators such as Brenner et al., (1959), Moody (1965, 1967a,b), Kellenberger et al. (1965), and Simon and Anderson (1967a, b), reviewed recently by Kozloff (1968), have led to the picture of T4 structure illustrated in figure 1a. In this figure a schematic illustration of a phage particle both before and after adsorption (with the tail in an extended and triggered configuration respectively) is shown. The phage head has the structure of an isomeric capsid with icosahedral symmetry in which the middle portion is extended. The head contains the DNA when the particle is in the extended tail form. The phage DNA is present as one double stranded molecule (Rubenstein et al., 1961; Davison et al., 1961), per phage head.

The phage head is attached to a tail containing at least eight morphologically different components. The longest portion of the tail is made of two hollow tubes, one inside the other, the core and the sheath respectively. The core is a hollow rod with a length of 1000 Å, an outside diameter of 80 Å, and an inside diameter of 25 Å. The sheath is also a hollow tube, but exists in different states in extended and

FIGURE 1: A schematic illustration of bacteriophage T4 structure and of the stages in the process of infection.

a) A wild type phage particle in an extended state and a contracted state showing both a side and a bottom view of the different components.

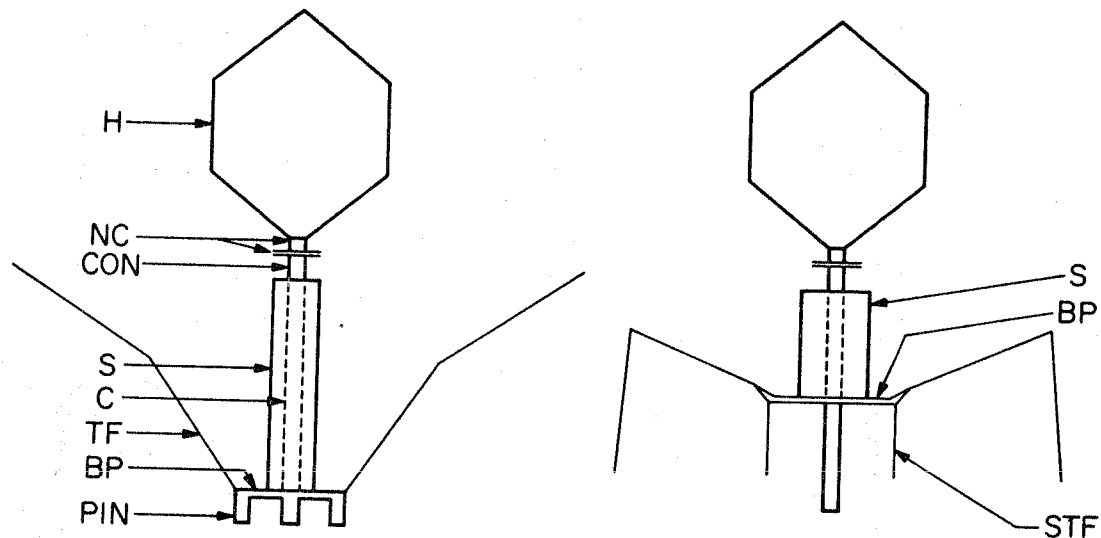
b) The sequence of steps observed in phage adsorption (from Simon and Anderson, 1967a). (1) An unattached particle. (2) A particle attached by the tips of the tail fibers to the cell wall. The opposite fibers attach to the cell wall about 1800 \AA apart, and are slightly bent in the center causing the baseplate to be about 1000 \AA from the cell wall. (3) The phage particle has moved closer to the cell wall and the tips of the tail pins have attached to the cell wall. (4) The sheath has contracted and the baseplate has changed configuration. The tail pins have lengthened into the short tail fibers; the baseplate is held to the cell wall by both the long tail fibers and the short tail fibers. The core has penetrated the cell wall allowing ejection of the DNA into the cell.

a

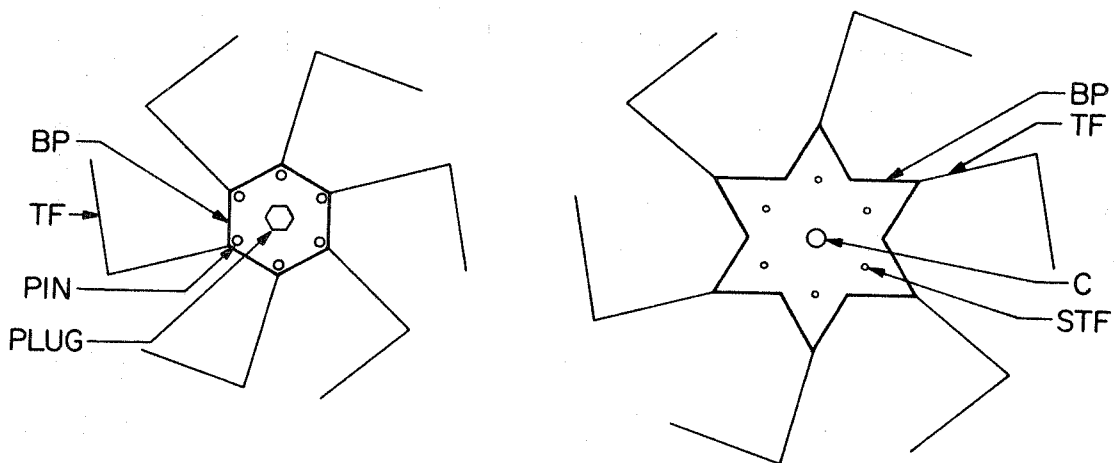
EXTENDED STATE

TRIGGERED STATE

SIDE VIEW



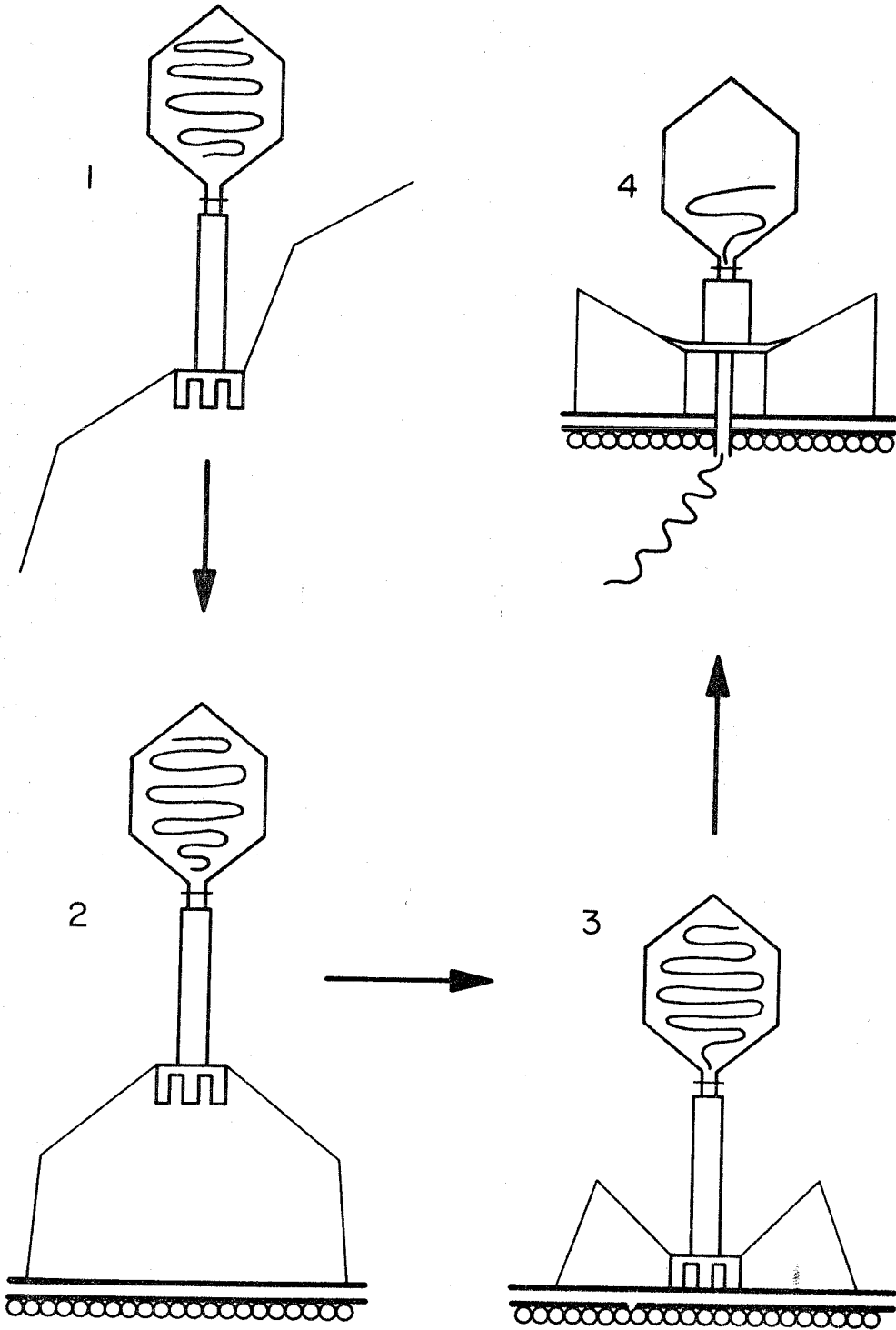
BOTTOM VIEW



SYMBOLS

- H = HEAD
- NC = NECK AND COLLAR
- CON = CONNECTOR
- C = CORE
- S = SHEATH
- PLUG = TAIL PLUG
- BP = BASEPLATE
- PIN = TAIL PIN
- STF = SHORT TAIL FIBER
- TF = TAIL FIBER

b



triggered particles. In extended particles the sheath is a hollow tube 1000 Å long with an outside diameter of 200 Å and an inside diameter of 100 Å; in triggered particles it is 500 Å long and 400 Å in outside diameter. A structure suggested for the extended sheath is one in which it is composed of 24 rings of 6 subunits each. Intercalation of subunits causes contraction of the sheath and results in a structure with 12 rows of 12 subunits each (Moody, 1967a, b). Extended sheath is thought to be in a high energy form since it is unstable and is irreversibly converted to the stable contracted form. Between the core-sheath portion of the tail and the head are three structures: the neck, the collar (a thin hexagonal plate about 260 Å in diameter), and the connector (King, 1968a). At the end of the tail is the baseplate which, like the sheath, has a different configuration in extended and triggered particles. Baseplates from extended tails have a hexagonal shape with a diameter of about 320 Å; baseplates from triggered particles have a six pointed star shape with the distance between tips being about 600 Å and a boat-like shape in profile caused by the tips making a 140° angle with the central region. Near the vertices in hexagonally shaped baseplates are six tail pins that are about 100 Å long; these tail pins are not found on baseplates in the six-pointed star shape, but in their place are six short tail fibers that are about 350 Å long. These short tail fibers should not be confused with the regular tail fibers, also six in number, which are attached to the tips of the vertices in both types of baseplates. These tail fibers are thin, rod-like structures about 1200 Å long with a characteristic kink in the center that is a 140° angle in the free tail fiber.

The role of the tail, this remarkably intricate protein aggregate, in the invasion of the host cell seems to be the puncture of the cell wall to allow the entry of the phage DNA. This role is clearly seen in the stages of the adsorption of the phage particle to bacterial cells as investigated by Simon and Anderson (1967a, b) who used both negative staining and thin sectioning to examine the details of how phage particles interact with the cell and how they change due to this interaction. A schematic illustration of the stages seen by Simon and Anderson (1967a) is given in figure 1b. The first stage involves the attachment of tail fibers to the cell wall. In the second stage the baseplate attaches to the cell wall as well. In the third stage the contraction of the sheath occurs so as to force the core through the cell wall and to inject the DNA into the cell.

The function of the tail is in some ways analogous to the action of a hypodermic syringe where the core represents the needle of the syringe and the DNA the material injected. The contraction of the sheath provides the mechanical energy to force the core through the cell wall.

A more detailed description of phage infection is the following series of steps:

- 1) Attachment of the tips of the tail fibers to the cell wall. Attachment of one of the fibers initially might be rate limiting compared to the subsequent attachment of the other five fibers.
- 2) Approach of the baseplate to the cell wall. Brownian motion might be sufficient to move the baseplate to within 100 \AA of the cell wall.

3) Attachment of the baseplate to the cell wall through the tips of the tail pins.

4) A change in the baseplate shape from the hexagonal configuration to the six pointed star configuration.

5) Bonds between the core and the baseplate are broken and a plug is removed from the center of the baseplate due to the change in the baseplate configuration. The end of the core is now open and the core itself is now free to slide through the baseplate.

6) Contraction of the sheath is induced by the change in the baseplate configuration. If the hexagonal form of the baseplate has six sites to which the sheath subunits attach while the six pointed star form has twelve sites, then the change in baseplate configuration could be the start of an intercalation process and initiate sheath contraction .

7) Penetration of the baseplate and the cell wall by the core is caused by contraction of the sheath. Apparently the core penetrates the cell wall but not necessarily the cell membrane.

8) Elongation of the tail pins into short tail fibers is caused by the contraction of the sheath and moves the baseplate to about 350 Å from the cell wall.

9) Release of phage DNA through the core into the space between the cell wall and membrane from which it is then taken up into the cell.

The structure of a phage particle and the mechanism by which it infects a cell is complex and appears a marvelous molecular mechanism in the intricacy and subtlety of its action. The problems of visual resolution limit the use of the electron microscope for the investigation

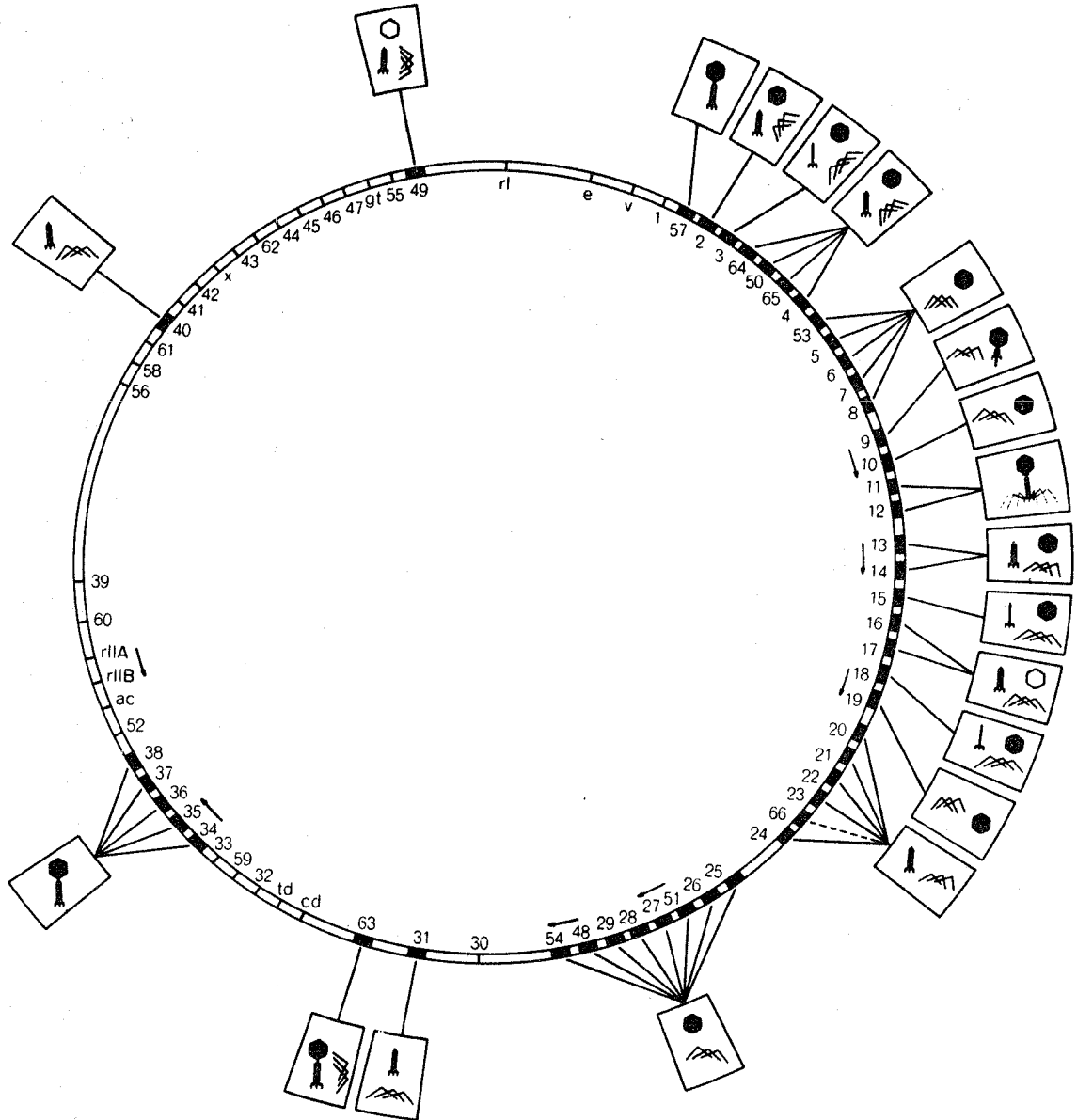
of phage structure, but genetic methods have provided a means of obtaining more insight into the structures of the phage. A determination of how many proteins are needed in the morphogenesis of the phage particle is possible through a study of conditional lethal mutants of the phage. Conditional lethal mutants are able to grow under permissive conditions where the gene product specified by the altered gene is active, but not under restrictive conditions where the active gene product is inactive or missing. Two types of conditional lethal mutants have been studied most thoroughly in phage T4: temperature sensitive mutants and amber mutants. Temperature sensitive mutants presumably cause the production of a thermolabile gene product since phage carrying such mutations are able to grow at 25°C, but not at 40°C. Amber mutations are characterized by the effect of bacterial suppressor genes on the expression of the altered gene in the phage; phage carrying such mutations are able to grow in bacteria having the suppressor gene (permissive strains), but not in bacteria lacking the suppressor gene (restrictive strains). The action of the suppressor gene carried by the bacterium seems to be at the level of protein synthesis. Under restrictive conditions only a fragment of the normal gene product is produced (Stretton et al., 1966), but under permissive conditions the entire protein is produced. One difference in the effect of these two types of mutations under restrictive conditions is that temperature sensitive mutations cause the formation of a non-functional protein while amber mutations cause the production of only a fragment of the normal protein. In principle both types of mutations should be found to occur in all phage genes which produce a protein essential in the phage life cycle.

Since conditional lethal mutants represent simple deficiencies of function under restrictive conditions, it is possible to classify them as being defective in the same or different genes on the basis of whether they complement under restrictive conditions. A map of phage T4 in which the genes are defined by complementation tests and the gene order determined by recombination analysis is illustrated in figure 2 (from Wood et al., 1968). The role the various gene products play in phage growth has been determined from studies of mutants from each of the different genes grown under restrictive conditions (Epstein et al., 1963). Of the 69 genes studied by this method 46 have been shown to play a role in the morphogenesis of the particle. These genes and the defective phenotypes of mutants blocked in morphogenesis are depicted schematically in the boxes in figure 2. These studies showed that most gene products needed in particle morphogenesis were concerned with either head, tail, or tail fiber formation, since only one of these structures was affected when a gene product was missing.

Biochemical analysis of the morphogenesis of the phage particle was rendered feasible with the development of an in vitro assembly system using the phage components that accumulate in mutant-infected cells (Edgar and Wood, 1966). An in vitro assay for any phage assembly step might be possible if there exist conditional lethal mutants defective in the gene product needed for the assembly step; addition of the missing gene product to the extract of the mutant-infected cells could result in the formation of active phage. Initially only a few assembly steps such as the attachment of tail fibers to fiberless particles,

FIGURE 2: A map of the genes of the bacteriophage T4 and the phenotype of conditional lethal mutants which are defective in genes concerned with particle morphogenesis.

The genes which have been identified as affecting particle morphogenesis are represented by broad black segments on the circular genetic map; narrow lines represent genes controlling phage "early functions" not directly involved in particle morphogenesis. Defective phenotypes are indicated by the boxed symbols, which represent the phage structural components observed in electron micrographs of defective lysates prepared under restrictive conditions with mutants representative of the various genes (from Wood et al., 1968).



head-tail union, and some steps in tail fiber assembly were observed, but now 15 of the 46 assembly steps in the morphogenesis of the phage particle have been found to occur in vitro (Wood et al., 1968).

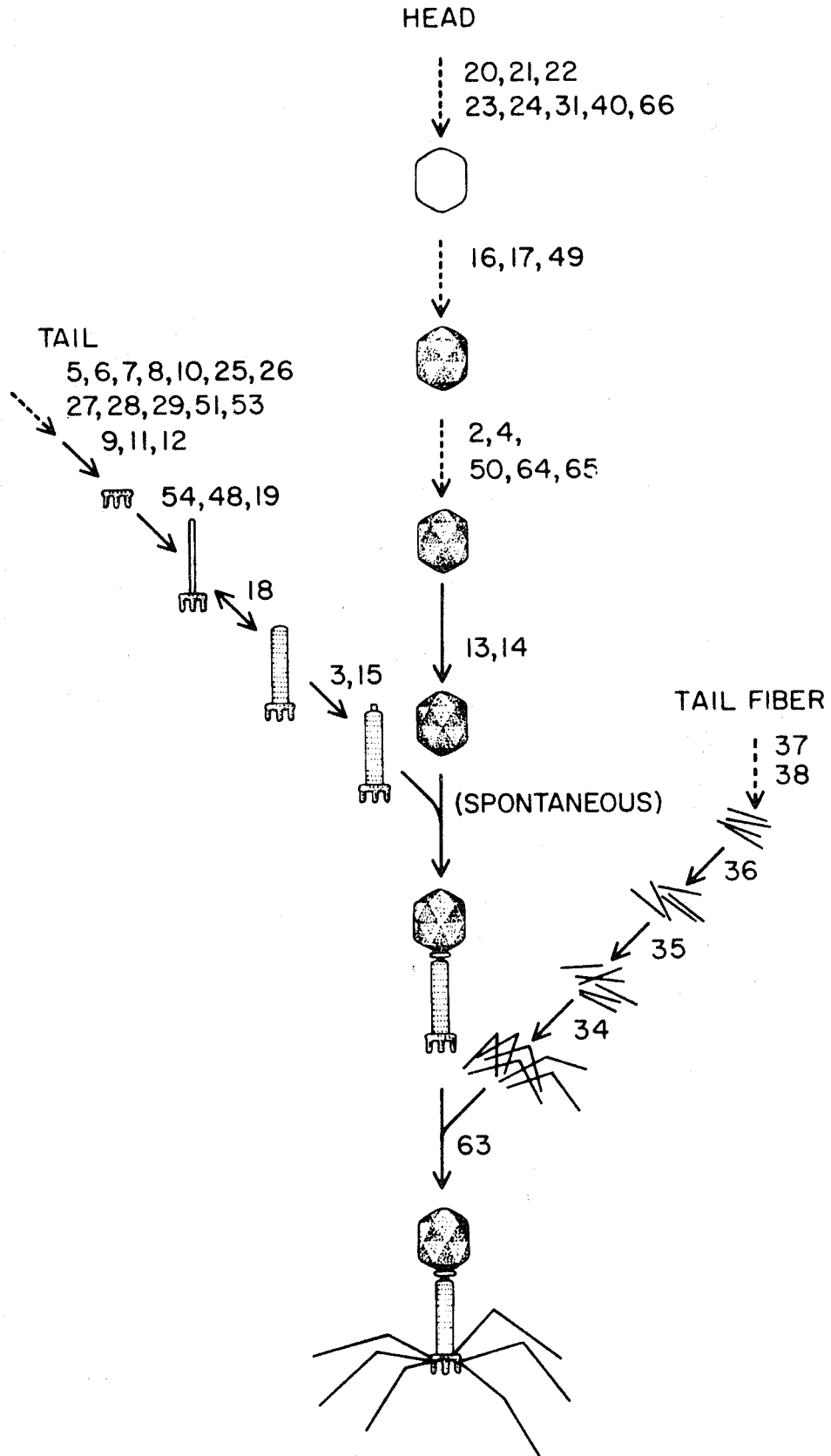
One striking feature of phage assembly is its division into three pathways; heads, tails, and tail fibers are constructed independently and then put together in the last two steps in assembly (Edgar and Lielausis, 1968; King, 1968). First the head and tail join to form a fiberless particle, and then tail fibers are added to this fiberless particle to form active phage. A pathway illustrating these assembly steps plus the other known steps in head, tail, and tail fiber formation is shown in figure 3. The sequence of steps in tail fiber assembly has been worked out by King and Wood (1968). Most of the steps in head assembly have not yet worked in vitro.

Some steps in tail assembly have been found to work in vitro (Edgar and Lielausis, 1968; King, 1968). The first steps in tail assembly lead to the production of baseplates which then serve as the substrates for the assembly of other tail components. The core is formed only on baseplates, and the sheath only on a core-baseplate structure. The absence of the gene 11 and 12 products during morphogenesis results in the production of defective particles which can be converted to active phage in vitro; the site of action of these two gene products has been shown to be the baseplate of the particle.

From this description the protein coat of the phage particle appears as a small, but complicated structure designed to penetrate the cell wall with the core in order to permit the injection of the phage DNA into the

FIGURE 3. The pathway for the assembly of the bacteriophage T4 particle.

The numbers next to an arrow indicate that the proteins specified by those genes are essential for the conversion of the structure before the arrow to the one following it, or the initial formation of a structure. The structures pictured schematically represent intermediates which have been found to occur in the assembly process. The solid lines represent steps which can occur in vitro; the dashed lines, steps which have not been observed to take place in vitro. The identification of the gene 9 product as being involved in baseplate assembly is based on the results presented later in this thesis (from Wood et al., 1968).



cell. As mentioned above, there appears to be a plug in the end of the core to insure that DNA is not released from the phage head until after the tail is triggered. If the plug were not released during the infection process, then the adsorbed particle would possess an altered baseplate, a contracted sheath, and a core penetrating the cell wall, but the DNA would remain inside the head. The appearance of such a particle would cause it to be classified as being arrested between particle 3 and 4 in the Simon and Anderson (1967a) model illustrated in figure 1b. Such phage particles have not been reported as occurring naturally, but defective particles unattached to cell walls have been observed among the progeny from cells infected with mutants defective in gene 9 (Epstein et al., 1963). These particles are defective not only in being in an improperly triggered form, but lack tail fibers as well (King, 1968) which means that such particles can not adsorb to bacterial cells. Studies of the function of the gene 9 product were initiated with the hope that the mechanism of its action would explain the operation of the system involved in triggering and DNA release.

MATERIALS AND METHODS

(a) Bacterial strains

All bacterial strains used as hosts for T4 were Escherichia coli strains from the collection of R.S. Edgar. E. coli CR63 was used as a strain permissive for both amber mutants and rII mutants. E. coli CR63(λ h) was used as a strain permissive for amber mutants, but restrictive for rII mutants. E. coli BB was used as a strain permissive for rII mutants and certain amber mutants (e.g. am E17 and am B25), but restrictive for other amber mutants (e.g. am B17). E. coli B/5, S/6/5, and B_c were used as strains permissive for rII mutants, but restrictive for all amber mutants; E. coli B_c in addition has the property (useful for extract making) that the phenotype of cells infected with rII mutants is indistinguishable from those infected with phage which are rII⁺ (Rutberg and Rutberg, 1964).

(b) Bacteriophage strains

All mutants were from the collection of R.S. Edgar or S. Brenner and had been derived from T4D (= wild type) and, except for some of the gene 9 mutants, had been described previously (Epstein et al., 1963; Edgar and Lielausis, 1964; Edgar and Wood, 1966). Amber mutants are able to form plaques with E. coli CR63 (a permissive host), but not with E. coli B/5 (a restrictive host). Similarly temperature sensitive mutants are able to form plaques at 25°C or 30°C (permissive temperatures),

but not at 42°C (a restrictive temperature). The rII mutation rdf41, which is a deletion covering both the rIIA and rIIB cistrons, was incorporated into certain strains to allow selective plating techniques to be used; strains carrying this mutation can form plaques with cells lacking a λ prophage, but not on cells carrying a λ prophage. Various multiple mutants used in these studies were either obtained from the collection of R.S. Edgar or were isolated as recombinants from crosses. In the latter case all genotypes were verified by backcrossing to the appropriate single mutants.

(c) Media and buffers

H broth contained 8 g Difco nutrient broth, 5 g Difco bacto-peptone, 5 g NaCl, 1 g glucose per 1000 ml distilled water, adjusted to pH 7.4 before autoclaving. EHA bottom agar contained 10 g Difco bacto-agar, 13 g Difco bacto-tryptone, 8 g NaCl, 2 g sodium citrate, 1.3 g glucose per 1000 ml distilled water. EHA top agar was the same as EHA bottom agar except that 6.5 g Difco bacto-agar and 3 g glucose was used. Slant agar contained 18 g Difco bacto-agar, 8 g Difco bacto-tryptone, 5 g NaCl, 1 g Difco yeast extract per 1000 ml distilled water, adjusted to pH 7.4 before autoclaving.

BU buffer contained 7 g Na_2HPO_4 , 3 g KH_2PO_4 , and 4 g NaCl per 1000 ml distilled water. Dilution buffer contained 7 g Na_2HPO_4 , 3 g KH_2PO_4 , 4 g NaCl, and 0.5 g gelatin per 1000 ml distilled water which was autoclaved before 1 ml of 1 M MgSO_4 was added. 0.1 M Tris pH 7.4 contained 12.1 g Tris (hydroxymethyl) aminomethane per 1000 ml distilled water,

adjusted to pH 7.4 before autoclaving. 0.01 M phosphate buffer contained 0.54 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ and 0.355 g Na_2HPO_4 per 1000 ml distilled water, adjusted to pH 7.0 if necessary before autoclaving. TNMG buffer was a mixture of 200 ml 0.1 M Tris pH 7.4, 30 ml 25% NaCl, 30 ml 1M MgSO_4 , and 10 ml 10% gelatin. BUM buffer was a mixture of 100 ml BU buffer, 20 ml 1 M MgSO_4 , and 880 ml distilled water.

(d) Chemicals

Chloroform was Mallinckrodt analytical grade. Sucrose for gradients was from Analar Limited. Dow Corning Antifoam A and K&K Laboratories propylene glycol monolaurate were used as antifoaming agents. Crystalline DNase I, used in preparing extracts, was obtained from Sigma Chemical Company. HyFlo Super cel is a purified form of diatomaceous earth obtained from the Johns Manville Co. as purified celite. All other chemicals were reagent grade.

(e) Preparations of bacteria, phage, and infected cells

Overnights were small cultures (~30 ml) of bacteria grown from a slant inoculum in H broth to a stationary phase. Overnights were used as a source of cells for starting other experiments and were usually prepared once a week.

Plating bacteria were cultures of cells which had grown $2\frac{1}{2}$ hours at 30°C or $1\ \&\ \frac{3}{4}$ hours at 37°C in H broth following a hundred fold dilution from an overnight culture and then concentrated ten fold in cold H broth by centrifugation. Plating bacteria were used for at most three days and were often prepared daily.

Small volume phage stocks were made by either the plate method or the liquid culture method.

The plate method. A plaque from a plate grown overnight at 30°C was picked with a sterile, hollow glass tube into a few ml of dilution buffer, a few drops of chloroform added, shaken to extract the phage, and then assayed for its titer. It was then diluted and plated under the conditions desired so that there would be approximately 10^5 plaques per plate. After an overnight incubation the surface of the agar on the plate was treated with a few ml of chloroform and then extracted with 5 ml of H broth per plate for 2 to 4 hours. The supernatant liquid from the plate was filtered through a Mandler candle filter and stored in the cold.

The liquid culture method. An overnight culture of the bacteria on which the stock was to be grown was diluted approximately one thousand fold in fresh H broth and the cells grown to a titer of approximately 5×10^7 /ml. To a culture of 25 to 40 ml was added an overnight plaque of the phage strain which was to be grown, the culture aerated until lysis or overnight, whichever came first, and then treated with chloroform. The unlysed cells and the bacterial debris were removed by filtration through a Mandler candle filter and the filtrate stored in the cold.

Large volume stocks were made by the following method. An overnight culture of the host bacteria to be used was diluted five hundred fold into fresh H broth and grown with asperger aeration at 30°C until the cells reached a titer of 2×10^8 /ml, the culture infected with a stock

of the phage strain desired at a multiplicity of 0.10 phage per cell, and aeration continued for 6 hours; during this six hour period the phage should go through at least two cycles of growth. The culture was then lysed by shaking with chloroform, some of the bacterial debris removed by means of a low speed centrifugation (2,500 rev/min for 20 min), the supernatant filtered through a Buchner funnel using HyFlo celite as a filter aid, and the filtrate stored in the cold as the stock. No loss in phage titer was seen in this filtration, and with this treatment virtually all bacterial debris which would scatter light was removed from the stock.

Extracts were made from cells infected under restrictive conditions. Overnight cultures of E. coli B/5 or B_c were diluted a hundred fold in fresh H broth and the cells grown with asparger aeration to a titer of 4×10^8 cells/ml (as determined by cell count), infected with a multiplicity of 5 phage per cell (incubation with asparger aeration at 30°C continued) and 20 min later superinfected with another 5 phage per cell of the same genotype; 50 min after infection the bottle containing the infected cells was placed in an ice-water bath and allowed to cool (still with asparger aeration) for approximately 25 min, by which time the temperature of the culture had dropped to 10°C. The aeration was then stopped and the cells collected by low speed centrifugation (2,500 rev/min for 10 min) in 250 ml centrifuge bottles, the supernatant discarded, and the bottles allowed to drain. The viscous pellet of infected cells was then resuspended with 1.0 ml of BUM buffer containing DNase (10 ug/ml) per 250 ml bottle and the resuspended pellet

frozen at -70°C in a acetone-dry ice bath and stored in a -70°C freezer until needed when it was thawed at 30°C .

In many experiments one type of extract (e.g. 9:23:27:rII extract) was used in large amounts. In order to achieve reproducibility, this extract was made in large batches, diluted, centrifuged to remove cell debris, and tested for the range of its activity before being refrozen. Aliquots could then be thawed as needed and used as a standard reagent in experiments.

Since the gene 9 product was found to have a sedimentation coefficient of about 5 S, extracts used as a source of gene 9 product in the two step procedures were centrifuged to remove cell debris and the supernatant rather than the whole extract used.

(f) Genetic methods

Crosses and quantitative complementation tests were performed in the following manner. An overnight culture was diluted a thousand fold in fresh H broth and the cells grown to an early logarithmic phase, collected by centrifugation, and resuspended at a concentration of $4 \times 10^8/\text{ml}$ in H broth containing 0.004 M KCN. Phage were adsorbed to these cells by mixing 0.50 ml of phage stock (at 6×10^9 phage/ml and containing equal numbers of the two phage strains to be tested) with 0.50 ml of this cell suspension; the adsorption tube was incubated at 30°C for 20 min and then a sample diluted ten thousand fold into fresh H broth to reverse the effects of the KCN and to allow the production of phage. In the final dilution the titer of infective centers

was determined (by assaying this dilution directly) and the titer of unadsorbed phage was determined (by assaying the dilution after it was shaken with chloroform), the difference between the two titers being the titer of infected cells. In those experiments where the unadsorbed phage might mask the yield of newly formed phage, anti-phage serum was added to the adsorption tube (to give a final serum titer of 1.0 min^{-1}) at 10 min after phage and cells were mixed, the adsorption tube incubated 10 min more before a sample was diluted to fresh H broth. When anti-phage serum was used, the adsorption tube was assayed for the titer of unadsorbed phage before the addition of serum (to verify that the phage has adsorbed) and after the incubation with serum (to verify that the unadsorbed phage had now been reduced to a negligible quantity). The bacterial strain used in crosses (E. coli CR63) was permissive for any mutations in the two phage strains, and the bacterial strain used for complementation tests (E. coli B_c) was restrictive for amber mutations.

Qualitative complementation tests were performed by means of spot tests in the following manner. Clear plastic trays containing depressions of 2 ml capacity were used for the test. Two drops of each mutant stock (at $\sim 10^8$ phage/ml) were placed in a depression and 1 ml of melted EHA top agar which contained 10% E. coli S/6/5 plating bacteria were added to each depression; the tray was then incubated overnight or until control tests which had received no phage became opaque from bacterial growth. The trays were then scored as to whether the agar in the depression was clear or opaque; clearing was taken as a sign that there was complementation between the two phage strains, while the lack of clearing was

taken as indicating the lack of complementation. Since such tests were qualitative, controls were performed in each set of experiments to show that both positive and negative results could be obtained.

(g) Isolation of phage components

Defective particles were purified from a lysate of cells infected with the desired phage strain by means of differential centrifugation in the following procedure. A defective lysate was prepared and $MgSO_4$ (to a final concentration of 0.005 M) and DNase (10 to 100 units per liter) added and the mixture incubated at 30° or 37°C for 2 to 4 hours; this procedure degraded most of the free DNA and made filtration in a later step easier. The lysate was then centrifuged for 15 min at 2,500 rev/min to remove some of the cell debris and the supernatant added to HyFlo celite (one teaspoon per 250 ml of lysate) and the slurry of lysate and celite filtered through a Buchner funnel and the filtrate saved; this celite had been found to remove cell debris, but not phage. Particles were then pelleted from the filtrate by a centrifugation for 2 hours at 11,000 rev/min. The pellet containing the defective particles was then resuspended in the appropriate buffer and dispersed by treatment on a vortex mixer; after being resuspended it was given another low speed centrifugation to provide a clear suspension. Most particle preparations were whitish-blue in color due to the phage, but sometimes contained a slight yellow tint due to contamination with broth from the original lysate. This yellowish tint could be removed by a second cycle of high and low speed centrifugation.

Sucrose gradients were made from Analar sucrose dissolved in BUM buffer and contained 5.0 ml of solution in which the sucrose concentration varied linearly from 20% to 5%. On such a gradient was layered 0.20 ml of the desired extract or lysate and the gradient centrifuged in a SW39 rotor in a Spinco model L centrifuge; the time of the run includes acceleration, but not deceleration. At the end of the run a hole was punctured at the bottom of the tube and 50 to 80 drops collected. In some cases the extract was centrifuged at low speed ($\sim 4,000$ rev/min for 10 min) and the supernatant fraction layered onto the gradient; this procedure removed cell debris and reduced the density of the extract below that of 5% sucrose.

(h) Electron microscopy

The procedures described here were adapted from those described by King (1968).

Grids used for electron microscopy were 200 mesh copper screen (Ladd Research Industries) onto which a film of carbon has been deposited directly (Towe, 1965).

Specimens were prepared by negative staining with saturated uranyl acetate (\sim pH 4.0). A drop of sample was applied to a carbon coated grid and after 1 min washed off with 2 drops of distilled water. The grid was washed with a drop of stain and then left in contact with a second drop for about 30 sec. This was washed off with one drop of water and the excess liquid blotted with filter paper. The specimens were observed in a Phillips EM 200 electron microscope fitted with an anticontamination device, at 60 or 80 kv. The microscope had double condenser illumination

with 300 μ condenser apertures. A 50 μ platinum objective aperture was used for most observations. Micrographs were taken on Kodak fine-grain positive film (P426) or Kodak high contrast projector slide plates.

Counts were made by selecting a well stained grid square and transverseing from one corner to the opposite corner and counting the various structures appearing in the transverse. In grids prepared as described above, both positively and negatively stained regions were sometimes found. All counts and photographs were of negatively stained regions only.

Magnifications were calculated from the tails, when present, using 1000 \AA as the length of the core, or from the manufacturer's calibration. Magnification factors are only approximate.

(i) Terminology

When a gene product is missing or is altered in an extract, lysate, or particle preparation, it is due to one of these mutations unless otherwise stated.

<u>gene</u>	<u>mutation in that gene</u>
8	am N132
9	am E17
10	am B255
11	am N128
12	am N69
18	am E18
23	am B17

<u>gene</u>	<u>mutation in that gene</u>
27	am N120
54	am H21
34	am B25 and/or am A455
35	am B252
37	am N52
38	am N262

An extract or lysate is identified by which genes in the infecting phage fail to function due to the presence of an amber mutation. Thus an extract derived from restrictive cells infected with am B17 (a gene 23 mutant) is referred to as a 23 extract; if a multiple mutant with defects in several genes was used, then the gene names are separated by colons in the description. Particles are identified by the lysate from which they were purified; they may be missing gene products other than the one included in their identification, due to the nature of that gene function.

Results 1: Genetic Studies with Gene 9 Mutants.

Introduction

It has been shown by Edgar and Wood (1966), Edgar and Lielausis (1968), and King (1968) that a number of the final assembly steps in the construction of phage T4 leading to the production of active phage can take place in vitro; the components from which these phage are assembled are obtained from cells infected with conditional lethal mutants under restrictive conditions. The components used in such studies are thus intermediates, or derived from intermediates, in the normal in vivo assembly of phage; and different components can be obtained by using mutants which eliminate different essential steps in phage assembly. Using this method assembly steps can be investigated if there exist conditional lethal mutants defective for a protein needed for such a step. At the present time it has not been possible to isolate components suitable for in vitro assembly studies by means of degrading and breaking apart normal phage particles. And since the components which can serve in the in vitro assembly studies are aggregates of similar or different types of protein molecules, it has not been possible to synthesize such components de novo by the methods of organic chemistry.

Since the components used in the in vitro assay for the gene 9 assembly step are obtained through the use of conditional lethal mutants blocked in the formation of an active protein responsible for this assembly step, it is important to show that all the mutants used for this

purpose are defective in the same gene and that they have no defects in other genes. If mutants classified as being defective in gene 9 are actually defective in different genes, then the same assembly step would not be measured with components extracted from cells infected with different mutants. If the mutants are defective in other genes as well, then the in vitro analysis of the gene 9 assembly step may also fortitously include a second and perhaps unrelated step.

The genetic experiments to be described indicate that the mutants previously designated as gene 9 mutants are in fact all defective in gene 9; in addition their behavior in complementation tests suggests that the gene 9 product may be active as a multimer and that gene 9 may be transcribed on the same messenger RNA molecule as gene 10. To show that there are no other defects in these mutants is a more difficult task; to do so completely it would be necessary to show that all other genes in the mutant strains are the same as in the parental strain from which the mutants were derived. However, no evidence for such second mutations has appeared either in crosses between the mutants in which additional mutations might have segregated, or from the phenotypic properties of such mutants under restrictive conditions since all the mutants appear to have similar properties.

Complementation Tests

An essential test to determine if mutants are defective in the same gene is their failure to complement one another in all pairwise combinations. Since many of the amber and temperature sensitive mutants were

classified as being defective in gene 9 only by their failure to complement in a simple, but sometimes misleading "spot test" similar to the one described in Materials and Methods, it seemed important to do quantitative complementation tests among various pairs of mutants to verify this preliminary classification. With T4, complementation between two conditional lethal mutants can be measured by determining the yield of phage from cells infected under restrictive conditions with both mutants and comparing it to the yield from cells infected with normal wild type phage under the same conditions. Such experiments were performed with a number of gene 9 mutants obtained from the collections of R.S. Edgar and S. Brenner. The results of such experiments are shown in tables 1, 2, and 3 for conditional lethal mutants defective in gene 9 and two mutants defective in two neighboring genes, 8 and 10.

The data in table 1 demonstrate that five amber mutants classified as being defective in gene 9 because of a negative spot test with an E17 fail to complement one another in all combinations tested, but do complement with mutants classified as being defective in two neighboring genes, 8 and 10. Since anti-phage serum was used in all complementation tests, the yield of phage measured is due to the net synthesis of phage and not to the presence of unadsorbed parental phage. This yield does not represent complementation, however, since it is similar to the yield of phage from cells infected with one or the other of the parental phages (see table 9), and is due to the low, but finite transmission of amber mutants in the restrictive host. Thus all these five amber mutants are defective in the same gene.

TABLE 1

Complementation Tests Between Gene 8, Gene 9,
and Gene 10 Amber Mutants

	<u>(8) am N132</u>	<u>(9) am E343</u>	<u>(9) am E17</u>	<u>(9) am C226</u>
(10) am B255	90.	24.	27.	84.
(9) am C226	110.	0.65	0.63	---
(9) am C215	---	---	0.17	---
(9) am E570	---	0.17	0.16	
(9) am E17	99.	---		
(9) am E343	135.			

The yield of phage from E. coli B_c cells infected with a pair of mutants in the manner described in Materials and Methods and lysed after 45 min. at 30° was determined. This yield is expressed as the percent of the yield using wild type phage under the same conditions (= 142. phage/cell). The number in parenthesis next to the mutant name is the gene in which the mutant defect is located.

Three mutants, ts N11, ts G16, and ts L54, were used by Edgar and Lielausis (1964) in their studies of T4 temperature sensitive mutants to define gene 9; two of these mutants, ts N11 and ts L54, had also been used in the physiological studies of Epstein et al. (1963) on the role of different genes in the life cycle of phage T4. To show that the amber mutants used in this study were defective in gene 9 as defined by these three temperature sensitive mutants, the complementation between a representative gene 9 amber mutant, am E17, and these temperature sensitive mutants was measured quantitatively. The data in table 2 demonstrate that there is no increase in the phage yield in cells infected with both E17 and one of these temperature sensitive mutants over the yield from cells infected with the temperature sensitive mutant alone. Thus am E17 is defective in gene 9 as it was defined by Edgar and Lielausis (1964). The other four temperature sensitive mutants have been isolated by R.S. Edgar subsequent to 1964 and had originally been classified in this gene only on the basis of a negative spot test with a gene 9 mutant; these data show that they have been correctly classified in gene 9.

As a further test to confirm that these seven temperature sensitive mutants are all defective in the same gene, complementation tests were performed in all pairwise combinations among them and the data reported in table 3. These data show that all of the mutants fail to complement one another in all combinations confirming that all seven mutants are defective in the same gene. The yield from mixedly infected cells sometimes showed a small increase over the yield from cells infected with only one or the other of the parental phage. This intragenic

TABLE 2

Complementation Tests and Crosses Between a Gene 9 Amber Mutant
and Gene 9 Temperature Sensitive Mutants

	Map Distance	Complementation Tests			
		30°C		39.5°C	
		x am E17	alone	x am E17	alone
ts P3	1.1	51.	67.	0.1	0.2
ts N11	1.8	170.	28.	0.1	0.1
ts CB16	1.5	79.	96.	0.1	0.1
ts G16	0.6	61.	81.	0.1	0.1
ts L54	2.2	81.	85.	0.1	0.3
ts A55	1.5	138.	37.	0.1	0.1
ts CB116	0.5	71.	84.	0.1	0.1

The yield of phage from E. coli B_c cells infected with a mutant or a pair of mutants in the manner described in Materials and Methods and lysed after either 30 min at 39.5°C or 45 min at 30°C is determined. This yield is expressed as the percent of the yield using wild type phage under the same conditions (= 103. phage/cell at 30°C and 24. phage/cell at 39.5°C). The map distance is the percent recombination between that ts mutant and am E17 determined in the yield from the complementation test performed at 30°C as determined in the manner described in the legend to Table 4.

TABLE 3

Complementation Tests Between Gene 9
Temperature Sensitive Mutants

	ts P3	ts N11	ts CB16	ts G16	ts L54	ts A55	ts CB116
ts CB116	0.16	0.028	0.96	0.74	1.1	0.85	0.09
ts A55	1.8	0.007	1.9	1.9	1.9	0.14	
ts L54	2.8	0.25	3.6	2.0	3.2		
ts G16	0.21	0.12	1.5	0.12			
ts CB16	2.6	6.5	0.84				
ts N11	11.1	0.33					
ts P3	1.7						

The yield of phage from E. coli B_c cells infected with a mutant or a pair of mutants in the manner described in Materials and Methods and lysed after 30 min at 39.5°C is determined. The yield is expressed as the percent of the yield using wild type phage under the same conditions (= 39. phage/cell).

complementation has been observed by Edgar, Denhardt, and Epstein (1964) to occur in other genes in T4 in complementation tests between some temperature sensitive mutants, but not in tests between amber mutants or amber mutants and temperature sensitive mutants.

Two phenomena are observed in the preceding data which modify the complementation behavior of these conditional lethal mutants so that such tests can not be classified as absolutely positive or negative: intragenic complementation between some temperature sensitive mutants, and polar effects of amber mutations in gene 9 on the expression of gene 10.

Intragenic Complementation

The yield of phage from cells infected with certain pairs of temperature sensitive mutants under restrictive conditions is greater than the yield from cells infected with either one of the mutants alone under restrictive conditions (c.f. table 3, ts P3 x ts N11). Intragenic complementation similar to this has been investigated in greater detail by Bernstein, Edgar, and Denhardt (1965) for genes 10, 12, 34, and 37 of phage T4. The intragenic complementation between gene 9 mutants shows two similarities to those found in these other four genes. First, there are examples of both positive (ts P3 x ts N11) complementation and negative (ts N11 x ts A55) complementation. Second, there is no complementation between amber mutants (tables 1 and 9), or between amber mutants and temperature sensitive mutants (table 2). The biochemical basis of intragenic complementation has been studied by Schlesinger and Levinthal

(1963) for the case of the bacterial enzyme alkaline phosphatase and has been found due to the formation of a hybrid dimer between polypeptide chains made by each of the mutant genes. This hybrid dimer has enzymatic activity whereas the dimer made of one or the other of the mutant polypeptides is inactive. Crick and Orgel (1964) have proposed that in general intragenic complementation is due to the formation of hybrid multimers made of polypeptides from each of the mutant genes. The polypeptide produced by a complementing type of mutant is one which is inactive in the multimeric state because its conformation is incorrect. When polypeptides from two different complementing mutants aggregate, they tend to impose upon one another a conformation similar to that found in the normal wild type multimer giving the hybrid multimer some activity. Such an explanation for intragenic complementation between the gene 9 temperature sensitive mutants would imply that the gene 9 product is active when it has aggregated to form a multimeric state.

Polarity Effects

Certain gene 9 amber mutants (am E17 and am E343) complement less well than another (am C226) in tests with am B255, a gene 10 mutant, indicating that they may reduce the expression of gene 10 function. None of the gene 9 mutants appears to reduce the expression of gene 8 as revealed by complementation tests with a mutant defective in that gene, am N132. Similar polarity effects have been found by Stahl, Murray, Nakata, and Craseman (1966) for amber mutations in gene 34

expression of gene 35. In the gene 9 - gene 10 case and the other cases the effects occur between adjacent gene pairs and only in one direction. In both ϕ phage system (Nakata and Stahl, 1966) and in a bacterial system (Imamoto, Ito, and Yanofsky, 1966) it has been found that there is a definite relationship between the location of an amber mutation in the gene and the extent to which it reduces the expression of the neighboring gene; the closer the mutation is to the affected gene, the less of a reduction is found in the expression of the affected gene. Since one of the gene 9 amber mutants (am C226) did not give reduced complementation with a gene 10 mutant, it would be expected that it is the closest of the amber mutations to gene 10. In table 4 the data for two and three factor crosses with gene 8, 9, and 10 mutants is given, and a map based on these data is shown in figure 4 in which am C226 is the closest gene 9 amber mutation to gene 10. The order of am C226 with respect to the other gene 9 amber mutations is based on three factor as well as two factor crosses. A cross between am E343 and the gene 9-gene 10 double mutant am E17 am B255 shows approximately the same frequency of recombination as the cross between am E343 and am E17; thus the order of the mutations is am E343 - am E17 - am B255. However, the cross between am C226 and the same double mutant shows a three fold lower recombination value than the cross between am C226 and am E17; thus the order of these mutations is am E17 - am C226 - am B255. Two factor crosses show that two other amber mutants identified as being defective in gene 9 fail to recombine with am E17, and may be considered as being identical to am E17.

TABLE 4

Recombination Values Between Phage Strains Containing
Amber Mutations in Genes 8, 9, and 10

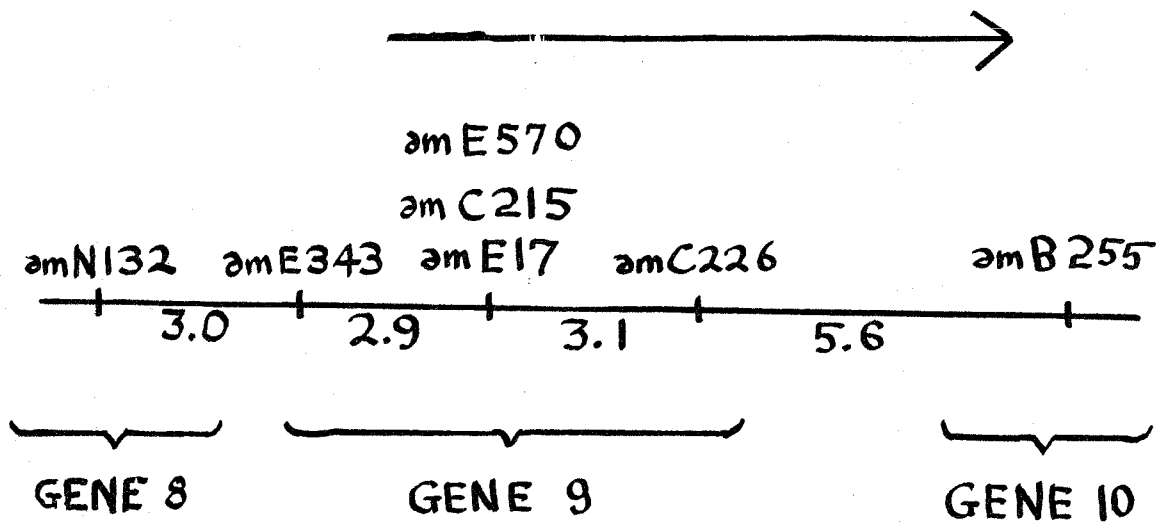
Two Factor Crosses						
	am N132	am E343	am E570	am E17	am C215	am C226
am B255	8.0 ²	8.3 ²		5.6 ⁵		5.6 ⁵
am C226	4.8 ²	4.4 ²	2.4 ¹	3.1 ⁵	3.9 ¹	
am C215	-	2.9 ¹	≤0.01 ²	≤0.01 ²		
am E17	3.8 ²	2.9 ⁵	≤0.01 ²			
am E570	-	3.1 ¹				
am E343	3.0 ²					

Three Factor Crosses						
am E343 x am E17 am B255 = 3.0 ¹						
am C226 x am E17 am B255 = 0.98 ¹						

The percent recombination found in the yield from E. coli CR 63 cells infected with a pair of mutant strains in the manner described in Materials and Methods and lysed after 45 min at 30°C was determined. The superscript is the number of determinations on which the value is based. Recombination percentage is calculated as twice the percentage of wild type progeny (assayed on the restrictive strain E. coli S/6/5) compared to total progeny (assayed on the permissive strain E. coli CR63).

FIGURE 4. The map positions of the gene 9 amber mutations relative to gene 8 and gene 10.

A map of the gene 9 amber mutations and their order relative to genes 8 and 10 as determined by both two factor and three factor crosses based on the data in table 4 is shown. The average map distance is shown for the shortest intervals. The arrow indicates the direction of the polarity effects shown by certain gene 9 amber mutations.



The location of the mutations which cause the temperature sensitive phenotype has not been determined, but from the recombination values given in table 2 it can be seen that they all map close to the reference amber mutation.

Evidence has been found by Imamoto, Ito, and Yanofsky (1966) that polarity effects such as this are due to two genes being transcribed on the same messenger RNA molecule. The existence of polarity effects between gene 9 and gene 10 thus implies that they are cotranscribed of the same messenger RNA molecule.

Summary

All the data presented in this section indicate that these five amber mutants and seven temperature sensitive mutants are defective in the same gene, and that they define gene 9 in phage T4. The existence of intragenic complementation between some temperature sensitive mutants suggests that the polypeptide specified by gene 9 is active when in a multimeric state. The polarity effects shown by some of the amber mutations on gene 10 expression indicate that gene 9 and gene 10 are cotranscribed onto the same messenger RNA molecule.

Results 2: The Assembly Defect Caused by Mutations in Gene 9

Introduction

The electron microscopic studies of Epstein et al. (1963) on the phenotype of cells infected with conditional lethal mutants under restrictive conditions indicated that the cells infected with mutants defective in gene 9 produced inactive phage particles of two types: one type similar in appearance to normal phage and the other type abnormal in that the particles have heads full of DNA but the sheath portion of the tail is in the contracted configuration (see plates 1, 2 and 3). The defect in gene 9 did not appear to affect other properties of the infected cell such as the production of phage DNA or serum blocking antigen. Lysis of the infected cell at the normal time indicated that the lytic mechanism as well as phage proteins needed for phage DNA synthesis and tail fiber formation were unaffected by the gene 9 defect.

King (1968) showed that both types of inactive particles produced by cells infected with a gene 9 mutant (and hereafter referred to as 9 particles or 9 defective particles) do not have tail fibers attached to them in spite of the production of normal amounts of tail fiber material. In these studies 9 particles were separated from the tail fiber material that is present in a 9 defective lysate by means of sucrose gradient centrifugation; when isolated in this way the 9 particles lack tail fibers as measured by their inability to block anti-tail fiber serum and when observed with negative staining in the electron microscope.

PLATE I. Fiberless particles from a 34 defective lysate. These particles have been acted on by the gene 9 product. The magnification of the larger picture is roughly 90,000 X while that of the insert pictures is roughly 220,000 X.

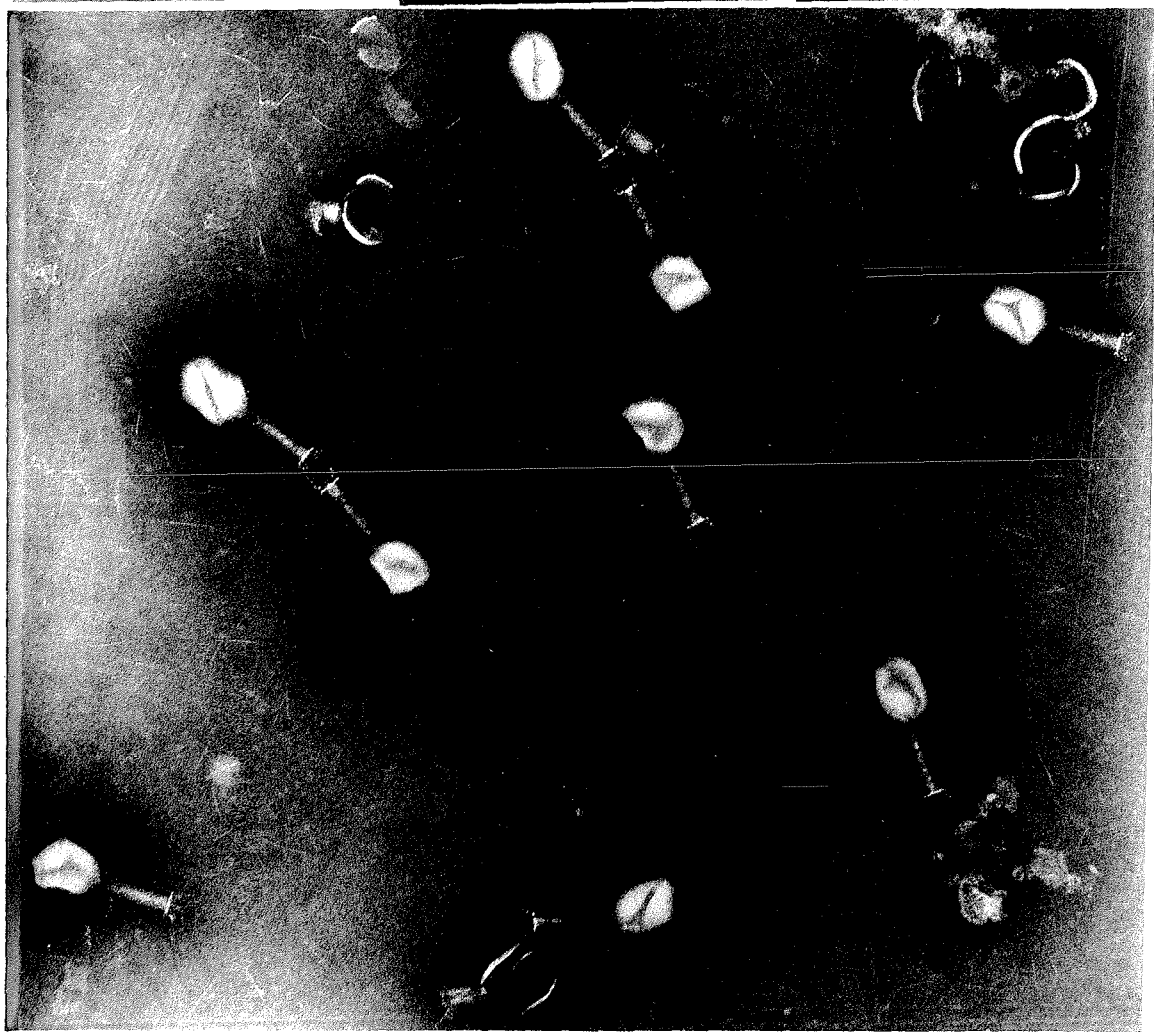
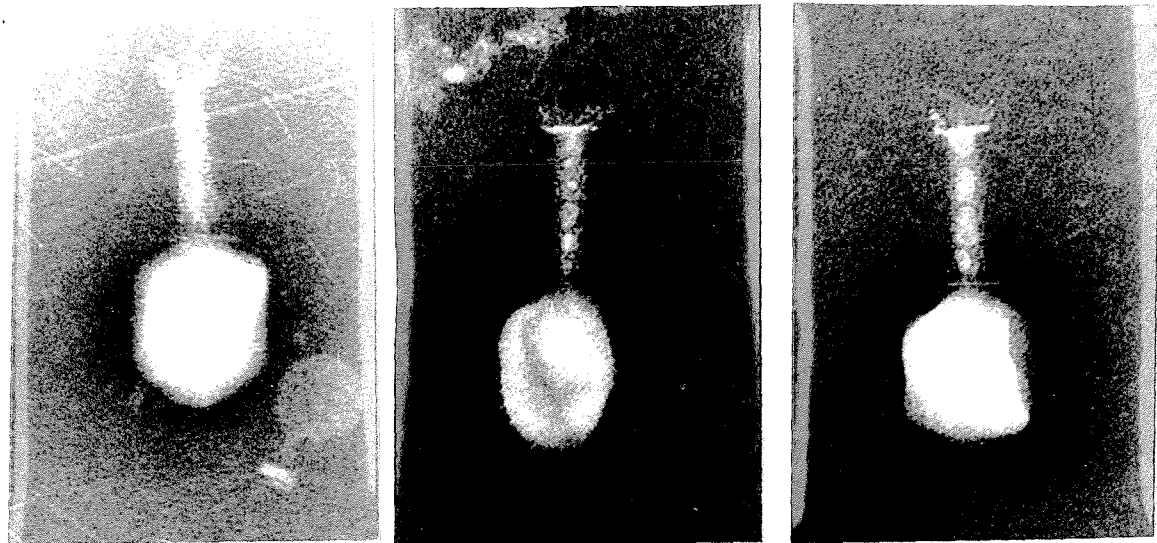


PLATE II. A mixture of extended and triggered particles from a 9 defective lysate with inserts showing a more detailed view of the extended particles. These particles have not been acted on by the gene 9 product. The magnification of the larger picture is roughly 150,000 X while that of the inserts is roughly 299,000 X.

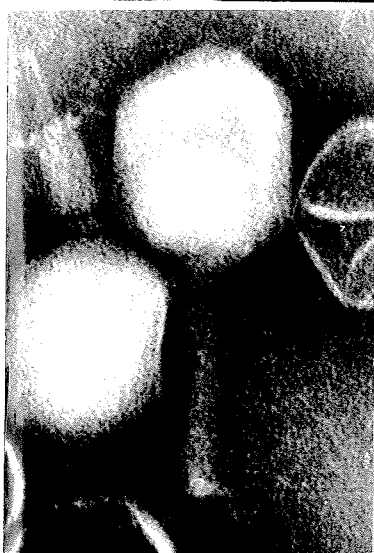
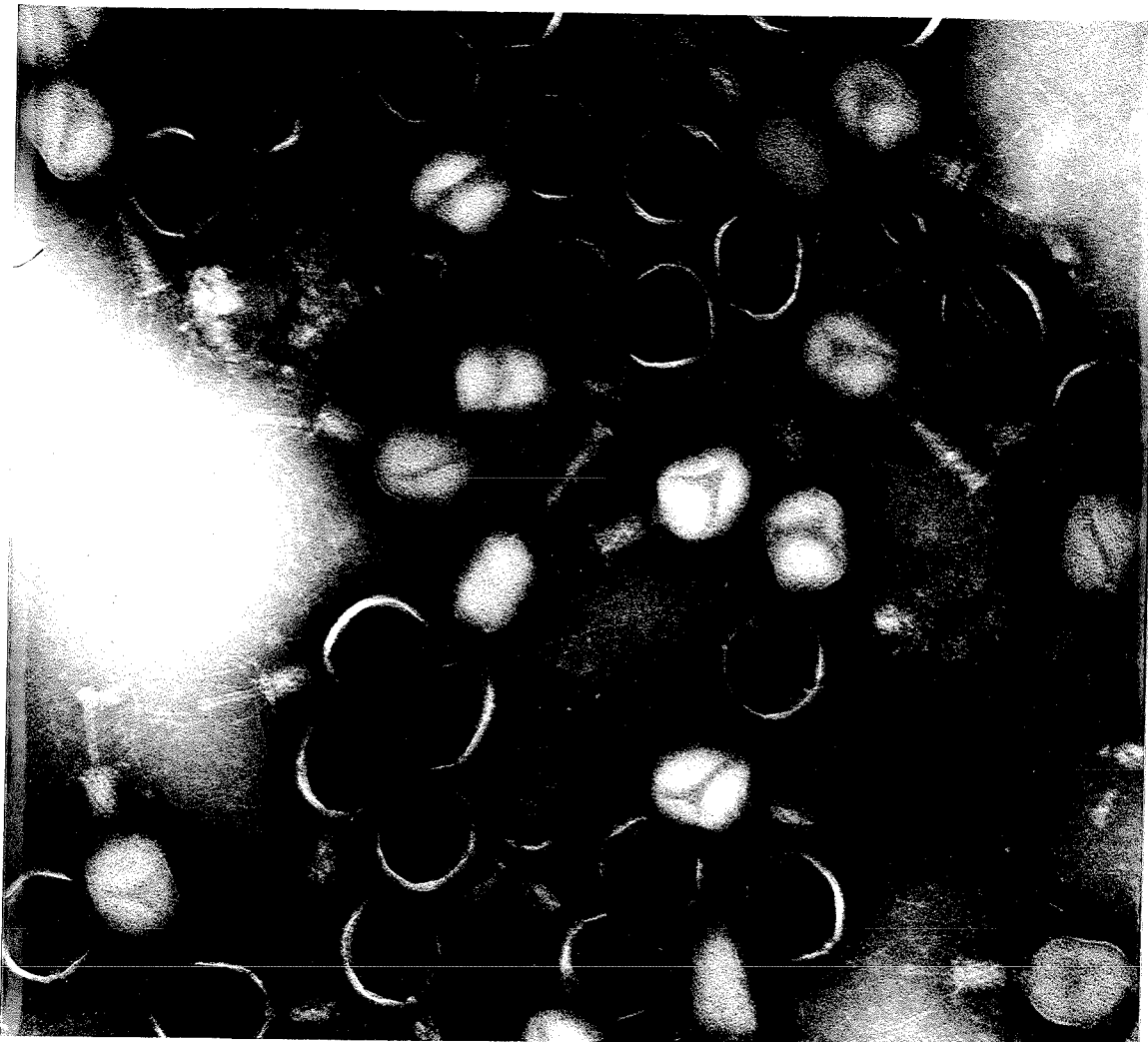
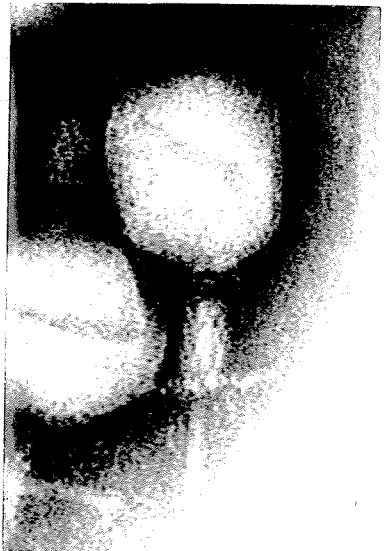
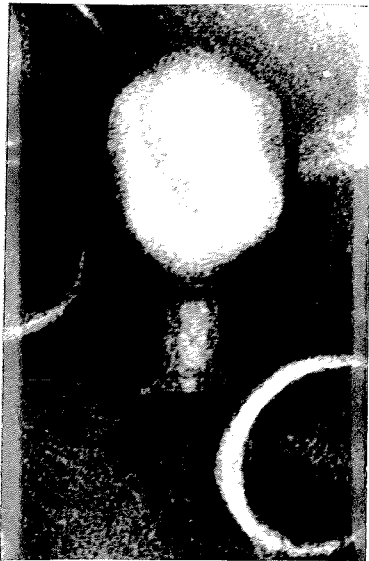
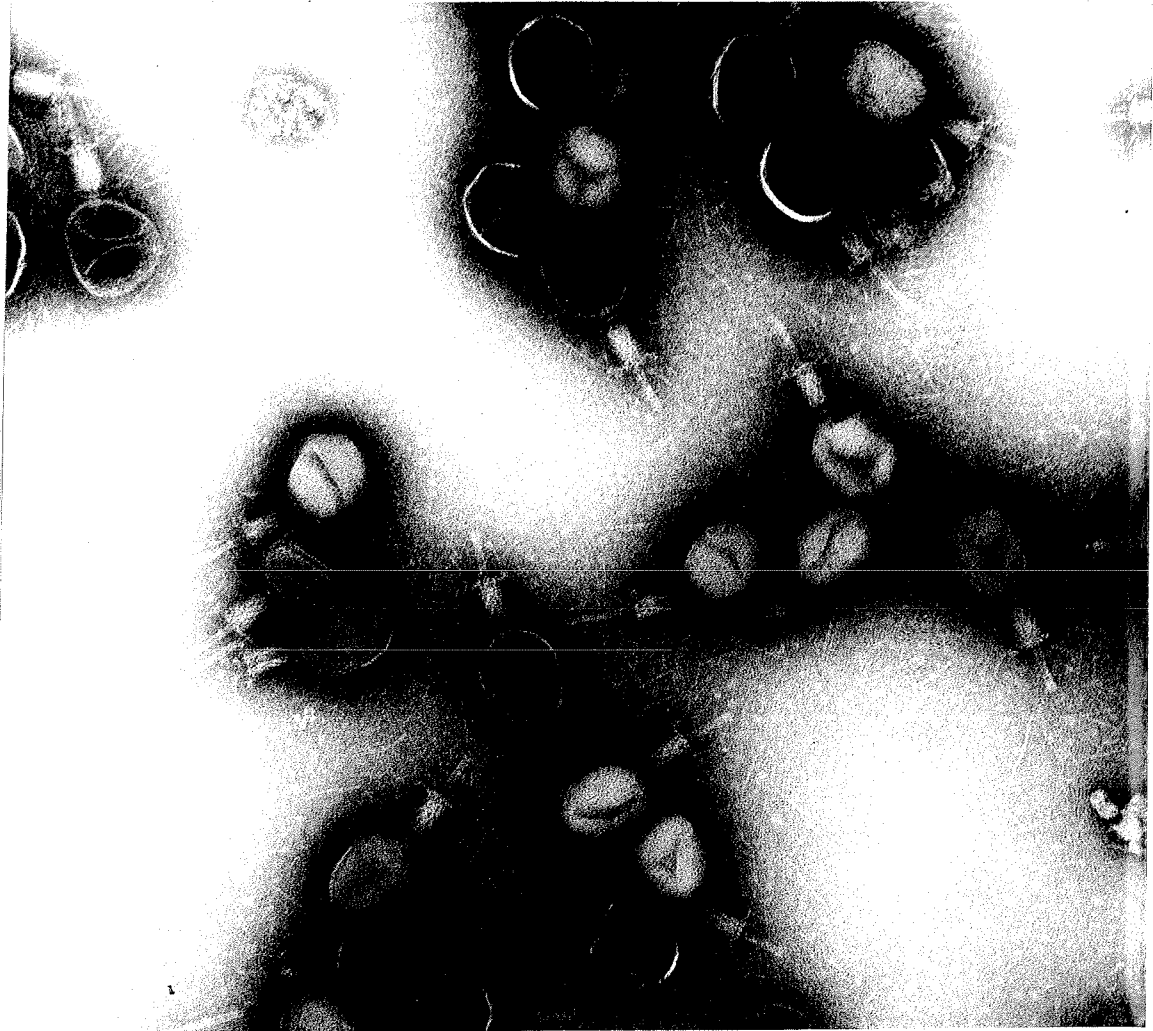


PLATE III. Triggered particles from a 9 defective lysate with inserts showing a more detailed view of them. These particles have not been acted on by the gene 9 product. The magnification of the larger picture is roughly 130,000 X while that of the insert pictures is roughly 290,000 X.



Two observations by Edgar and Wood (1966) suggested that under appropriate conditions ϕ particles might be converted to active phage in vitro. First, fiberless particles derived from cells infected with a mutant blocked in tail fiber formation can be extracted and purified from such cells and then converted to active phage quantitatively when added to an extract not defective in tail fiber formation. Second, that head-tail union can take place in vitro and that using such a system extracts of mutant infected cells could be classified as either head donors or tail donors. Since a ϕ extract served as both a head donor and a tail donor, this seemed to imply that a structure such as the ϕ particle was being converted in both of these complementation experiments to active phage.

Using these observations as a starting point, the properties of ϕ particles have been investigated in order to gain insight into the nature of the gene ϕ assembly step.

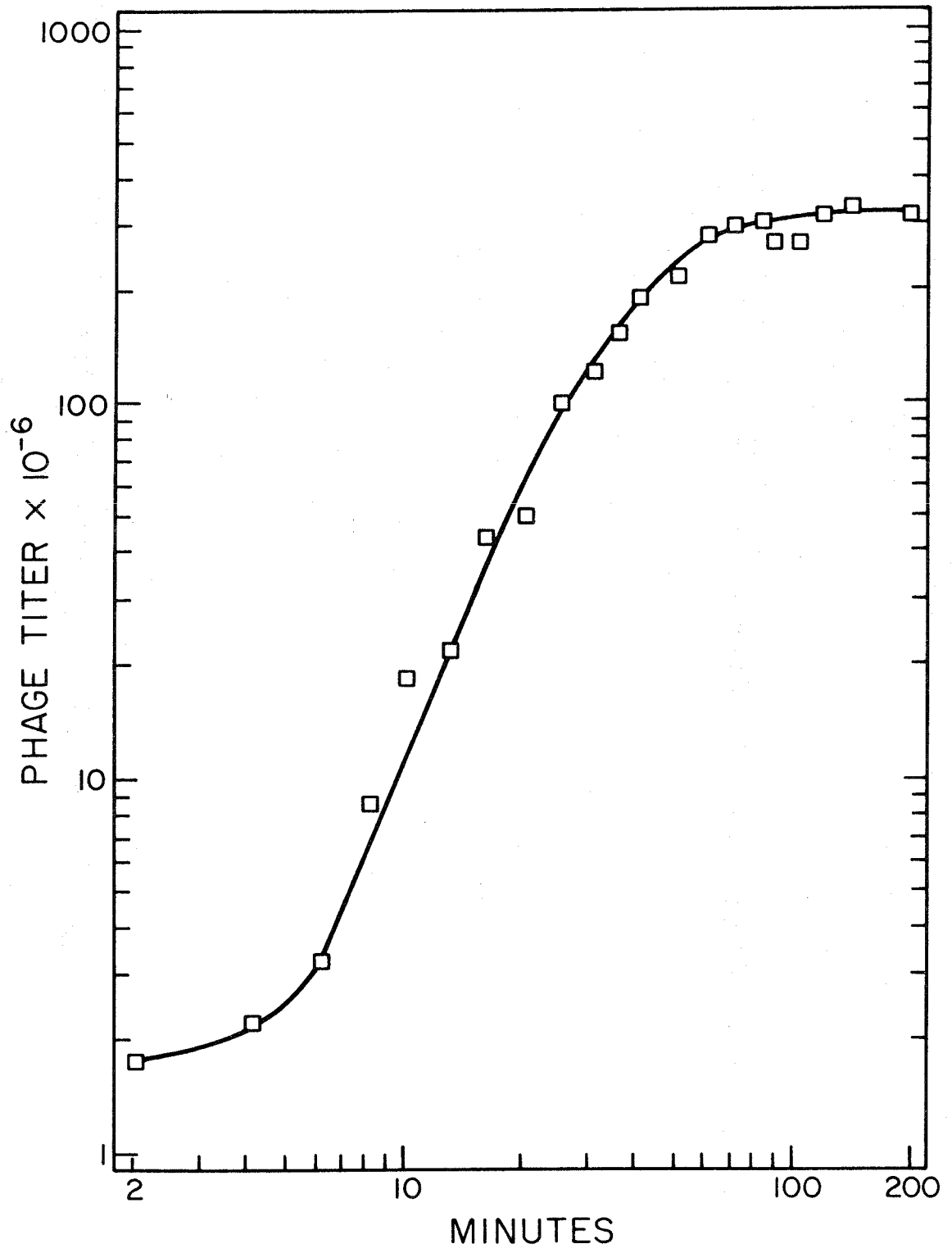
ϕ Particles Can Be Converted to Active Phage

1. Rescue of the mutant genome from a ϕ lysate.

In an experiment similar to the one for fiberless particles reported by Edgar and Wood (1966), the production of active phage from ϕ particles was found to take place when a ϕ lysate was mixed with a 23:27:rII extract; the kinetics of this production are illustrated in figure 5. Since the 23:27:rII extract is derived from infected cells in which the formation of both heads and tails has been blocked by amber mutations in two genes, one (27) essential for tail formation and the other (23) for head formation, the ϕ lysate must contribute both of

FIGURE 5. The kinetics of the conversion of the defective particles in a 9 lysate to active phage by incubation in a 23:27 extract.

A mixture of 0.50 ml of 23:27:rII extract and 0.025 ml of a 9 lysate (both prepared as described in Materials and Methods) was incubated at 30°C and sampled at different times for the titer of rII⁺ phage. The abscissa is the logarithm of the time after mixing and the ordinate the logarithm of the increase in the rII⁺ phage titer.



these structures to the active phage being produced. In this experiment to insure that no background normal phage derived from the 23:27:rII extract are measured, a selective plating system was used taking advantage of the fact that the mutant strain used to make the ϕ lysate was rII⁺, while that used to make the 23:27:rII extract contained an rII deletion mutation. By plating samples from the in vitro reaction mixture on CR63 (λ) where the amber mutants can grow, but the rII mutants can not, only those phage with the genome of the gene 9 mutant will form plaques. This technique enables the conversion of ϕ particles to active phage to be measured no matter what the background titer of normal phage from the 23:27:rII extract is. In this experiment the titer of active phage derived from ϕ particles increases over 200 fold over the titer of background phage in the ϕ particle preparation (due to the leakage of the gene 9 mutant and due to unadsorbed phage in making the ϕ lysate) before reaching a plateau value. A series of other experiments have shown that the plateau level of active phage is directly proportional to the amount of ϕ lysate added to the reaction mixture; hence the titer of the plateau level can be used as a quantitative measure of the number of ϕ particles originally present in a sample. The shape of the conversion curve when plotted on a log-log scale indicates that the conversion process does not obey simple linear kinetics: this conversion process will be examined in more detail in a later section.

2. The mutant genome is contained in a fiberless particle.

A further proof that it is the ϕ particle which is being converted to active phage in such an in vitro system is that the structure from the

gene 9 lysate whose activity is measured in vitro sediments in a sucrose gradient slightly faster than normal phage, in a manner very similar to fiberless particles (see figure 6). Such a result rules out the possibility that the 9 lysate is contributing both heads and tails to the reaction mixture, and that only in the presence of the 23:27:rII can the two be joined and go to form active phage. If that were the case, it would be expected that no activity could be recovered from the sucrose gradient as such a sedimentation procedure will very cleanly separate heads from tails (Edgar and Lielausis, 1968). The finding that the 9 particle actually sediments slightly faster than the normal active phage particles which were used as a control marker is in agreement with the finding of Kellenberger et al. (1965) that particles without tail fibers have a slightly greater sedimentation coefficient than particles with tail fibers. Also the observation (see table 8) that extracts which lack a tail fiber component are unable to convert 9 particles to active phage is in agreement with the finding that 9 particles lack tail fibers. Any extract used to convert the 9 particles to active phage must be able to supply the tail fiber attachment step as well as the gene 9 step.

Properties of the 9 Particles

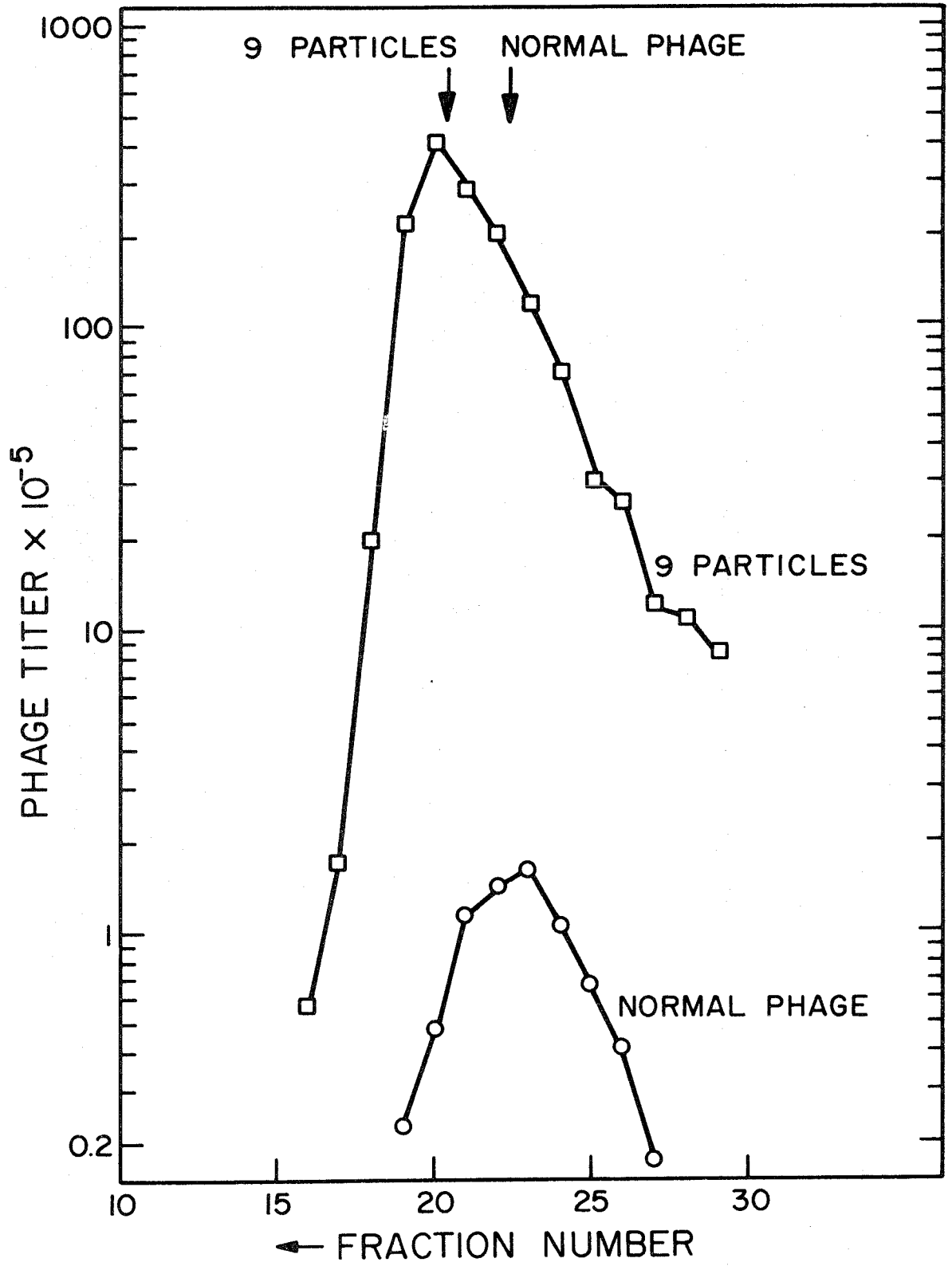
1. Stability studies on 9 particles.

An early observation in this study was that 9 particles were much more labile than normal phage. That is, defective particles in 9 lysates or extracts lose their potential to be converted to active phage when stored under conditions that do not affect the titer of background viable

FIGURE 6. A comparison between the sedimentation of normal phage and 9 particles in a sucrose gradient.

A 0.20 ml sample of a 9 lysate was layered on a sucrose gradient and centrifuged at 19,400 rev/min, 20°C, for 12.5 min. Thirty fractions of two drops each were collected into 2.0 ml samples of dilution buffer. The samples were assayed directly for normal background phage and after incubation with 23:27:rII extract for active rII⁺ phage derived from the 9 particles. The abscissa is the fraction number (the arrow indicates the direction of sedimentation) and the ordinate the logarithm of the particle titer in the collected fractions. The midpoint of the peaks at 50% of maximum activity are shown by the arrows at the top of the figure.

○ normal phage
□ 9 particles



phage in the preparations. This instability of the 9 particle relative to normal phage is a handicap in doing many experiments since the potential titer obtainable in an experiment is subject to this variable. In order to explore this property of 9 particles and to determine what factors, if any, will stabilize their potential to be activated, a series of experiments were carried out in which the activability of 9 particles was measured as a function of time in a variety of ionic conditions. The particles from a 9 lysate were first concentrated and purified by means of differential centrifugation as described in Materials and Methods. The concentrated particles were then diluted into the solution to be tested and both the background viable phage and the particles activable in vitro by 23:27:rII extract were measured as a function of time. The titer of background phage served as a control for the stability of normal phage under these conditions. Since selective plating conditions (like those used in the experiment described for figure 5) were used to insure that only those particles with the genome of the gene 9 mutant were assayed, it was possible to do these experiments at low ($\sim 10^8$ /ml) phage concentrations. These experiments have been summarized in table 5. Two factors have been found sufficient to confer stability to 9 particles: high salt concentrations and moderate temperatures. The 9 particles were found to be cold labile; in any given solution they are more stable at 30°C than 0°C. The salt requirement is quite strong; only $MgSO_4$ and NaCl have been tested and both are required in concentrations greater than 0.10 M to insure that over a three day period the particles do not decline significantly in activability.

TABLE 5

The Stability of 9 Particles as a Function of the
Ionic Conditions and the Temperature

Solution	Fraction remaining after 19 hours	
	0°C	30°C
0.10 M tris	0.003	0.02
0.10 M tris + 0.10 M MgSO ₄	0.22	0.98
0.10 M tris + 1.0 M MgSO ₄	0.90	0.95

Solution	Fraction remaining after 24 hours	
	0°C	30°C
0.01 M phosphate	0.025	0.05
0.01 M phosphate + 0.10 M NaCl	0.25	1.05
0.01 M phosphate + 1.0 M NaCl	0.68	1.1

The fraction of 9 particles remaining is the ratio of the titer of 9 particles after incubation under the conditions given in the table to the initial titer of 9 particles. The titer of 9 particles is determined by the increase in rII⁺ phage titer after a two hour incubation at 30°C with a 23:27:rII extract. Under the conditions used here there is no significant change in the titer of background viable phage.

These salt concentrations are considerably higher than those required for the stability of normal phage. Some preliminary experiments indicated that EDTA stabilizes the activability of ϕ particles, but this effect was not observed with purified particle preparations. In Hershey Broth the ϕ particles were found to lose their activity rapidly; at 0°C all activable particles had vanished within a day. The instability of ϕ particles in Hershey Broth accounts for the loss of activity in ϕ lysates and extracts that had been stored.

2. All ϕ particles can become active phage.

If all the ϕ particles in a given preparation can be converted into active phage, then physical observations made on such particles will measure the properties of particles which are actual precursors to active phage. To establish that in fully activable preparations of ϕ particles all the particles can be converted to active phage, it is necessary to show that the titer of particles as measured by some physical method is the same as the titer determined by the conversion of these particles to active phage. A convenient physical method is provided by the relationship found by Winkler, Johns, and Kellenberger (1962) between the optical density at 265 m μ and the titer in a suspension of phage particles: 1.0 O.D. unit = 1.2×10^{11} particles. Since the ϕ particles are labile under some conditions, it is necessary to take care in the preparation of concentrated ϕ particles so that there is no loss in activity. It was found that the method of differential centrifugation described in Materials and Methods combined with the conditions found in

the previous section for 9 particle stability resulted in preparations with little loss of activity, since the ratio of 9 particles to active phage before and after the purification procedure remained the same. Fiberless particles were purified in a manner similar to the 9 particles from a lysate of a mutant (am B25 defective in gene 34) blocked in tail fiber formation and were used as a control in these experiments. The data from this experiment, shown in table 6, indicate that the majority if not all the 9 particles are capable of serving as in vitro precursors to active phage. The titer of active phage derived from the preparation of 9 particles is roughly one-half of the particle titer based on the optical density of the preparation, but this result obtained for the fiberless particles as well. Edgar and Wood (1966) found that all the particles in a preparation of fiberless particles could be converted to active phage in vitro. The somewhat lower ratio of activable particles to O.D. found in this experiment may well be due to optical impurities in the particle preparations. This experiment indicates that the 9 particles do not differ appreciably from fiberless particles in terms of their in vitro activability. We may safely conclude that the majority of the 9 particles may serve as precursors of active phage in the in vitro reaction.

3. 9 particle lability as the conversion of normal particles to contracted particles.

As mentioned in the introduction, 9 lysates were found to contain two types of particles: those similar to normal phage in that the tails

TABLE 6

A Comparison Between the Titer of Defective Particles Determined
by Optical Density and by Conversion to Active Phage

	Titer x 10 ⁻¹¹			Total Particles by O.D.	B/P
	Activable particles				
	background with no extract	9 ⁻⁺ F ⁺ extract			
9 particles	0.098	0.12	25.	55.	0.45
9:11:12 particles	0.020	0.048	2.2	6.5	0.34
fiberless 34 particles	0.0012	7.4	5.0	11.0	0.45

The titer of activable particles was measured by mixing 0.10 ml of particles with 0.40 ml of extract, incubating at 30°C, and determining the average titer after the conversion to active phage had reached a plateau value. The titer of total particles was measured by determining the optical density at 265 mμ of the particle suspension and using the relationship of Winkler, Johns, and Kellenberger (1962) that 1.0 O.D. unit = 1.2×10^{11} particles. B/P is the ratio of particles activable with 9⁺⁺F⁺ extract to the total number of particles. 9⁺⁺F⁺ extract = 23:27:rII extract; 9⁻⁺F⁺ extract = 9:23:27:rII extract.

have an extended sheath and others in which the tail has triggered resulting in a contracted sheath, but in which the DNA has remained inside the head. Since sheath contraction is thought to be irreversible (Moody, 1967b), it would be expected that the particles with extended sheath are precursors to active phage while those with contracted sheath are incapable of being converted to active phage. This explanation would account for the lability of the 9 particles; the lability being the conversion of particles with normal tails to those with contracted sheath tails. In order to test this idea, both the titer of activable 9 particles and the fraction of particles of each type as determined from electron microscopic observations were measured in samples taken at different times from a preparation of 9 particles being inactivated in a low salt buffer kept in the cold (a solution of 0.01 M phosphate buffer pH 7.0 at 0°C). The titer of activable particles was measured by the titer of phage produced after incubation with a 23:27:rII extract. The fraction of particles in the two states was measured by preparing an electron microscope grid of a sample and counting the number of particles of each type in several fields of view using the method described in Materials and Methods. The data from this experiment, presented in table 7, show that an inactive sample of 9 particles consists largely of particles with contracted sheath tails, while an active sample consists of a mixture of particles with extended sheath tails and contracted sheath tails. This observation supports the notion that only particles with an extended sheath tail are activable and that the extended sheath form is unstable, contracting and being inactivated under conditions of low salt and low temperature. It thus seems plausible that

TABLE 7

A Correlation Between the Conversion of 9 Particles to the Contracted Sheath Form and Their Inability to be Converted to Active Phage

Time	Percent of Maximum Titer	Extended Sheath Particles	Contracted Sheath Particles	Total Particles
0 Hours	100.	41	18	59
8 Hours	20.	2	50	52
48 Hours	4.0	1	38	39

A preparation of 9 particles was diluted into 0.01 M phosphate buffer pH 7.0 at 0°C, and sampled at different times. In each sample the titer of activable 9 particles was determined by the method described in the legend to table 5, and the number of particles of each type was determined by observation in the electron microscope using the technique as described in Materials and Methods.

the cell infected with a gene 9 mutant does not produce a mixture of the two particle types. Rather the product of the defective assembly process is a particle with an extended sheath; only after the particle is made is it subject to sheath contraction. The 9 particle may thus be considered an unstable intermediate in phage assembly. Whether or not it is a necessary intermediate will be considered later.

4. Summary of the properties of the 9 particle.

9 particles are most probably assembled in the extended sheath form and as such are an intermediate in the assembly process that can be quantitatively converted to active phage. Their stability properties are different from viable phage, however, requiring high salt and moderate temperatures to prevent conversion to a contracted sheath form that can not be converted to active phage.

The Steps in the Conversion of 9 Particles to Active Phage

1. Active tail fibers in the absence of gene 9 function.

The conversion of 9 particles to active phage requires the function of the gene 9 product as well as the addition of tail fibers. The defect in phage formation in cells infected with a gene 9 mutant might thus be due to the production of a particle which is unable to have tail fibers attached to it, or to the production of tail fibers which can not be attached to a fiberless particle. An extract lacking tail fibers, but containing the gene 9 product is observed to complement in vitro a 9 extract suggesting there is nothing wrong with the fiber

attachment steps in the 9 extract. More likely the 9 particle is unable to act as a fiberless particle suitable to tail fiber attachment, and the gene 9 assembly step which is taking place in vitro converts the 9 particle into a form that is now suitable for tail fiber attachment.

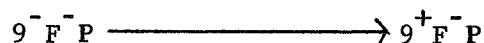
In order to examine the tail fiber assembly and attachment steps in the absence of the gene 9 product, fiberless particles isolated from a lysate of a mutant defective in tail fiber formation were tested for their ability to be converted to active phage by 9 extract. The fiberless particles were isolated from a lysate of an B25 (defective in gene 34) by two cycles of high and low speed centrifugation as described in Materials and Methods. Two extracts were used to convert these fiberless particles to active phage: one a 23:27:rII extract which has the gene 9 product as well as all tail fiber components and the other a 9:23:27:rII extract which lacks the gene 9 product but has all the tail fiber components. Since the fiberless particles are derived from a strain which is rII⁺, the selective plating technique described in connection with the experiment presented in figure 5 can be employed to insure that only phage derived from the fiberless particles are measured. The titers given in table 6 are an average of samples taken after no further increase in active phage is detected. The data show that the presence of the gene 9 product is not essential for the conversion of fiberless particles to active phage; the absence of the gene 9 product does not cause defective tail fibers to be formed or the tail fiber attachment system to be affected. It would seem that the defect in cells infected with a gene 9 mutant is the production of particles unable to accept tail fibers.

2. Conversion of 9 particles to active phage in a two step process.

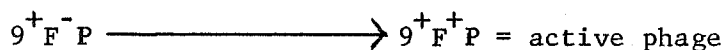
Apparently the gene 9 step in vitro concerns the conversion of 9 particles to a form that can accept tail fibers. This notion suggests an intermediate with the properties of fiberless particles should occur in the conversion of 9 particles to active phage. To explore this possibility an in vitro complementation test was performed under conditions where such an intermediate should accumulate. These conditions should obtain an in vitro reaction between 9 particles and an extract that contains the gene 9 product, but lacks a tail fiber component. The suspected intermediate would have the properties of fiberless particles in that it would not be active until tail fibers have been attached to it. This final step was carried out by means of an in vitro reaction between the suspected intermediate and an extract that lacks the gene 9 product, but contains all the tail fiber components. A 23:27:34:rII extract was used as a source of gene 9 product, but lacking tail fibers (due to the amber mutation in gene 34) and a 9:23:27:rII extract was used as a source of tail fiber components, but lacking the gene 9 product. Amber mutations in genes 23 and 27 were used respectively to block head and tail formation and an rII deletion mutation to enable a selective plating system to be used to measure phage derived from 9 particles (which contain an rII⁺ genome). The following scheme was used in the two step procedure: 9 particles were mixed and incubated for various lengths of time with one of the two extracts and then diluted in buffer; a sample of this dilution was subsequently mixed and incubated with the second extract. The final incubation was stopped by dilution into buffer. Both sequences

of extract were used (i.e. the $9^{+}F^{-}$ extract first and then the $9^{-}F^{+}$ extract, as well as the $9^{-}F^{+}$ extract first and then the $9^{+}F^{-}$ extract) and the titer of phage derived from the 9 particles measured both before extract treatment and after incubation in one extract and then the other. As an additional control, a reconstruction experiment was performed in which the particles and extract were diluted separately before being mixed and added to a second extract. The titers presented in table 8 have been corrected for the dilutions made in this experiment so that all values are relative to the original preparation of 9 particles and increases represent conversion of 9 particles to active phage.

The data presented in table 8 show that the 9 particles can be converted to active phage in a reaction that has two sequential steps. If 9 particles are represented as $9^{-}F^{-}P$, then the first step is



and the second step is



These results show that the tail fiber addition step must take place after the gene 9 step. There is an obligatory sequence between the action of the gene 9 product and the addition of tail fibers.

3. An in vitro comparison of the $9^{+}F^{-}$ particle and the fiberless particle.

The intermediate in the conversion of 9 particles to active phage, the $9^{+}F^{-}$ particle, should be identical to the fiberless particle if the gene 9 step and the tail fiber addition step are the only two needed

TABLE 8

A Two Step System for the Conversion of
9 Particles to Active Phage

	Titer x 10 ⁻⁹			
	Sequence of Extract in Reaction Procedure			
First Incubation	9 ⁺ F ⁻		9 ⁻ F ⁺	
Second Incubation	9 ⁻ F ⁺		9 ⁺ F ⁻	
	9 Particles	Fiberless 34 Particles	9 Particles	Fiberless 34 Particles
Original titer	1.4	1.5	1.4	1.0
After first incubation	1.3	2.5	2.0	600.
After second incubation	650.	600.	1.8	
Reconstruction experiment	1.3			

9 particles were incubated sequentially in two different extracts by the following procedure: 0.10 ml of particles was mixed with 0.40 ml of extract, incubated 2 hours at 30°C, and then the reaction mixture diluted 500 fold in dilution buffer; then 0.10 ml of this dilution and 0.40 ml of the second extract were mixed, incubated 2 hours at 30°C, and then this reaction mixture diluted 500 fold in dilution buffer. The titer of active phage from the 9 particles, corrected for any dilution that was made, is given after each incubation procedure. The reconstruction experiment was performed by diluting the particles and the first extract separately before adding them to the second extract. As a control for the fiber addition step, fiberless 34 particles were used in a parallel experiment. 9⁺F⁻ extract is 18:23:27:34:rII extract and 9⁻F⁺ extract is 9:23:27:rII extract.

in this conversion. Therefore both 9^+F^- particles and fiberless particles should be converted to active phage at the same rate in the second step where tail fiber addition, but not the gene 9 step can occur. If there were more steps needed to convert the 9^+F^- particle to active phage than just tail fiber addition, then the conversion of fiberless particles to active phage might be more rapid than that of the 9^+F^- particle. That the two rates are identical is seen in an experiment presented in figure 7 in which the time course of the conversion of particles to active phage in a 9:23:27:rII extract is measured. In this experiment one of the samples of 9^+F^- particles was taken from a reaction mixture in which only 10% of the 9 particles had been converted to the 9^+F^- form, the other sample was one in which 100% of the 9 particles were converted to the 9^+F^- form; no difference is seen in the conversion process with these two samples.

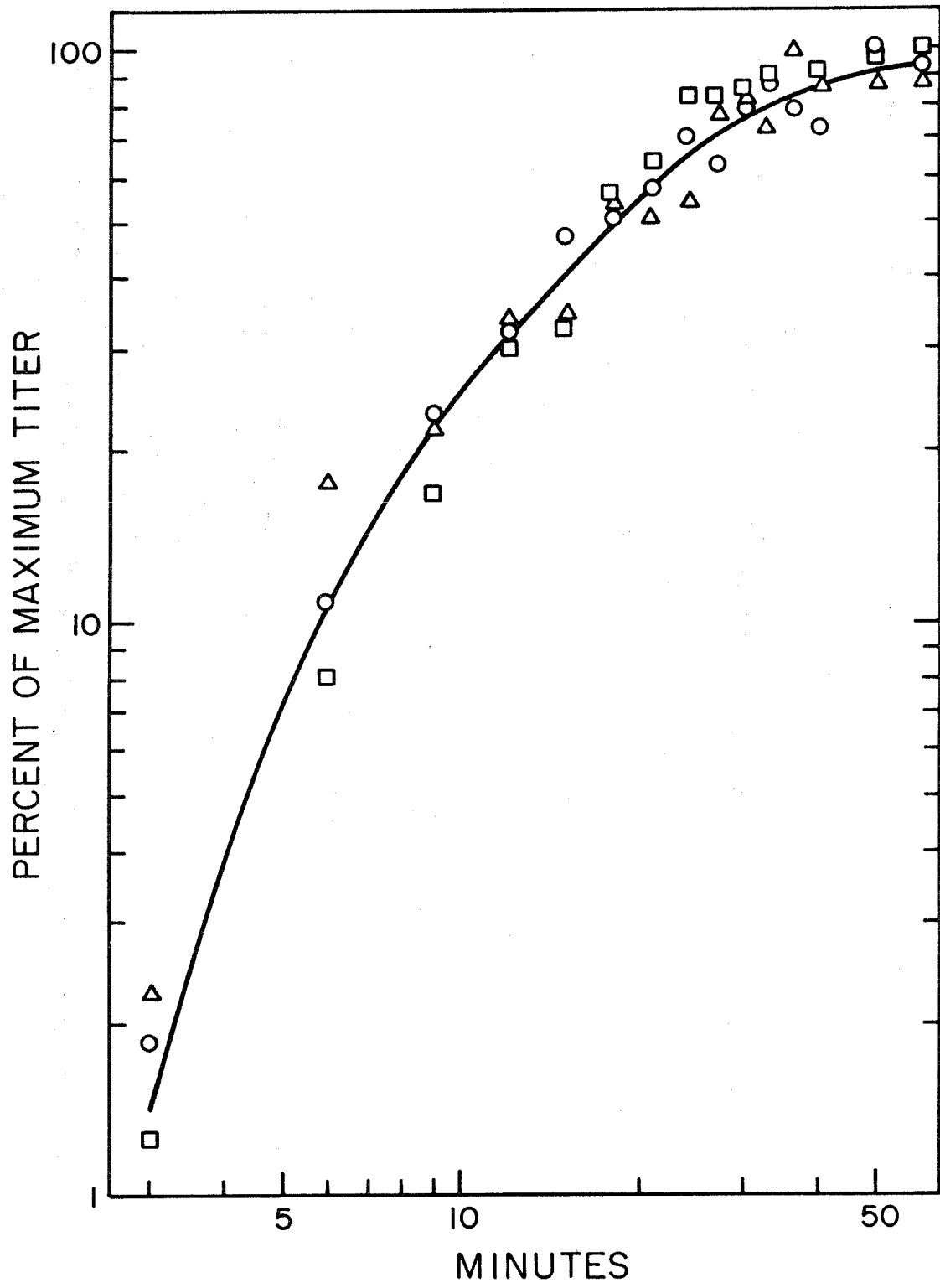
4. Implications of the two step system.

With this two step procedure for converting 9 particles to active phage, it is possible to set up a system for studying specifically the gene 9 step in vitro. By assaying samples from a reaction mixture for the titer of 9^+F^- particles, using the incubation with 9:23:27:rII extract to convert them to active phage, it is possible to eliminate the requirement for the fiber addition step from the kinetics. In this way the complications of analyzing a multistep reaction can be avoided.

FIGURE 7. The kinetics of the conversion to active phage of 9^+F^- particles derived from 9 particles.

Mixtures of 0.90 ml of 9:23:27:rII extract and 0.10 ml of particles were incubated at $30^{\circ}C$ and sampled to determine the titer of active phage. The abscissa is the logarithm of the time of the reaction and the ordinate the logarithm of the maximum number of active rII⁺ phage obtainable with that particle preparation.

- ▲ 9 particles : 10% conversion to a 9^+F^- state
- 0 9 particles : 100% conversion to a 9^+F^- state
- fiberless 34 particles



Defective Particles Produced Using Different Gene 9 Mutants

1. Introduction.

The experiments presented thus far have shown that the 9 particles produced under restrictive conditions in cells infected with the gene 9 mutant am E17 can be quantitatively converted to active phage and that this conversion requires sequentially first the action of the gene 9 product and then the addition of tail fibers. These experiments, however, do not say whether the properties of the 9 particles are solely due to the lack of gene 9 function or whether the 9 particles are a major product of the phage assembly process in the mutant infected cell. To show that these properties are solely due to the absence of the gene 9 function in the infected cell and not due to a second defect caused by an additional mutation in the am E17 phage strain, properties of 9 particles derived from other mutants were shown to be identical to the properties found using am E17. To show that the 9 particles are not a minor by-product of an assembly process deranged by the absence of gene 9 function, the burst size of 9 particles from mutant infected cells is shown to be comparable to the burst size from wild type infected cells.

2. 9 particles from different mutants.

Defective lysates of different gene 9 mutants were tested for the presence of particles that could be converted to active phage. In these experiments three in vitro complementation tests were performed with each lysate: one with an extract containing both gene 9 product and tail

fibers (a 23:27:rII extract); another with an extract containing gene 9 product, but not tail fibers (a 23:34:rII extract). The selective plating system described in connection with the experiment presented in figure 5 was used to insure that all the phage measured contained the genome of the mutant used to make the 9 lysate. As can be seen in table 9, all 9 lysates contain defective particles which can be converted to active phage, and this conversion requires both the action of the gene 9 product and the addition of tail fibers. Being converted to active phage in a 23:27:rII extract which lacks both heads and tails demonstrates that the structure from the 9 lysate must be a particle; it is a type of fiberless particle since it does not become active in the absence of tail fibers. It would seem unlikely that a second mutation is present in the am E17 phage strain as the same defect would also have had to occur independently in all the other gene 9 mutants. This result thus indicates that the defective particle produced in am E17 infected cells is typical of the particles produced in the absence of gene 9 function.

3. The burst size of defective particles from mutant infected cells.

If the burst size of 9 particles from mutant infected cells was comparable to the burst size of normal phage from a wild type infected cell, then it would seem likely that the 9 particle was a true intermediate in the phage assembly process or closely related to such an intermediate. But if the 9 particle were produced in minor quantities, then it would seem less likely that this particle is a significant

TABLE 9

The Yield of 9 Particles From Cells Infected
With Different Gene 9 Mutants

Mutant	Phage per Cell	
	before 23:27:rII extract	after 23:27:rII extract
am E17	0.18	36.
am C215	0.072	26.
am E570	0.15	37.
am E343	0.074	30.
am C226	0.030	141.
wild type	210.	185.

The yield of phage from E. coli B_C, infected in the manner described in Materials and Methods with the mutants listed in the table and lysed after 45 min. at 30°C, is determined. The yield of phage is determined by measuring the titer in the lysate directly and by measuring the average titer of active rII⁺ phage in a reaction mixture of 0.10 ml of lysate and 0.40 ml of 23:27:rII extract incubated at 30°C until the conversion of 9 particles to active phage had reached a plateau value. Both am C215 and am E570 failed to recombine with amE17 (see table 4) and the amber mutations in these three strains are probably identical.

intermediate in phage assembly, and it might be merely an aberrant product of the usual assembly process. Since 9 particles can be quantitatively converted to active phage, the burst size of 9 particles can be measured by converting them to active phage in vitro and measuring the titer of active phage. The burst size in a one step growth experiment with mutants defective in each of the three sites in gene 9 at which amber mutations can occur is shown in table 9. One of the gene 9 amber mutants, am C226, produces as many defective particles as the wild type control, but the other two mutants, am E17 and am E343, produce reduced amounts of particles. The reduction in the production of 9 particles in cells infected with these last two mutants correlates with the reduced expression of gene 10 due to the polar influence of the gene 9 amber mutations in these mutant strains. Since gene 10 is known to produce a component essential for the construction of the tail (Epstein et al., 1963), these strains with reduced expression of gene 10 would not produce enough tails to make a normal yield of particles.

These experiments demonstrate that the 9 particles are produced in substantial quantities in mutant infected cells, in quantities comparable to the number of particles produced in wild type infected cells. In addition there is direct evidence that the polar effect of two gene 9 amber mutations, which was noted in the previous section, results in reduced production of defective particles in cells infected with such mutants.

TABLE 10

Properties of 9 Particles From Cells Infected
With Different Gene 9 Mutants

Mutants	Phage per Cell				
	25°C	39.5°C			
	before extract	before extract	after 9 ⁺ F ⁺ extract	after 9 ⁺ F ⁻ extract	after 9 ⁻ F ⁺ extract
ts P3	59.	0.3	48.	0.3	0.6
ts N11	31.	0.2	39.	0.3	0.3
ts CB16	33.	0.2	40.	0.3	0.3
ts G16	94.	0.2	85.	0.3	0.3
ts L54	82.	0.8	71.	0.5	0.6
ts A55	105.	0.2	82.	0.3	0.3
ts CB116	94.	0.2	70.	0.3	0.3
am E17	0.2	0.2	50.	0.3	0.3
wild type	117.	106.	99.	107.	129.

The yield of phage from E. coli B_c, infected in the manner described in Materials and Methods with the mutants listed in the table and lysed after 30 min. at 39.5°C or 90 min. at 25°C, is determined. The yield of phage is determined by measuring the titer of active rII⁺ phage directly in the lysates and after a 2 hour incubation at 30°C with different types of extract. 9⁺F⁺ extract = 23:27:rII extract. 9⁺F⁻ extract = 23:27:34:rII extract. 9⁻F⁺ extract = 9:23:27:rII extract.

Summary

Phage mutants defective in gene 9 cause the production of defective particles (= 9 particles) when used for one step growth experiments under restrictive conditions. Some properties of these particles and their production are the following:

1. 9 particles can be quantitatively converted to active phage in extracts containing both gene 9 product and tail fibers; 9 particles produced by different gene 9 mutants show the requirement for both gene 9 product and tail fibers.

2. This conversion to active phage takes place in two distinct steps: action of the gene 9 product on the particle to convert it into a 9⁺ state to which tail fibers can then be added.

3. The same number of 9 particles are produced in cells infected with gene 9 mutants as normal phage are produced in cells infected with wild type phage.

4. 9 particles are both cold labile and low salt labile, conditions which do not affect the stability of normal phage; they retain their ability to be converted to active phage if kept at high salt concentrations at room temperature.

5. The cold and low salt lability is due to the conversion of particles from a form resembling fiberless phage to one which has a contracted sheath, but in which the phage DNA is still retained in the head.

Results 3: The Site of Action of the Gene 9 Product on the Particle.

Introduction

The results described thus far show that the gene 9 assembly step can take place in vitro to convert the gene 9 defective particles to fiberless particles which are then a suitable substrate for the second reaction of tail fiber addition. What component of the 9 particle is the substrate for the gene 9 product: the head, the baseplate, or the neck? Conceivably differences in the appearance of particles that have undergone the gene 9 step compared to ones that have not might be revealed by electron microscopic studies. An examination of fiberless particles (from cells infected with a mutant defective in gene 34, essential for tail fiber formation) and 9 particles in the electron microscope revealed no obvious differences in the appearance of the two types of particles in either the normal or contracted sheath state (except for the presence of DNA in the heads of contracted 9 particles); examples of the two types of particles can be seen in plates 1, 2, and 3. Apparently the conversion of the 9 particle into a fiberless particle must produce an alteration too subtle to be seen in the electron microscope. Another approach is needed to determine at what site on the particle the gene 9 step acts.

A number of phage components accumulate in cells infected with mutants defective in phage assembly such as heads, baseplates, naked tails, and sheathed tails; completed heads and sheathed tails have been shown to be obligatory intermediates in the assembly of active phage

(Edgar and Lielausis, 1968; King, 1968). If an obligatory intermediate in assembly can be isolated free from gene 9 product and still complement in vitro with an extract lacking both that intermediate and the gene 9 product, then the gene 9 assembly step must act on the components that are present in this intermediate and not on those which are missing. This approach, if successful, could eliminate all but a few components as being the site of action of the gene 9 step.

The Relation of the Gene 9 Step to the Precursors of
the Fiberless Particle

1. Introduction

A final step in assembly is the attachment of tail fibers to a fiberless particle (King, 1968) and in table 6 it was shown that such fiberless particles can be converted to active phage in the absence of the gene 9 product, implying that the fiberless particle has already undergone the gene 9 assembly step. Edgar and Lielausis (1968) have shown that two precursors in the formation of fiberless particles are heads and sheathed tails; these two unite spontaneously to form a fiberless particle. If the gene 9 product can act only after the head and the tail have united to form a particle, then it should be possible to convert neither the head nor the sheathed tail to active phage in the absence of the gene 9 product. If, however, the gene 9 product can act on one of these precursor components, then it should be possible to convert that component to active phage in the absence of gene 9 product.

This approach was used successfully by Edgar and Lielausis (1968) in determining the site of action of the products of genes 11 and 12.

The basic experiment used to test this possibility is a modification of the experiment described by Edgar and Wood (1966) in which in vitro complementation was observed between extracts lacking heads and extracts lacking sheathed tails. In this modification the component supplied by one of these extracts which was essential for in vitro complementation with the other extract was separated from free gene 9 product and tested for its ability to complement in vitro with an extract missing that component as well as the gene 9 product. As a control this component was also tested for its ability to complement in vitro an extract missing the component, but containing the gene 9 product. If the isolated component is able to complement both tester extracts, then it must already have been acted on by the gene 9 product in such a way that the action of the gene 9 product is no longer necessary in subsequent conversion steps to active phage. If, however, the component is able to complement the tester extract containing gene 9 product but not the tester extract lacking it, then there is still a requirement for the action of the gene 9 product in the subsequent conversion of that component to active phage.

Before either heads or sheathed tails can be tested to see whether they have undergone the gene 9 step, they must be isolated free from gene 9 product present in the extract. With the fiberless particles isolation by means of differential centrifugation was possible since the particles have the same stability properties as normal phage and the large

difference in the sedimentation properties of gene 9 product and phage particles could be exploited. Since less was known about the stability of free heads and tails, separation of these components was carried out by exploiting the difference in sedimentation properties using sucrose gradient centrifugation instead of differential centrifugation.

2. Heads require gene 9 product in their conversion to active phage.

A 27 extract (lacking tails due to a failure to make baseplates) was used as a source of heads produced in the presence of gene 9 product. These heads were separated from gene 9 product by means of sucrose gradient centrifugation and tested for their ability to be converted to active phage by means of in vitro complementation with tester extracts that either contain or lack the gene 9 product. The data in table 11 show that a fraction from this sucrose gradient which contains heads can be converted to active phage in a 23 extract which contains the gene 9 product, but can not be converted to active phage in a 9:23 extract which lacks the gene 9 product (23 extracts lack heads due to the absence of the major structural component of the capsid). Thus the free head is not the site of action of the gene 9 product.

3. Sheathed tails do not require gene 9 product in their conversion to active phage.

A 23 extract was used as a source of sheathed tails made in vivo in the presence of gene 9 product. These sheathed tails were then separated from gene 9 product by sedimenting the extract through a sucrose gradient; fractions from this gradient were then tested for their ability

TABLE 11

A Requirement for the Gene 9 Product in the Conversion
of Heads to Active Phage

	<u>Control</u>	<u>27 Extract</u>	<u>27 Heads</u>
control		0.36	0.050
23 extract	0.051	100.	19.6
9:23 extract	0.37	93.	0.56

An aliquot of 27 extract which serves as a source of heads was layered on a sucrose gradient and centrifuged at 19,600 rev/min, 20°C, for 15 min. Five fractions of nine drops each were collected and the second fraction (= 27 heads) was the source of heads separated from gene 9 product. The value in the table is the titer of active phage (in percent of the maximum titer found) from in vitro complementation tests in which equal volumes of samples had been mixed and incubated for 2½ hours at 30°C.

to complement in vitro tester extracts that either contain or lack the gene 9 product. The data presented in figure 8 show that the fractions from this gradient are able to complement both a 27 extract which contains gene 9 product as well as 9:27 extract which lacks gene 9 product. This result indicates that sheathed tails made in vivo in the presence of the gene 9 product have already undergone the gene 9 assembly step.

The result of this second experiment shows that the gene 9 step does not act only on a single structure which is an intermediate in the assembly of the phage; both the sheathed tail and the 9 particle are suitable substrates for this assembly step. Apparently under conditions where the gene 9 step can not take place, an abnormal 9 defective tail is produced which while still able to attach to the head is not a substrate of tail fiber attachment once attached to the head. When active gene 9 product is present in vivo, the gene 9 step occurs prior to head-tail union.

The Component of the Sheathed Tail on which the Gene 9 Product Acts.

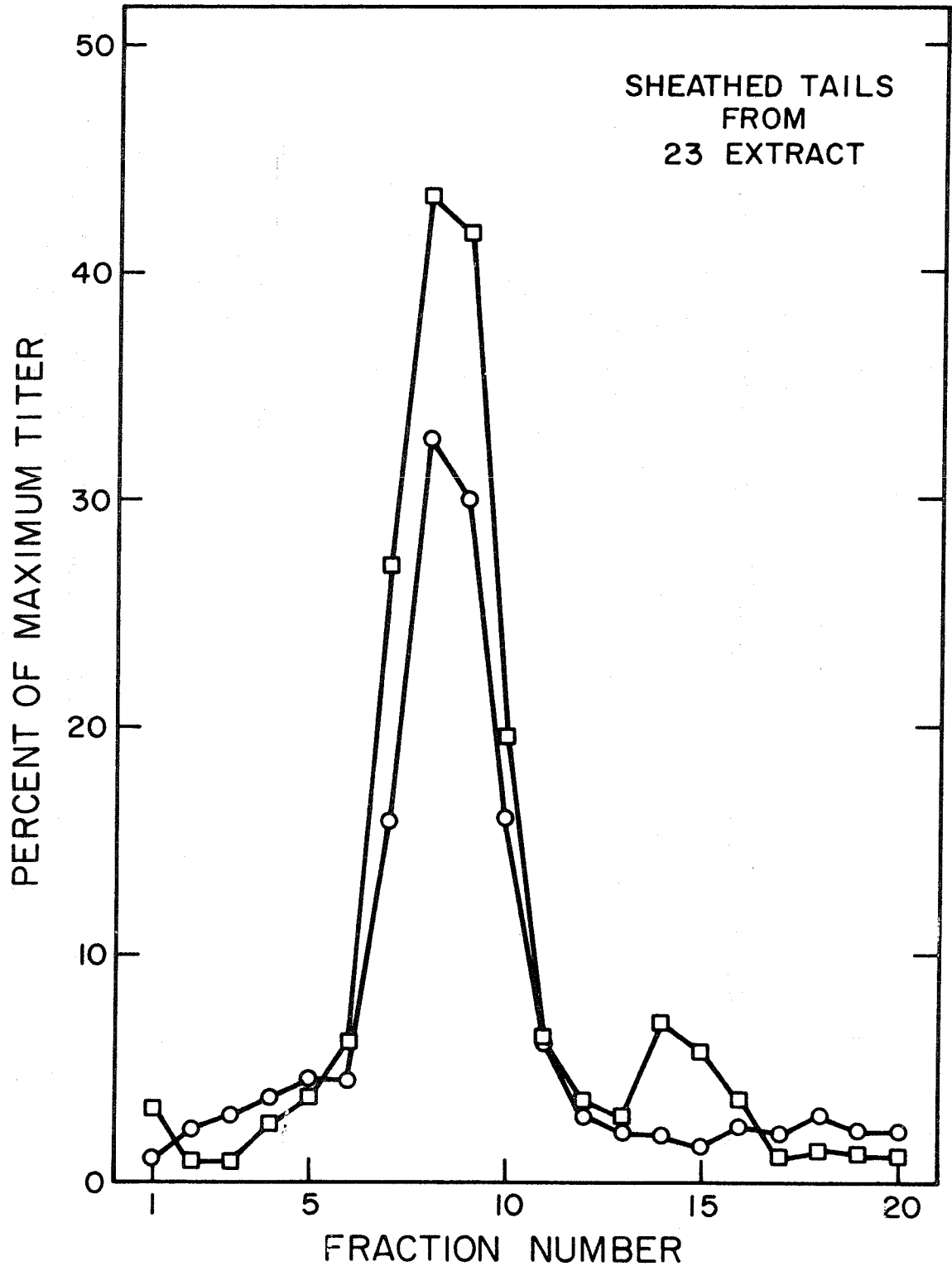
1. Introduction

The experiment presented in the previous section has demonstrated that the gene 9 product acts on the tail of the phage particle; since the tail is composed of several different components, it might be possible to determine on which one the gene 9 product acts by means of the method used in the previous section. If an intermediate in tail assembly lacking a given component can be converted to active phage in the absence

FIGURE 8. The absence of a requirement for gene 9 product in the conversion of sheathed tails to active phage.

An aliquot of 23 extract (a source of sheathed tails) was layered on a sucrose gradient and centrifuged at 37,300 rev/min, 20°C, for 35 min. Twenty fractions of three drops each were collected and assayed for their ability to complement in vitro with a 27 extract and a 9:27 extract by incubating two drops of the gradient fraction with three drops of the tester extract for two hours at 30°C. The abscissa is the fraction number and the ordinate the percent of the titer of the complementation test relative to the maximum titer obtainable with that extract.

- assayed with 27 extract
- assayed with 9:27 extract



of gene 9 product, then the missing component is not the site of action of the gene 9 product; the gene 9 product must therefore act on one of the remaining components of the intermediate.

Some of the steps in tail assembly have been worked out by Edgar and Lielausis (1968) and King (1968). They have shown that at least two fairly stable intermediates accumulate in mutant infected cells (where the mutation blocks some aspect of tail assembly): the baseplate and the core-baseplate (or naked tail). From these studies the baseplate appears as the end product of a number of steps that have not been clarified. On the baseplate the core is assembled to produce the naked tail. On the naked tail the sheath is then added to form the sheathed tail that is now able to combine with a completed head. One interesting feature of these two steps is that both the core and the sheath are added onto the tail as subunits rather than as pre-assembled structures. This feature permits the separation of the baseplate or naked tail from free core or sheath subunits by exploiting the large difference in sedimentation coefficient between these structures and the subunits of the core and of the sheath. Since gene 9 product has sedimentation properties similar to core and sheath subunits, baseplates and naked tails can also be separated from gene 9 product by this method.

A number of genes have been identified as specifying proteins necessary for either core formation (genes 19, 48, and 54) or sheath formation (genes 3, 15, and 18), and both core formation and sheath formation are assembly steps which can be carried out in vitro. Both baseplates (which accumulate in cells infected with mutants defective in core for-

mation) and naked tails (which accumulate in cells with mutants defective in sheath formation) can be converted to active phage by complementation in vitro with an extract derived from cells infected with a mutant defective in baseplate formation. A 27 extract was used as an extract lacking baseplates, but containing heads, tail fibers, core, and sheath material; addition of baseplates in the form of sheathed tails, naked tails, or baseplates results in the assembly of active phage. To test whether a structure has already undergone the gene 9 assembly step, it was first isolated from free gene 9 product and tested for its ability to complement in vitro with both 9:27 extracts as well as 27 extracts.

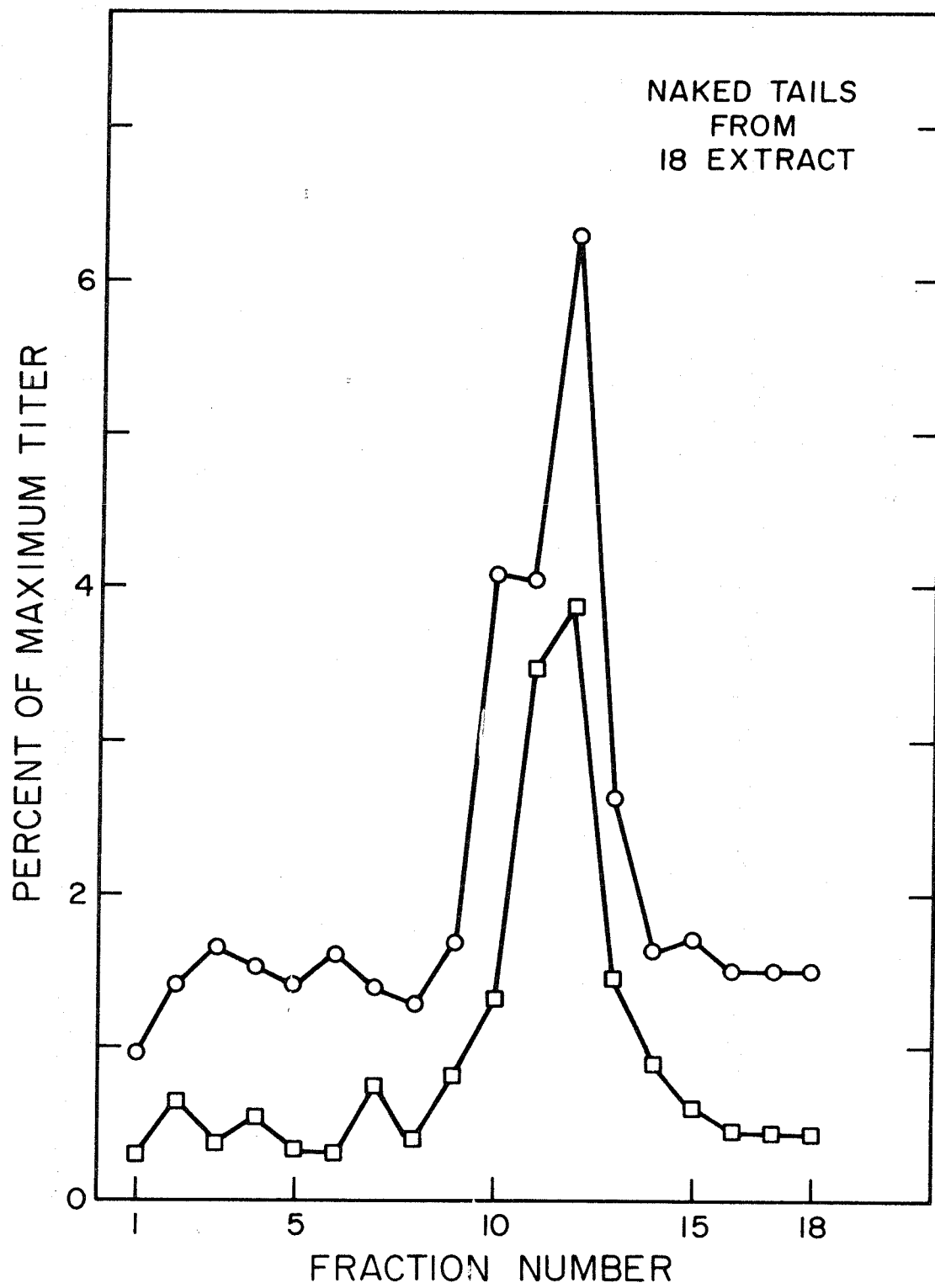
2. Naked tails do not require gene 9 product in their conversion to active phage.

In figure 9 are illustrated the results of an experiment which shows that naked tails can be converted to active phage in the absence of gene 9 product. An 18 extract was used as the source of naked tails made in the presence of gene 9 product (gene 18 specifies the major structural component of the sheath) and these naked tails were separated from gene 9 product by means of sedimentation through a sucrose gradient using the conditions described by King (1968). A peak of activity is found in the gradient at about 80 S which contains material able to complement both 27 extract and 9:27 extract; slightly less activity is found with 9:27 extract than with 27 extract. Since naked tails have been shown by Edgar and Lielausis (1968) and King (1968) to sediment in this region of the gradient, it is concluded that the

FIGURE 9. The absence of a requirement for gene 9 product in the conversion of naked tails to active phage.

An aliquot of 18 extract (a source of naked tails) was layered on a sucrose gradient and centrifuged at 34,500 rev/min, 20°C, for 40 min. Eighteen fractions of four drops each were collected and assayed for their ability to complement in vitro with a 27 extract and a 9:27 extract by incubating two drops of the gradient fraction with three drops of the tester extract for two hours at 30°C. The abscissa is the fraction number and the ordinate the percent of the titer of the complementation test relative to the maximum titer obtainable with that extract.

- assayed with 27 extract
- ◻ assayed with 9:27 extract



structure able to complement both extracts is the naked tail. Hence it seems that the naked tail behaves in vitro as though it had already been acted on by gene 9 product. Such an explanation rules out the sheath as being the component on which the gene 9 product acts.

3. Baseplates do not require gene 9 product in their conversion to active phage.

Baseplates were tested in a manner similar to naked tails and the results illustrated in figure 10 show that they can be converted to active phage in the absence of gene 9 product. A 54 extract was used as the source of baseplates made in the presence of gene 9 product (gene 54 specifies a protein needed in core assembly). When 54 extract was sedimented on a sucrose gradient, a peak of activity was found at about 80 S which complemented with both 27 and 9:27 extract; in this case more activity was found with the 9:27 extract than with the 27 extract. Since baseplates have been shown by King (1968) to sediment at about this position, it is concluded that the structure able to complement both extracts is the baseplate. Hence it seems that the baseplate behaves in vitro as though it had already been acted on by gene 9 product. Such an explanation rules out the core as being the component on which the gene 9 product acts.

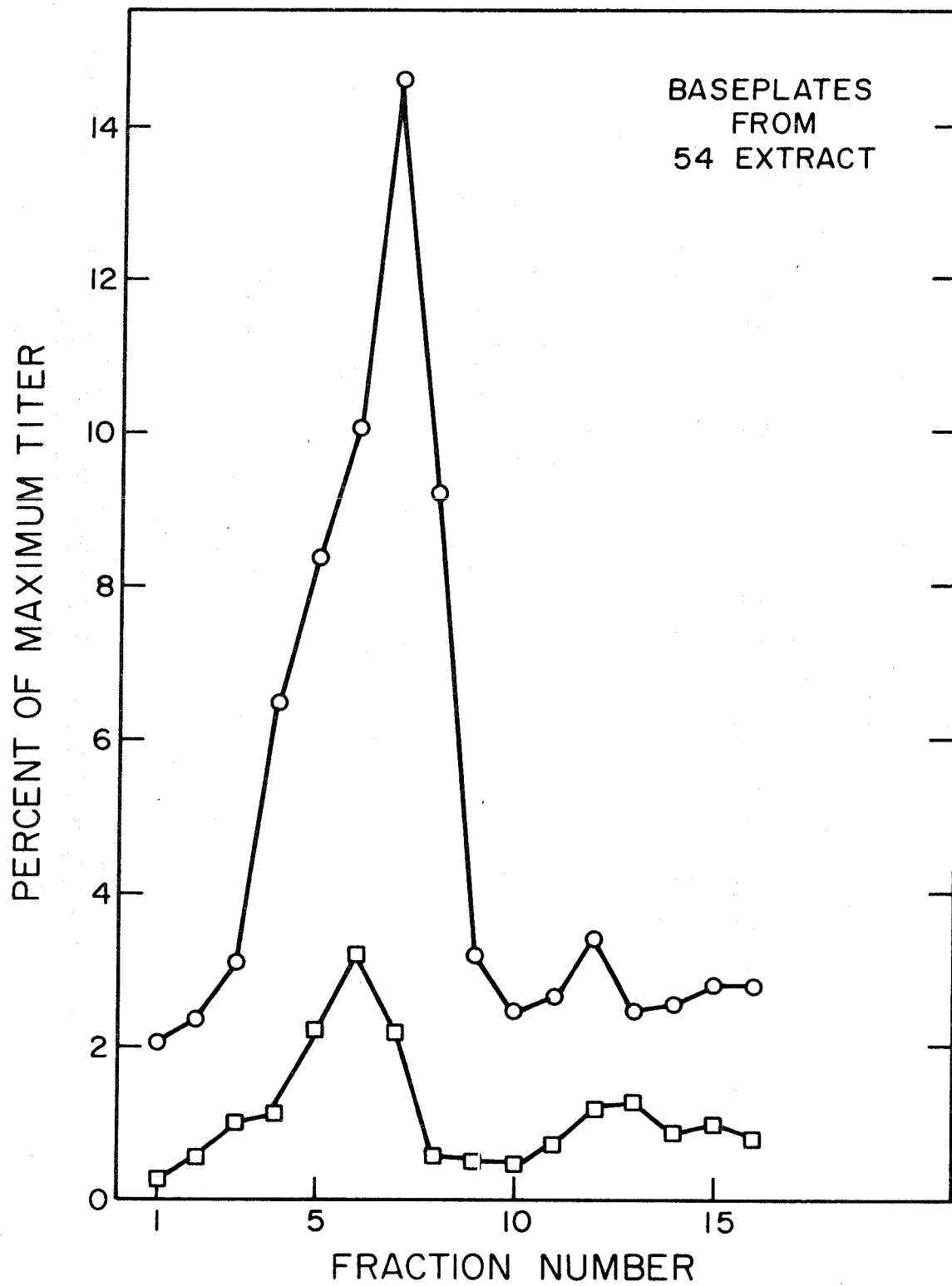
4. The fraction of tail components in a 9^+ state.

In the assay for baseplates, naked tails, and sheathed tails the phage titer was measured at only one time in these in vitro complementation tests. The assay is thus qualitative because it is not known

FIGURE 10. The absence of a requirement for gene 9 product in the conversion of baseplates to active phage.

An aliquot of 54 extract (a source of baseplates) was layered on a sucrose gradient and centrifuged at 37,300 rev/min, 20°C, for 45 min. Sixteen fractions of four drops each were collected and assayed for their ability to complement in vitro with a 27 extract and a 9:27 extract by incubating two drops of the gradient fraction with three drops of the tester extract for two hours at 30°C. The abscissa is the fraction number and the ordinate the percent of the titer of the complementation test relative to the maximum titer obtained with that extract.

- assayed with 27 extract.
- assayed with 9:27 extract.



whether this titer represents an endpoint or a rate, so that quantitative comparisons of the ability of a sample to complement two different tester extracts can not be made exactly. The observation that gradient fractions of naked tails and sheathed tails give about one half the activity with 9:27 as compared to 27 extracts suggests that the fraction of components which are 9^+ is not minor. In addition the observation that baseplate fractions showed four fold more activity with 9:27 extract as compared to 27 extract suggests that in this case a rate may be measured rather than an endpoint. Since such assays show a strong concentration dependence, differences in the concentration of infected cells used to make such extracts may cause rate differences which account for the different degree of complementation found for baseplates. In any case, none of the data suggest that the fraction of a component which is 9^+ under these conditions is minor, rather the 9^+ state is typical of the majority of the components.

5. No contamination of the isolated tail intermediates with free gene 9 product.

My interpretation of the experiments with sheathed tails, naked tails, and baseplates depends upon the assumption that free gene 9 product does not cosediment with these tail components. Contamination of the fractions containing these components with gene 9 product would render invalid the conclusion that the various tail components have actually undergone the gene 9 step. This possibility was explored by sedimenting a sample of 23:27 extract (as a source of free gene 9 product)

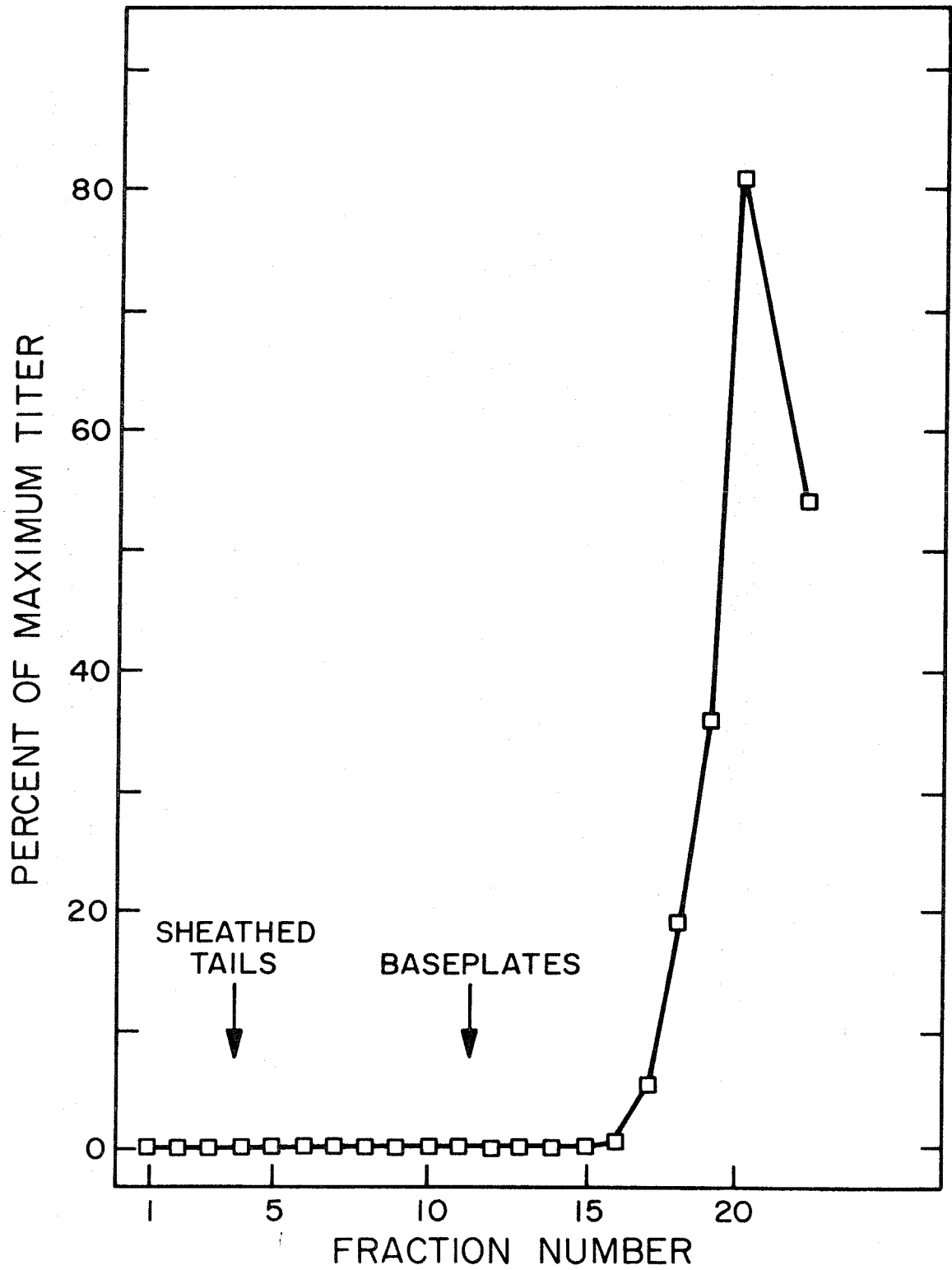
on a sucrose gradient under conditions used to isolate these tail components. Fractions from this gradient were then tested for their ability to complement a 9 extract, i.e. to supply free gene 9 product; as controls sheathed tails and baseplates were sedimented in parallel experiments under the same conditions to determine at which region in the gradient they would be found. The data from this experiment is shown in figure 11 where it is seen that there is a peak of activity of gene 9 product only at the top of the gradient. At the positions at which sheathed tails and baseplates would sediment, indicated by arrows in the figure, there is no gene 9 product activity. Thus the 9⁺ property of these structures is not due to contamination with gene 9 product.

No Functional Connection Between the Gene 9 Step
and the Gene 11 and 12 Steps

Genes 11 and 12 were found by Edgar and Lielausis (1968) to act in a manner similar to gene 9 in the role they play in the construction of phage; in the absence of either the gene 11 or gene 12 product, defective particles are produced. In vitro the gene 11 and 12 products are able to convert these defective particles to active phage, but under non-defective in vivo conditions the site of action is the tail, presumably the baseplate. As in the case for the gene 9 assembly step, the absence of the gene product does not block the assembly process at an early stage; assembly may proceed and defective particles are produced which lack the action of that particular assembly step. Two

FIGURE 11. The sedimentation of gene 9 product in relation to sheathed tails and baseplates.

An aliquot of 23:27 extract (a source of gene 9 product) was layered on a sucrose gradient and centrifuged at 37,200 rev/min, 20°C, for 45 min. Twenty-one fractions of three drops each were collected and assayed for their ability to complement in vitro with a 9 extract by incubating one drop of the gradient fraction with two drops of the tester extract for four hours at 30°C. The abscissa is the fraction number and the ordinate the percent of the titer of the complementation test relative to the maximum titer obtainable with the tester extract. The two arrows indicate the position to which sheathed tails and baseplates sedimented in a parallel experiment.



differences have been found between the gene 9 step and the gene 11 and 12 steps. First, the 11 particles and the 12 particles both have tail fibers attached to the baseplate; neither of these assembly steps is necessary for the attachment of tail fibers to the baseplate of fiberless particles. Second, there is a functional connection between the gene 11 and 12 assembly steps. Edgar and Lielausis (1968) have shown that particles defective for the gene 11 function are also defective for the gene 12 function even though they were produced in cells that contain gene 12 product; however particles defective for gene 12 function are not defective for gene 11 function. This functional connection suggests that the action of the gene 11 product on the baseplate is necessary before the action of the gene 12 product can take place.

The similarity in the action of these three assembly steps suggested that all three might be functionally connected. By determining if particles defective for one of these assembly steps can be converted to active phage in the absence of the gene product necessary for one of the other assembly steps, it should be possible to determine whether the particle is defective for more than one gene function. The data presented in table 12 show that 11 and 12 particles can be converted to active phage in the absence of gene 9 product; all the particles are converted to active phage in both 23:27:rII extract which contains gene 9 product as well as in 9:23:27:rII extract which lacks gene 9 product. Similarly the data presented in table 13 show that 9 particles can be converted to active phage in the absence of either the gene 11 product

TABLE 12

The Absence of a Requirement for Gene 9 Product in the Conversion
of 11 Particles and 12 Particles to Active Phage

	No Extract	Titer x 10 ⁻⁹	
		23:27:rII extract	9:23:27:rII extract
11 particles	0.094	3.8	3.9
12 particles	0.010	15.	14.
9:11:12 particles	1.4	80.	1.5

The titer of active phage of a rII⁺ genotype derived from the defective particles by in vitro complementation tests is given. In such experiments 0.20 ml of extract was mixed with 0.05 ml of particles, incubated at 30°C, and the average titer determined after the conversion of defective particles to active phage had reached a plateau value.

TABLE 13

The Absence of a Requirement for the Gene 11 Product or the Gene 12 Product in the Conversion of 9 Particles to Active Phage

	Titer x 10 ⁻⁹	
	9 particles	9:11:12 particles
No extract	6.3	1.9
23:27:rII extract	1700.	230.
11:23:27:rII extract	2100.	5.7
12:23:27:rII extract	2000.	4.8

The titer of active phage of a rII⁺ genotype derived from defective particles by in vitro complementation tests is given. In such tests 0.20 ml of extract was mixed with 0.05 ml of particles, incubated at 30°C, and the average titer determined after the conversion of defective particles to active phage had reached a plateau value.

or the gene 12 product; all 9 particles can be converted to active phage in either 11:23:27:rII extract or 12:23:27:rII extract as well as 23:27:rII extract. In both of these experiments 9:11:12 particles, which are missing all three gene functions, were used as a control to show that a negative result was possible. These particles were prepared from cells infected with a triple mutant defective in all three genes.

These results indicate that while there is a similarity in the manner in which the gene 11 and 12 assembly steps work compared to the gene 9 assembly step, there is no functional connection between these steps in the assembly of phage particles.

Tail Fibers as a Possible Site of Action of Gene 9 Product.

The previous experiments have shown that the gene 9 product acts on some part of the baseplate so that after the union of the head and the tail, the tail fibers can be attached to the baseplate. Not excluded however, is the possibility that the gene 9 product can act either on the baseplate or on the tail fiber, and that it need only to act at one of these two sites in order for phage to be assembled properly. If this were the case, two different types of tail fibers are possible: those which have undergone the gene 9 step (= 9^+ tail fibers) from infected cells containing gene 9 product, and those which have not undergone the gene 9 step (= 9^- tail fibers) from infected cells lacking the gene 9 product. On this model both types of tail fibers could participate in the conversion of fiberless particles to active phage;

however, 9^+ tail fibers could convert 9 particles to active phage directly, while 9^- tail fibers would not interact with 9 particles until one component or the other had been converted to a 9^+ state. The gene 9 assembly step could thus take place either on the particle or on the tail fiber. The two step activation procedure described on page 65 showed that fiberless particles were an intermediate in the conversion of 9 particles to active phage; however, this experiment did not test whether they were an obligatory intermediate in this conversion and so did not rule out the possibility outlined above.

A direct method of determining if the special class of 9^+ tail fibers exists would be to isolate tail fibers from extracts of infected cells containing gene 9 product by means of sucrose gradient centrifugation and to measure their ability to complement a defective extract missing gene 9 product as well as tail fibers. However, two technical problems make this approach unworkable. First, infected cells accumulate only a small fraction of tail fiber material as completed tail fibers; most of the tail fiber material is in the form of precursor half fibers (King, 1968a). Second, the whole tail fiber and the precursor components sediment at positions in a sucrose gradient very close to one another. Any tail fiber activity seen in fractions from such a sucrose gradient might be due either to tail fibers or to the overlap between the peaks of the two major precursors. Since gene 9 product has roughly the same sedimentation properties as tail fiber material, co-sedimentation of tail fibers and gene 9 product can not be tested under these conditions because the peaks of the two types of activity will overlap in the gradient.

Tail fibers and gene 9 product from a 23:27 extract when sedimented through a sucrose gradient do show peaks of activity (illustrated in figure 12) which nearly overlap in the gradient. Assuming that the tail fiber activity is due to the overlap of the peaks of A and BC tail fiber precursors which have sedimentation coefficients of 8 and 9S respectively, the sedimentation coefficient of gene 9 product can be estimated to be 5 to 7 S (the uncertainty being due to the large size of the fractions collected from this gradient).

In order to circumvent these problems an indirect approach was attempted. If 9^+ tail fibers exist, then in a reaction involving the mixed activation in the presence of such tail fibers of fiberless particles and 9 particles, both types of particles must undergo the same number of steps before they become active phage. If, however, the gene 9 step must take place before the tail fibers are added to the particle, in such a reaction 9 particles will undergo more steps before they become active phage than fiberless particles will, and the relative rates of appearance of active phage from the two types of particles should differ. The results of such an experiment are shown in figure 13 where it is seen that fiberless particles are converted to active phage at a faster rate than 9 particles, evidence that 9 particles must undergo at least one more reaction than fiberless particles in their activation. This observation, although indirect, argues against a special class of 9^+ tail fibers. It thus appears that the baseplate is the only site of action of gene 9 product.

FIGURE 12. The sedimentation of gene 9 product in relation to tail fiber activity.

An aliquot of 23:27 extract (a source of gene 9 product and tail fibers) was layered on a sucrose gradient and centrifuged at 37,100 rev/min, 4°C, for 14.5 hours. Eight fractions of eight drops each were collected and assayed for their ability to complement in vitro with a 9 extract and with a 34:35:37:38 extract by incubating one drop of the gradient fraction with two drops of tester extract for four hours at 30°C. The abscissa is the fraction number and the ordinate the percent of the titer of the complementation test relative to the maximum titer obtainable with one of the gradient fractions.

○ assayed with 9 extract.

□ assayed with 34:35:37:38 extract

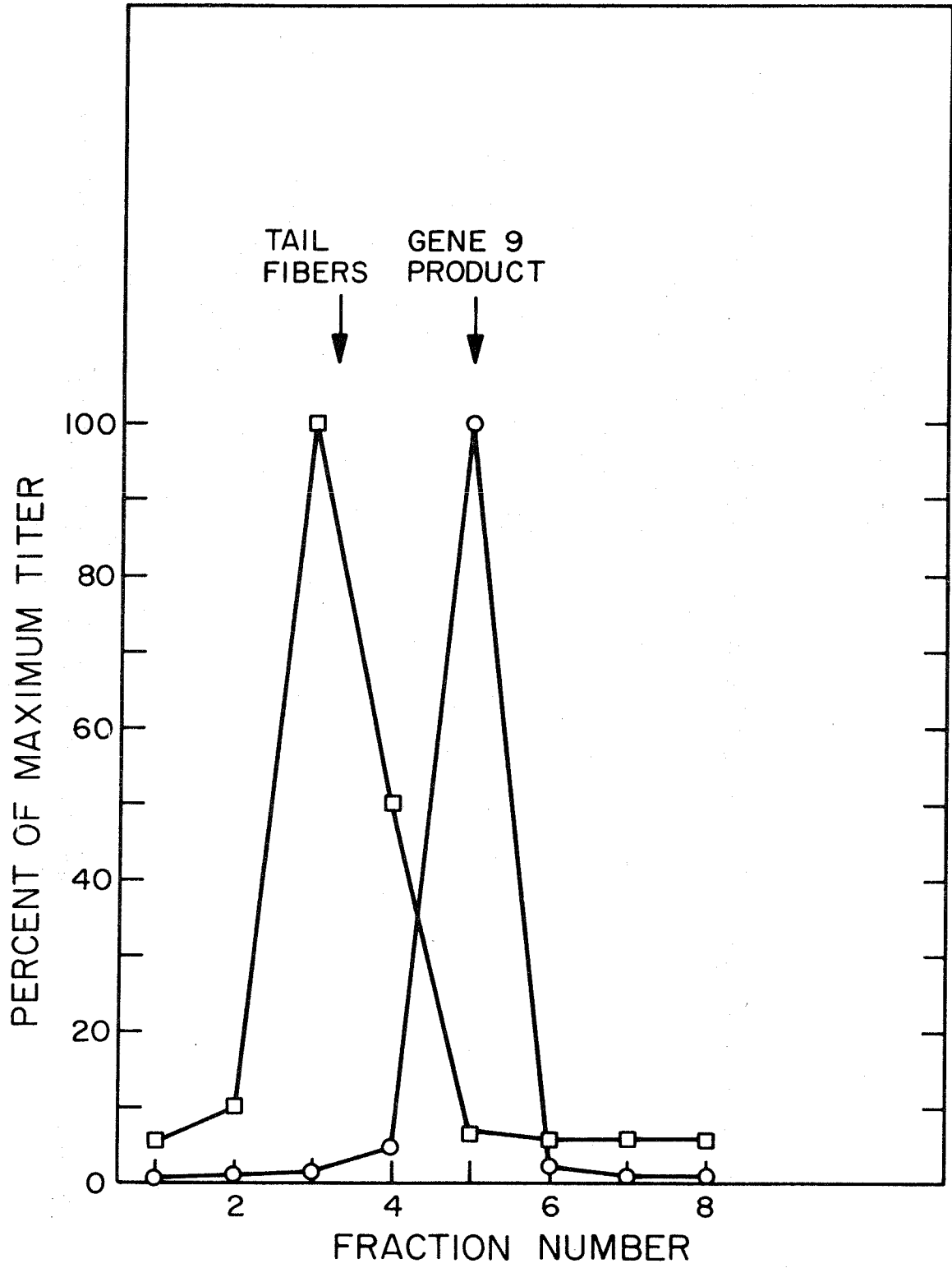
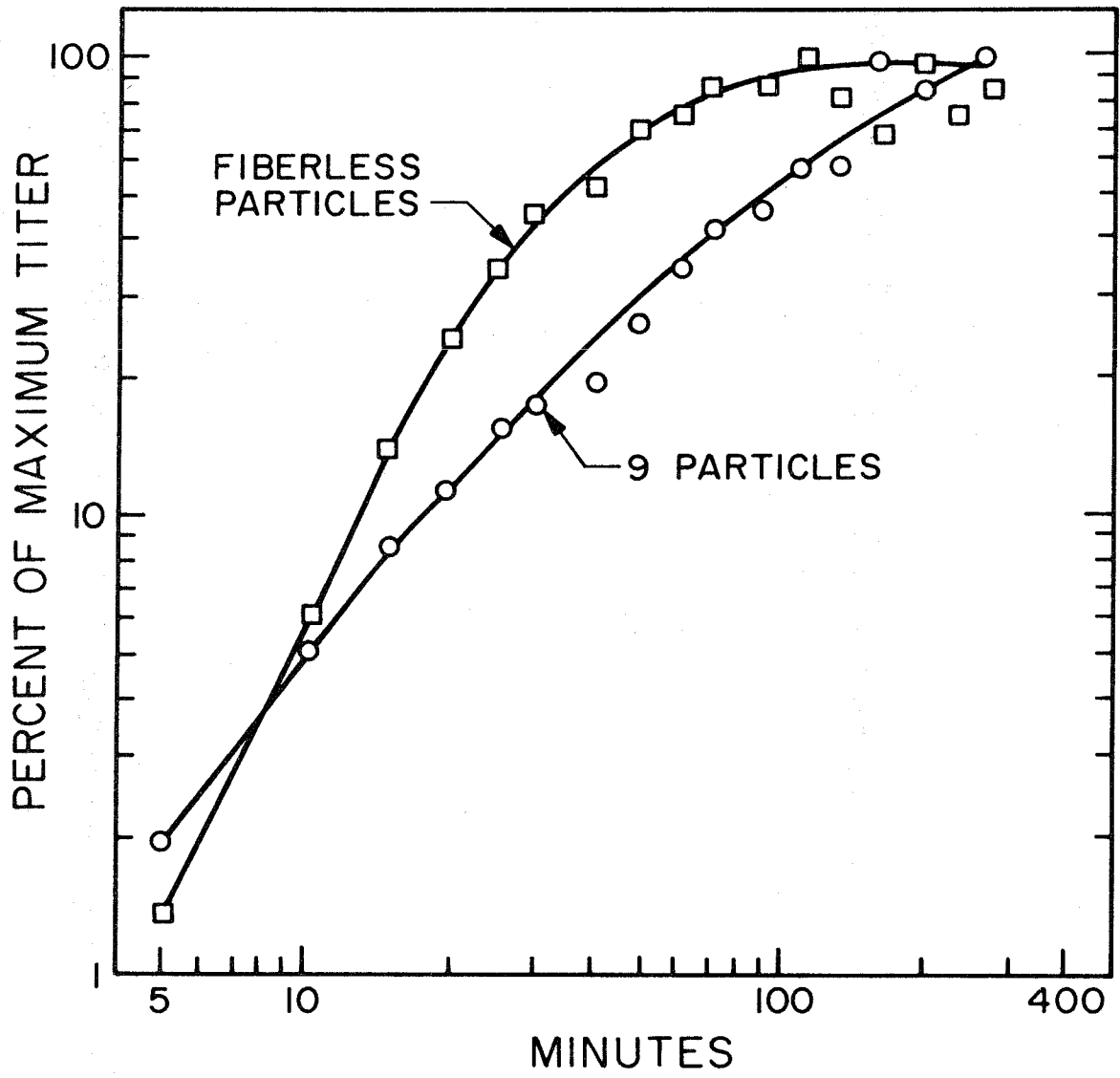


FIGURE 13. A difference between the rate of conversion of 9 particles and fiberless particles to active phage in a 23:27 extract.

A reaction mixture of 0.50 ml of 23:27:rII extract, 0.02 ml of 9:rII particles and 0.02 ml of 34:35:37:38 particles was incubated at 30°C; samples were taken at different times after mixing and assayed for active phage by plating with CR(λ) at 25°C (where only those phage derived from 34:35:37:38 particles will form plaques) and by plating with BB at 40°C (where only those phage derived from 9 particles will form plaques); under these plating conditions no active phage of the genotype 23:27:rII will form plaques. The abscissa is the logarithm of the time at which the sample was taken and the ordinate the logarithm of the percent of the titer of active phage derived from either the 34:35:37:38 particles or the 9 particles, both relative to the maximum titer of phage of that type eventually produced.

- 9 particles
- ◻ 34:35:37:38 particles



Summary

These series of experiments have illustrated several features about the gene 9 assembly step. The site of action of the gene 9 product is the baseplate, and in the gene 9 step the baseplate is modified so that tail fibers can attach after head-tail union occurs. Premature triggering of the tail is also prevented, thus increasing the stability of the particle. The gene 9 product can act early in assembly since baseplates made in the presence of the gene 9 product have already undergone the gene 9 assembly step; structures which contain a baseplate and which were made in the presence of gene 9 product (naked tails, sheathed tails, and fiberless particles) also have undergone the gene 9 assembly step. The absence of the gene 9 product does not block assembly at the point in the sequence at which the gene 9 product can first act; assembly proceeds until a 9⁻ fiberless particle is produced and further assembly is blocked at that point. The behavior of the gene 9 product is similar to that of the gene 11 and 12 products in assembly; no functional connection between the gene 9 step and the gene 11 or 12 steps was detected, however. The gene 9 product does not act on the tail fibers. The sedimentation coefficient of the gene 9 product is between 5 and 7S.

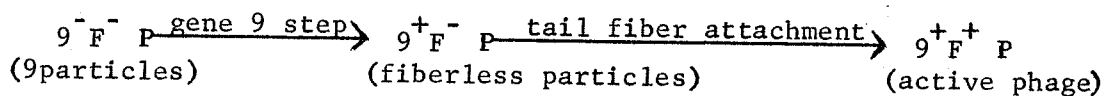
Results 4: Properties of the Gene 9 Assembly Step

Introduction

The gene 9 assembly step takes place at some site on the baseplate. Two approaches have been used in an attempt to determine the nature of the change the baseplate undergoes by conversion from a 9^- state to a 9^+ state: a study of the kinetics of the assembly step and the factors which influence its rate, and an attempt to see if in the reaction between 9 particles and gene 9 product a stoichiometric or catalytic relationship holds. The first approach might reveal what the important rate limiting factors in the reaction are and any requirement for the reaction in terms of ionic conditions and cofactors. The second approach in principle could show whether the gene 9 product is incorporated into the baseplate or whether it catalytically alters some structure on the baseplate without itself being used up in the reaction.

Use of the Two Step Activation System for Measuring the Gene 9 Step

The conversion of 9 particles to active phage has been shown earlier to require at least two different steps: action of the gene 9 product on the 9 particle to produce a fiberless particle, followed by the attachment of tail fibers to this fiberless particle to produce active phage. This conversion may be represented in the following two reactions:



Measuring the conversion of ϕ particles to active phage is not a simple index of the gene 9 step, but measuring the conversion of ϕ particles to fiberless particles is. This measurement can be done by using the two step system described on page 65 . The first reaction can be stopped by dilution and all of the fiberless particles which are in a ϕ^+ state converted to active phage in a second reaction which lacks gene 9 product, but contains tail fibers.

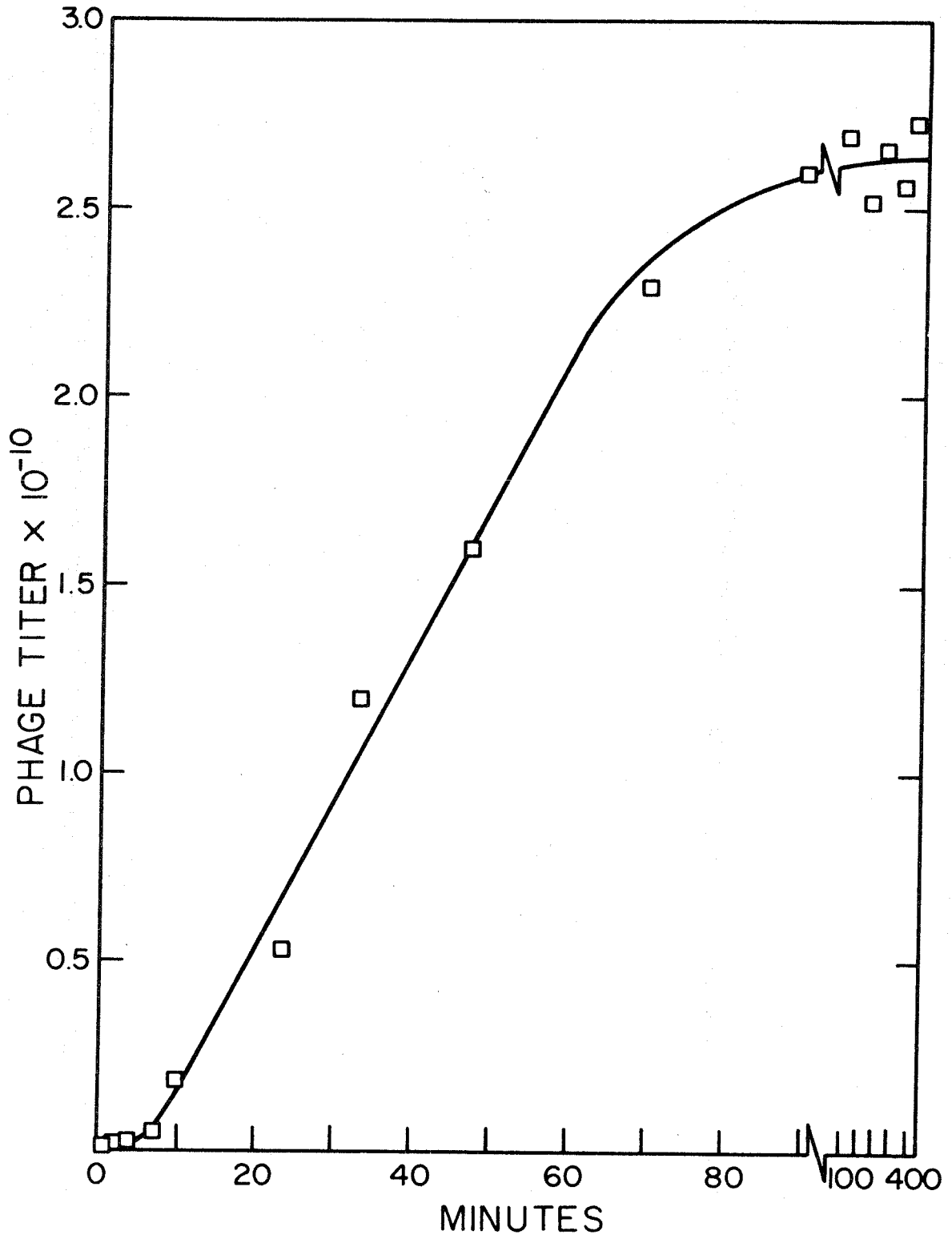
In addition the selective plating system described on page 65 was used to insure that the active phage were all derived from the ϕ particles and were not background viable phage present in the extracts used for either the first or second reaction. Preparations of ϕ particles contain some active phage due both to unadsorbed phage in the infection of restrictive cells and also due to the finite leakage of the amber mutant in the restrictive cells. In the experiments reported in this section the background viable phage titer in the ϕ particle preparations have been subtracted from the titer found after the second reaction, so that the titers represent the number of ϕ^+ particles produced during the course of the reaction.

The Kinetics of the Gene 9 Reaction

The increase in titer of ϕ^+ particles in the gene 9 reaction is shown in figure 14 as a function of the time after mixing ϕ particles with an extract containing gene 9 product; in the figure both the titer of ϕ^+ particles and the time are graphed on linear axes. Two features of this curve should be noted: there is a limit to the titer of ϕ^+

FIGURE 14. The conversion of 9 particles to a 9^+ state.

A mixture of 0.05 ml of 9 particles and 0.50 ml of 18:23:27:34:rII extract (a source of gene 9 product) was incubated at 30°C and sampled at different times for the titer of 9^+ particles. The assay for 9^+ particles was an in vitro complementation test where 0.05 ml particles were incubated with 0.20 ml of 9:23:27:rII extract for two hours and the titer of rII^+ phage determined. The abscissa is the time and the ordinate the titer of 9^+ particles in the first reaction mixture. The solid line is the curve for equation 8 on page 115.



particles produced, and there is a lag period before the reaction proceeds rapidly. The limit to the titer of 9^+ particles is set by the number of 9^- particles added. On page 58 it was shown that all 9 particles could be converted to active phage; as would be expected, this is the result here as well. The titer of 9 particles in the reaction mixture as measured by optical density was 3.0×10^{10} /ml and the titer of the 9^+ particles produced is 2.6×10^{10} /ml, which represents thus about an 87% conversion. In the gene 9 step all of the 9 particles can be converted to a 9^+ state in the absence of the attachment of tail fibers. That the lag period is an important feature of the reaction can be seen in figure 15 where the same data as in figure 14 are plotted only the logarithm of the increase in 9^+ particles is plotted as a function of the logarithm of the time. Here it is seen that the initial points fall on a straight line and that only at late times does this straight line become curved and reach a plateau value due to the exhaustion of 9 particles from the reaction mixture.

An Interpretation of the Kinetics of the Gene 9 Reaction

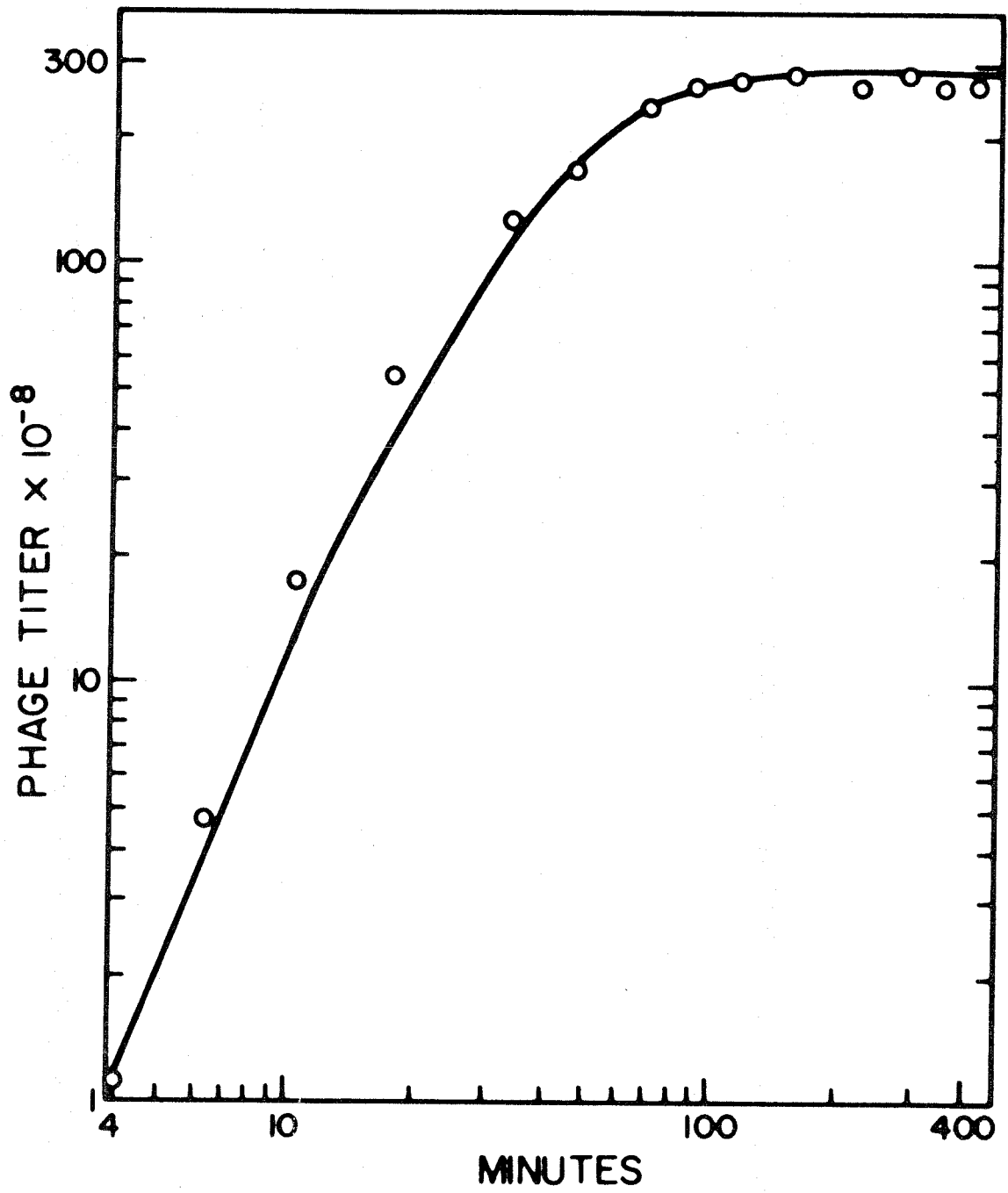
One feature of the initial phase of the gene 9 reaction is a linear relationship between the logarithm of the fraction of particles converted to a 9^+ state and the logarithm of the time of incubation of the reaction. If T is the titer of 9^+ particles at a time t and if T_m is the maximum titer of 9^+ particles possible, then this linear relationship can be expressed as

$$\log (T/T_m) = N \cdot \log t \quad \log a \quad (1)$$

where a and N are constants. This equation (1) can be converted to the form

FIGURE 15. The conversion of 9 particles to a 9^+ state.

The same data as in figure 14 except that the abscissa is the logarithm of the time and the ordinate the logarithm of the titer of 9^+ particles.



$$T/T_m = a \cdot t^N \quad (2)$$

showing that the initial increase in the titer of 9^+ particles is a power function of the time after mixing the reactants. The constants a and N in this relationship can be used to characterize the initial rate of the reaction so that different experiments can be compared quantitatively. For the straight line portion of the curve in figure 15 these values are

$$a = 1.4 \times 10^{-4} \text{ min}^{-2.6}$$

$$N = 2.6$$

A relationship of this type can be derived by considering the gene 9 reaction in terms of a target theory in which the 9^- defective particle is a target which contains several sites that must be modified by the action of the gene 9 product in order for the particle to be converted to a 9^+ state. The following assumptions are made in order to derive an expression for the rate of the gene 9 reaction:

- a) there are n sites on the 9^- defective particle which can be modified from a 9^- to a 9^+ condition by the action of the gene 9 product,
- b) all n sites on the particle must be modified for the particle to be in a 9^+ state,
- c) the sites on the particle at which the gene 9 product acts are equivalent and can be modified independently of one another at a rate k,
- d) in the reaction mixture there is more gene 9 product than sites on the particle to be modified so that the rate at which sites can be modified will not change during the course of the reaction.

If the gene 9 reaction behaves in this manner, then the fraction of sites at a time \underline{t} which have not been modified by the action of the gene 9 product is

$$S/S_0 = e^{-kt} \quad (3)$$

The fraction of sites at this same time \underline{t} which have been modified by the action of the gene 9 product is therefore

$$1 - S/S_0 = 1 - e^{-kt} \quad (4)$$

Since it was assumed that the sites on a particle can be modified independently of one another, the fraction of particles which are in a 9^+ state because all \underline{n} sites have been modified is

$$T/T_m = (1 - S/S_0)^n = (1 - e^{-kt})^n \quad (5)$$

During the initial phase of the reaction when \underline{kt} is small, the approximation

$$1 - e^{-kt} \approx kt \quad (6)$$

can be used to simplify equation (5) to the form

$$T/T_m \approx (kt)^n = k^n t^n \quad (7)$$

which now has the same form as equation (2), and which is observed to fit the initial phase of the reaction.

If the empirical equation (2) is interpreted in terms of this target theory model, then \underline{N} is equal to \underline{n} and is the number of sites on the particle at which the gene 9 product must act for the particle to be in a 9^+ state; and \underline{a} is equal to \underline{k}^n and thus is related to the rate at which sites on the 9 defective particle are modified by the action of the gene 9 product. The curve for equation (5) has been drawn in figure 15 using the following values for \underline{k} and \underline{n} :

$$n = 2.6$$

$$k = (a)^{1/2.6} = 3.3 \times 10^{-2} \text{ min}^{-1}$$

The agreement between this curve and the experimental points is good except in the region between the linear portion and the plateau portion of the curve. The 9 defective particles are not observed experimentally to have an integral target number. If modification of two sites on the particle is sometimes sufficient for the particle to be converted to a 9^+ state, while modification of three sites always converts the particle to a 9^+ state, then a target number between two and three would be expected.

The conversion of fiberless particles to active phage by the attachment of tail fibers shown kinetics similar to the gene 9 reaction and an equation for the rate of appearance of active phage in the former reaction has been formulated by Wood and Henniger (1968), using a set of assumptions similar to those used to analyze the gene 9 reaction and the knowledge that there are six sites on the particle for the attachment of tail fibers. The equation derived by Wood and Henniger (1968) contains parameters that predict what fraction of the particles with different numbers of tail fibers are active; these parameters were determined by fitting the equation to the experimental data. The same approach could be used to analyze the gene 9 reaction and to determine what fraction of the 9 defective particles which have different number of sites modified by the action of the gene 9 product are in a 9^+ state. However, more extensive data on the time

course of the gene 9 reaction would be needed to determine all the unknown parameters in such an equation.

Kinetics of the Gene 9 Reaction at Different Particle Concentrations

If the only factor that causes the gene 9 step to slow down and to reach a plateau value is the exhaustion of 9^- particles from the reaction mixture, then curves similar to the one shown in figure 15 should be obtained when the particle concentration in the reaction mixture differs. When reactions were performed in which the 9^- particle concentration is different but in which the concentration of extract containing the gene 9 product remains constant, then the curves for all reaction mixtures were identical if T/T_m were plotted as a function of the time. This result is seen more clearly if the data is presented in the following manner. The straight line with a slope of 1.0 in figure 16 shows that the T_m (= maximum titer) of the 9^+ particles in a reaction mixture is directly proportional to the relative concentration of 9 particles added, at least in this concentration. The three sets of points, all with approximately the same value, in figure 17 show that the T/T_m ratio for these different reaction mixtures are the same at any given time; there is no systematic variation in these values with the concentration of the 9 particles added.

Two points should be noted concerning this last experiment. In the range tested the concentration of 9 particles never limited the rate of

FIGURE 16. The final yield of 9^+ particles from the gene 9 reaction in vitro as a function of the relative concentration of 9 particles used.

Gene 9 reactions similar to the one described in the legend to figure 14 were performed and the final titer of 9^+ particles determined. The abscissa is the logarithm of the relative concentration of 9 particles added to the reaction (based on how much they were diluted) and the ordinate the logarithm of the final yield of 9^+ particles found in such a reaction mixture.

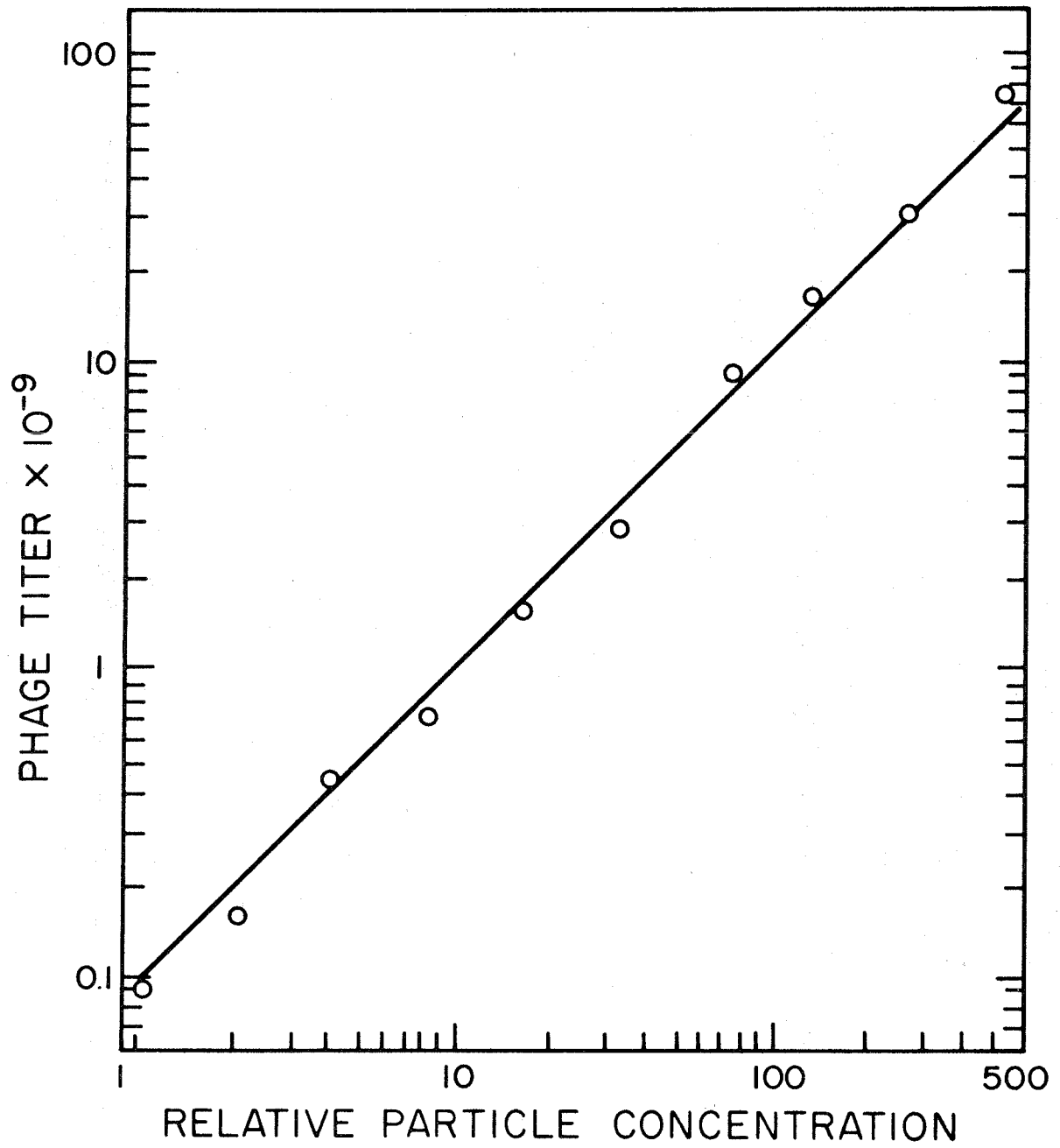
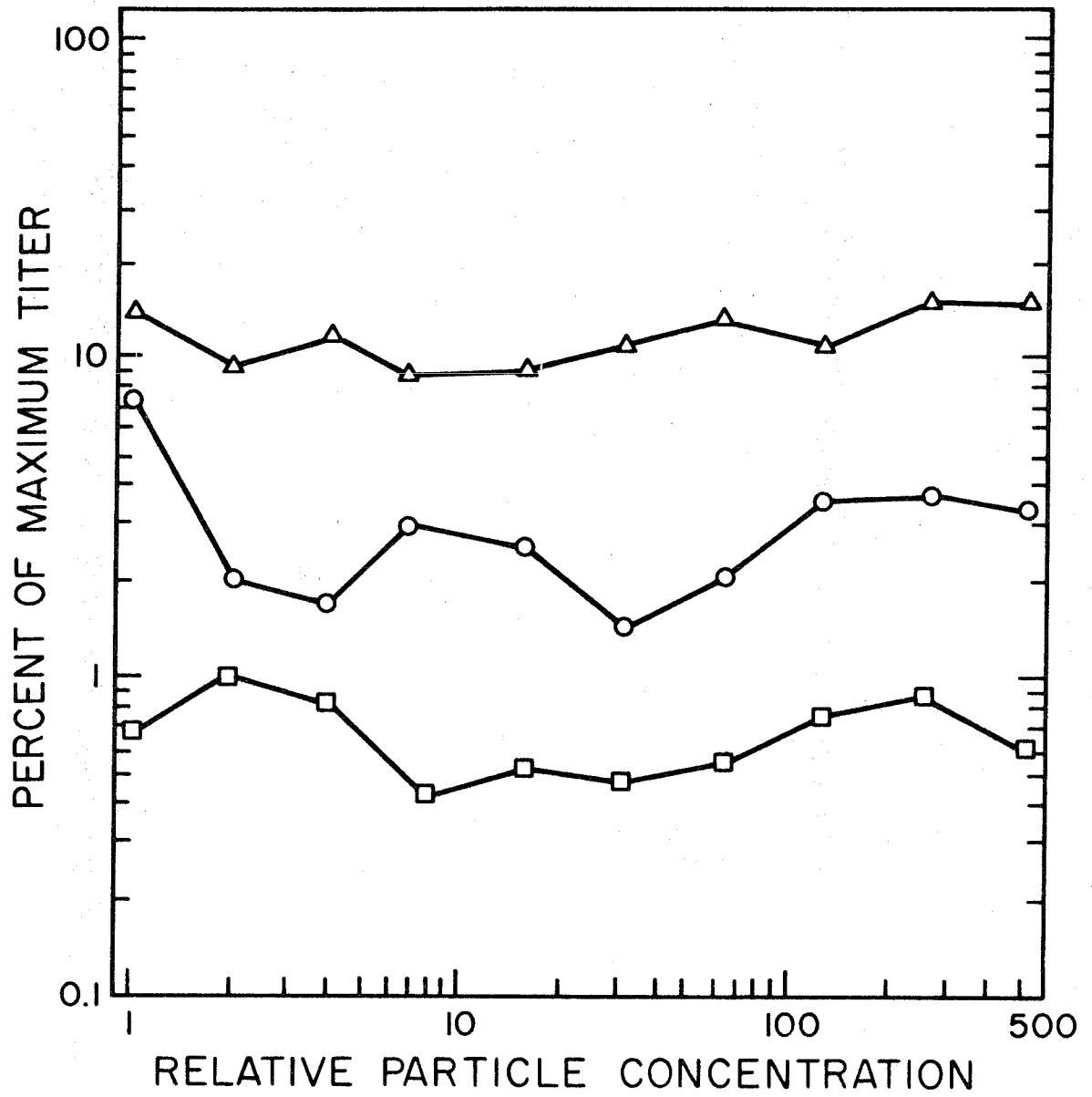


FIGURE 17. The rate of conversion of 9 particles to a 9^+ state as a function of the relative concentration of 9 particles used.

The data are from the reaction mixtures described in the legend to figure 16. The abscissa is the logarithm of the relative concentration of 9 particles added to the reaction mixture and the ordinate the logarithm of the titer of 9^+ particles in a reaction mixture at different times in terms of the percent of the final titer of 9^+ particles.

- 10 min.
- 20 min.
- △ 40 min.



the gene 9 reaction since the same fraction of 9 particles are converted to a 9^+ state in a given time no matter what the absolute particle concentration. This result is in accord with the implicit assumption made in formulating the target theory interpretation that $f(t)$ depends on the time, but not on the 9 particle concentration. The second point, really a corollary to the first, is that \underline{a} and \underline{N} are independent of the 9 particle concentration used in the reaction mixture, and experiments in which the 9 particle concentration differs can be compared with respect to the parameters \underline{a} and \underline{N} .

By analogy with other experimental systems a physical interpretation could be suggested for the parameters \underline{a} and \underline{N} . \underline{N} , the number of "hits" could relate to the number of sites on the baseplate that the gene 9 product must modify to change a 9 particle to a 9^+ state. \underline{a} , the "cross section" of the 9 particle could represent the probability of the 9 particle encountering and interacting with one gene 9 product, i.e. an affinity constant.

Conditions Which Influence the Rate of the Gene 9 Step

1. Introduction

The target theory model for the conversion of a 9^- particle to a 9^+ state can be further tested by seeing whether the parameters \underline{a} and \underline{N} are sensitive to the conditions under which the reaction is carried out. If \underline{N} represents the average number of times a 9^- particle must interact with gene 9 product before being converted to a 9^+ state, then this parameter should be independent of the concentration of gene 9 product,

the temperature, or the ionic conditions under which the reaction is carried out. But \underline{a} should be related to the rate at which 9^- particles interact with gene 9 product and should be strongly affected by these variables. In the next series of experiments to be described each of these variables was checked for its effect on the \underline{a} and \underline{N} parameters of the gene 9 step.

2. The effect of concentration of the gene 9 product.

The rate of the gene 9 step was measured in several reactions similar to the one described for figure 14 except that the concentration of extract containing gene 9 product was varied. The \underline{a} and \underline{N} parameters measured from these reactions are given in table 14. In accord with the interpretation presented earlier, there is no significant change in \underline{N} when the concentration of gene 9 product is varied by a factor of five. However, extremely different values of \underline{a} are found in these different reaction mixtures, the \underline{a} value increasing monotonically but not linearly with the extract concentration. The value of \underline{a} seems roughly to follow some power function of the concentration of gene 9 product since a plot of $\log(\underline{a})$ vs. $\log(\text{gene 9 product})$ gives a straight line with a slope of 2.8

3. The effect of temperature.

The next variable tested for its effect on the gene 9 step was the temperature at which the reaction was carried out. The rate of the gene 9 step was measured in five different reaction mixtures that differed only in the temperature at which they were incubated. The data in table 15

TABLE 14

The Kinetics of the Gene 9 Reaction at Different
 9^+ Extract Concentrations

Relative Extract Concentration	a	N
1.0	$1.7 \times 10^{-6} \text{ min}^{-2.4}$	2.4
2.0	$1.1 \times 10^{-5} \text{ min}^{-2.6}$	2.6
3.0	$6.0 \times 10^{-5} \text{ min}^{-2.5}$	2.5
5.0	$1.4 \times 10^{-4} \text{ min}^{-2.6}$	2.6

An 18:23:27:34:rII extract which serves as a source of gene 9 product was diluted in BUM buffer and the dilutions used in reaction mixtures similar to the one described in the legend to figure 13. The a and N parameters from the rate of the gene 9 reaction are given as a function of the 9^+ extract concentration, where the most diluted sample was given a value of 1.0.

TABLE 15

The Kinetics of the Gene 9 Reaction at Different Temperatures

Temperature °C	a	N
0	$1.4 \times 10^{-6} \text{ min}^{-1.5}$	1.5
22	$7.9 \times 10^{-5} \text{ min}^{-1.9}$	1.9
30	$1.7 \times 10^{-4} \text{ min}^{-1.9}$	1.9
37	$1.3 \times 10^{-4} \text{ min}^{-1.9}$	1.9
46	$4.8 \times 10^{-5} \text{ min}^{-1.8}$	1.8

The kinetics of the gene 9 reaction was measured as described in the legend to figure 13, except that the reaction mixtures were incubated at different temperatures. The a and N parameters calculated from the initial part of the reaction are given as a function of the temperature.

give the values of \underline{a} and \underline{N} for the different temperatures: \underline{N} is insensitive to the temperature as predicted, but \underline{a} shows a marked dependence on the temperature with an optimum in the 30 to 37°C range.

The values of \underline{N} reported in these experiments (1.9) is significantly different from those reported in the previous series of experiments (2.6). Such variation in the value of \underline{N} from one set of experiments to another was often noted, but the cause never determined. Within one set of experiments, the same value of \underline{N} was always observed.

3. The effect on ionic conditions.

In order to test for a salt requirement for the gene 9 step, it is necessary to remove what salt is already present in the extract so that the salt concentration in the reaction mixture can be adjusted exactly. This was done by dialyzing the extract overnight against 0.01 M phosphate buffer pH 7.0 at 4°C; the dialyzed material was then used as a source of extract for the gene 9 reaction. A reaction mixture similar to the one described in figure 14 was used except that salt solutions of different concentrations were added as well as 9⁻ particles and dialyzed extract. The \underline{a} and \underline{N} parameters for these reaction mixtures are given in table 16 for the two different salts tested, MgSO₄ and NaCl. The dialyzed extract lacks activity but regains it when salt is added. Both salts will restore activity, but the Mg salt is more effective on a molar basis than the Na salt. This result parallels the finding that a divalent cation is more effective than a monovalent cation in satisfying the ionic requirements for tail fiber attachment (Wood and Henninger, 1968).

TABLE 16

The Kinetics of the Gene 9 Reaction Under Different Ionic Conditions

I. Effect of MgSO_4

Molar Concentration	\underline{a}	\underline{N}
0.20	$5.0 \times 10^{-5} \text{ min}^{-1.6}$	1.6
0.080	$1.3 \times 10^{-4} \text{ min}^{-1.9}$	1.9
0.040	$4.4 \times 10^{-5} \text{ min}^{-1.8}$	1.8
0.020	$1.4 \times 10^{-5} \text{ min}^{-1.6}$	1.6
0.008	$9.2 \times 10^{-6} \text{ min}^{-1.9}$	1.9
blank	$< 10^{-6} \text{ min}^{-1.9}$	if $N = 1.9$

II. Effect of NaCl

Molar Concentration	\underline{a}	\underline{N}
0.20	$4.4 \times 10^{-5} \text{ min}^{-2.05}$	2.05
0.050	$3.7 \times 10^{-7} \text{ min}^{-2.0}$	2.0
0.020	$< 6.0 \times 10^{-9} \text{ min}^{-2.0}$	if $N = 2.0$
blank	$< 8.0 \times 10^{-9} \text{ min}^{-2.0}$	if $N = 2.0$

The \underline{a} and \underline{N} parameters for the initial rate of the gene 9 reaction are given as a function of the salt concentration of the reaction mixture. The parameters for the NaCl experiment have been normalized to the MgSO_4 parameters by means of a control experiment using 0.020 M MgSO_4 and multiplying the \underline{a} values for the NaCl experiment by the ratio of the \underline{a} values for 0.020 M MgSO_4 in the two experiments. In these experiments 0.20 ml of dialyzed 18:23:27:34:rII extract was added to 0.05 ml of salt solution and 0.005 ml of 9 particles freshly diluted in 0.01 M phosphate buffer pH 7.0, incubated at 30°C, and sampled for the titer of 9^+ particles which were then assayed by the method described in the legend to figure 13.

\bar{N} as measured in these reactions is insensitive to changes in salt concentration, but \underline{a} is markedly dependent on how much salt is present. With MgSO_4 there seems to be an optimum \underline{a} value at 0.08 M; but with NaCl there was no optimum, the highest concentration tested being the most effective.

These experiments on the salt requirement of the gene 9 reaction reveal another property of this assembly step: there is no soluble cofactor requirement for this assembly step. The 9 particles were isolated from a lysate by means of differential centrifugation and this process would have resulted in the loss of any soluble cofactor not bound to the particle. Similarly any soluble factor not bound to some protein in the extract would be lost in the dialysis of the extract. It can therefore be concluded that the gene 9 reaction shows no requirement for a low molecular weight cofactor other than salt.

4. Summary

This series of experiments has shown that when the gene 9 reaction takes place under different conditions, the number of sites on the 9^- particle changed in order to convert it to a 9^+ state is invariant, but the rate at which these sites are modified is very strongly dependent on the conditions under which the reaction occurs.

Stoichiometry in the Gene 9 Reaction

It should be possible to decide whether the gene 9 product acts in a stoichiometric or a catalytic manner from the behavior of the reaction under conditions where the relative rate of the production of 9^+ particles of the final titer of 9^+ particles in the reaction is affected by the concentration of both 9 particles and gene 9 product. If the gene 9 product acts in a stoichiometric manner, for example by becoming incorporated into the baseplate, then it should be possible to limit the extent of the reaction, i.e. the number of particles converted to a 9^+ state, by using a reaction mixture which contains more 9 particles than gene 9 product. If only one gene 9 product could be incorporated into the 9 particle when it was converted to a 9^+ state, then the final titer of 9^+ particles should be directly proportional to the concentration of gene 9 product. But if more than one gene 9 product had to be incorporated into the 9 particle to convert it into a 9^+ state and if gene 9 products were incorporated independently of one another, then the final titer of 9^+ particles in the reaction would depend upon the molar ratio between gene 9 product and 9 particles as well as the concentration of gene 9 product. If the average number of gene 9 products incorporated per 9 particle in a reaction is less than the number needed to convert a 9 particle to a 9^+ state, then not all the 9 particles which have incorporated gene 9 product will be 9^+ . Those 9 particles in which more than the critical number of gene 9 products were incorporated will be 9^+ , the rest of the 9 particles which have incorporated fewer gene 9 products than the critical number will remain 9^- .

However, if the gene 9 product acts in a catalytic manner, then the rate of the reaction could be affected by the concentration of both gene 9 product and 9 particle, but the final titer of 9^+ particles should be determined solely by the concentration of 9 particles in the reaction. If the gene 9 product had to act on the particle only once to convert it to a 9^+ state, then there should be a maximum rate to the reaction determined by the concentration of gene 9 product and not of 9 particles. But if several "hits" (= action of the gene 9 product on the particle) were needed to convert a 9 particle to a 9^+ state and if these "hits" occurred independently of one another, then the rate would depend on both the concentration of gene 9 product and the ratio between gene 9 product and 9 particles. At the beginning of the reaction the average number of "hits" per 9 particle is less than the critical number needed to convert the particle to a 9^+ state, so very few of the particles which have received "hits" by gene 9 product will be converted to a 9^+ state; only when many 9 particles have received one less than the critical number of "hits" will the rate of production of 9^+ particle increase markedly. The rate of production of 9^+ particles during the course of the reaction would first accelerate due to the multi-hit nature of the process and then would decline due to the exhaustion of 9 particles from the reaction.

The results from an experiment to test these possibilities suggest that the gene 9 product acts in a stoichiometric rather than a catalytic manner. The production of 9^+ particles was measured in reactions similar to the one described in connection with figure 14 except that the concen-

tration of 9 particles was increased and of gene 9 product decreased; the data are illustrated in figure 18 (a&b). The curves shown in figure 18 (a) are for reactions containing the same concentration of gene 9 product, but containing concentration of 9 particles which increase in ten fold steps going from reaction 1 to 3. The curves shown in figure 18 (b) are for reactions containing a concentration of 9 particles equal to that used in reaction 3 of figure 18 (a) but containing concentrations of gene 9 product which increase in roughly two fold steps going from reaction 3 to 5. The data in figure 18 (a) show that the yield in reaction 3 is slightly less than from reaction 2, even though 3 had ten times as many particles as 2; such a result suggests an exhaustion of gene 9 product in reaction 3 has occurred and implies a stoichiometric manner of action for gene 9 product. That this exhaustion is not solely an artifact due to the high concentration of 9 particles used in reaction 3 is shown by the data in figure 18 (b); reactions 4 and 5 with the same concentration of 9 particles as reaction 3, but increased concentrations of gene 9 product show a nearly quantitative conversion of 9 particles to a 9^+ state.

The results from these experiments are not compatible with a catalytic manner of action of gene 9 product since the final yield from a reaction can be a function of the gene 9 product concentration as well as of the 9 particle concentration. Instead these results suggest a stoichiometric manner of action and hint that more than one gene 9 product must be used in the conversion process since the yield of 9^+ particles is depressed by a decrease in the ratio of gene 9 product to

FIGURE 18. The final yield of 9^+ particles in the gene 9 reaction can be limited by the amount of gene 9 product used.

The titer of 9^+ particles was measured in reaction mixtures similar to the one described in the legend to figure 14 except that the 9 particles and 9^+ extract were prepared in TNMG buffer. The abscissa is the logarithm of the time and the ordinate the logarithm of the titer of 9^+ particles in a reaction mixture.

a) different concentrations of 9 particles, but a constant dilution of 9^+ extract (a 5 x dilution of 18:23:27:34:rII extract)

(3) 10^{12} 9 particles/ml

(2) 10^{11} 9 particles/ml

(1) 10^{10} 9 particles/ml

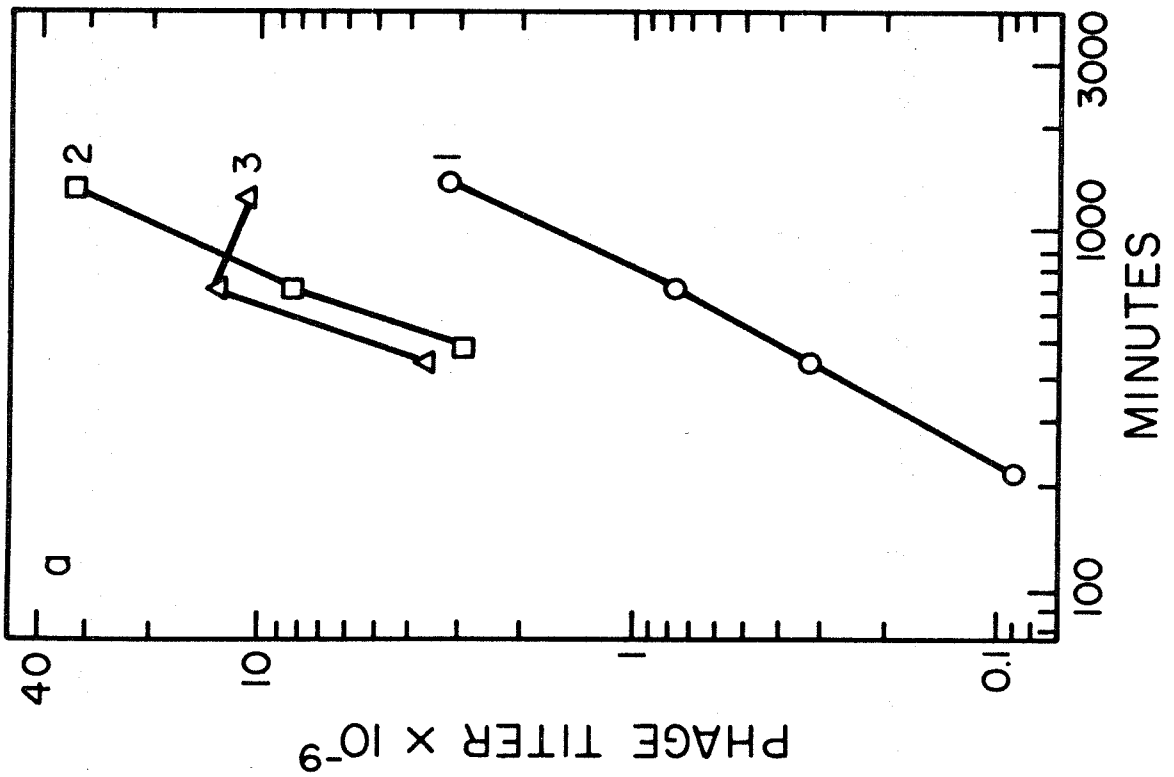
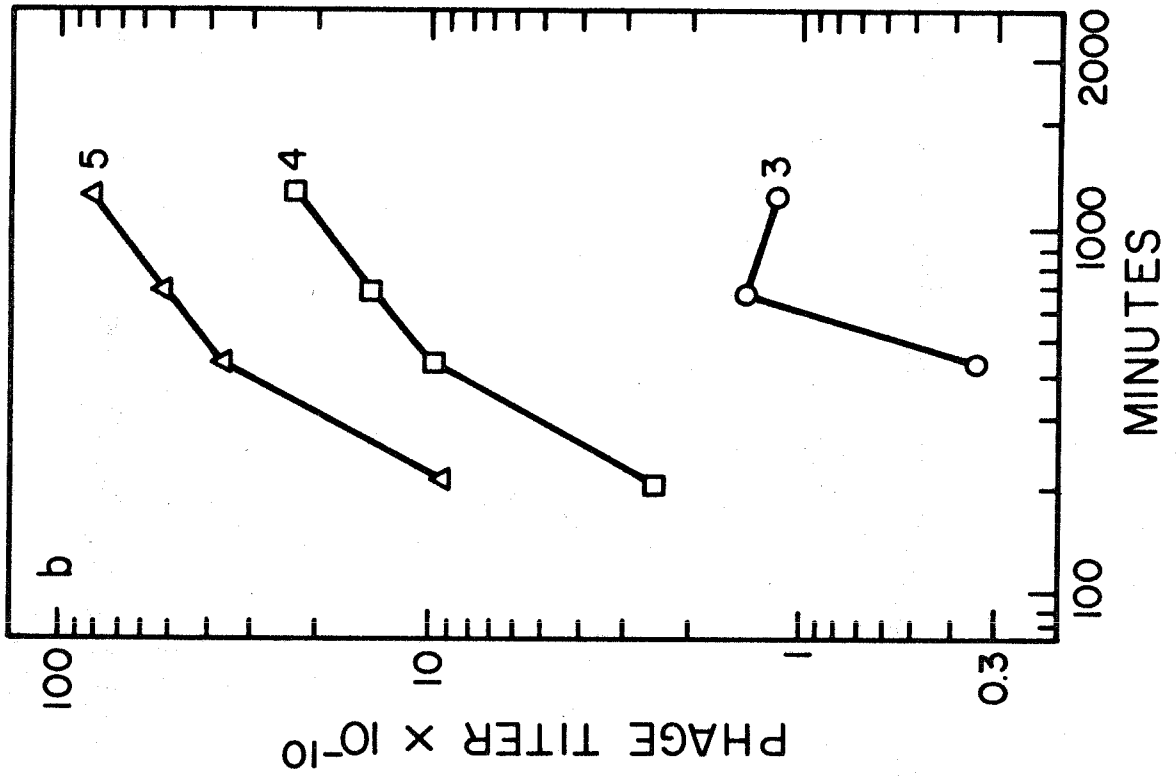
b) different dilutions of 9^+ extract (18:23:27:34:4II extract)

but a constant concentration of 9 particles (10^{12} 9 particles/ml)

(5) 1 x dilution of 9^+ extract

(4) 2 x dilution of 9^+ extract

(3) 5 x dilution of 9^+ extract



9 particles as between reaction 3 and reaction 2. However, to determine the number of gene 9 products which were used in converting the particle to a 9^+ state from the decrease in the yield as a function of this ratio of gene 9 product to 9 particles would require more measurements at a number of different ratios. While these experiments do not indicate what mechanism might cause the exhaustion of gene 9 product in a reaction, one possibility is that the gene 9 product is incorporated into the baseplate of the phage. Another possibility is that the gene 9 product modifies the baseplate without itself being incorporated into the phage and in doing so is irreversibly altered so that it can not function again.

Summary

Even though these experiments do not reveal the nature of the gene 9 step, several facts have been established concerning the manner in which this assembly step takes place.

1. The gene 9 product must act on the particle more than once to convert it to a 9^+ state.
2. The gene 9 step in vitro normally takes place under conditions where the gene 9 product is in excess, but under special conditions the amount of gene 9 product can limit the extent of the reaction indicating that it may act by becoming incorporated into the baseplate.
3. There is no low molecular weight cofactor other than a monovalent or divalent salt participating in the gene 9 step in vitro.

DISCUSSION

Properties of the Gene 9 Defective Particles

Infected cells missing the gene 9 product are blocked in particle morphogenesis since they accumulate non-infective, fiberless particles and free tail fibers. These gene 9 defective particles can be quantitatively converted to active phage particles in vitro by the sequential action of the gene 9 product and the addition of tail fibers, showing that the gene 9 defective particle can serve as an intermediate in the assembly of normal phage particles and is not merely an aberrant and defective by-product of morphogenesis. The tail fiber is not affected by the absence of the gene 9 product during its assembly since it can be attached both to fiberless particles (formed in cells missing a gene product essential for tail fiber formation) and to gene 9 defective particles which have been acted on by the gene 9 product. The gene 9 defective particles are apparently fiberless because the attachment sites on the particle for the tail fibers are missing; not because there are no normal tail fibers in an infected cell missing the gene 9 product. Action of the gene 9 product on the gene 9 defective particle thus provides sites for the attachment of tail fibers.

The gene 9 defective particles are unstable in that they irreversibly lose their ability to be converted to active phage when stored in low salt solutions or at low temperatures, conditions that do not affect the stability of either normal phage particles or of fiberless particles formed in infected cells missing only tail fibers. The gene 9 defect,

in addition to preventing tail fiber attachment, has made these particles more sensitive to their environment. The loss of activability of the gene 9 defective particles is correlated with the conversion of the tail from a normal extended state to a triggered state in which the DNA is not released from the head even though the sheath and the baseplate have changed configuration. These triggered particles resemble an intermediate that might be expected between particles 3 and 4 in figure 1b in the scheme of Simon and Anderson (1967a) except that there are no tail fibers on these particles so that they can not attach to a cell wall. This observation indicates that the release of DNA from the head of the phage is not an obligatory consequence of the triggering of the tail, but of some other mechanism which requires the role of the gene 9 product.

The action of the gene 9 product on the particle is pleiotropic since it is needed for three apparently different functions:

- 1) To provide an attachment site on the baseplate for the tail fibers,
- 2) To stabilize the tail against triggering by environmental conditions, and
- 3) To help set up the system for DNA release in the infection process.

Site of Action of the Gene 9 Assembly Step on the Particle

The substrate for the action of the gene 9 product was shown by an indirect procedure to be the baseplate of the phage particle. Base-

plates can be used in the assembly of active phage in the absence of the gene 9 product if isolated from cells that contain the gene 9 product; this indicates that there are no other sites on the particle at which the action of the gene 9 product is essential except on the baseplate. The behavior of the gene 9 product is thus similar to that of the gene 11 and 12 products (Edgar and Lielausis, 1968) in its site of action and its role in assembly. The action of these three gene products is needed in the formation of the baseplate of an active phage, but their absence does not block later assembly steps and defective particles are produced. Since these gene products are able to act on several different structures, their role in assembly can not be described in terms of a unique step in a pathway for phage morphogenesis (see figure 3).

Properties of the Gene 9 Assembly Step

An analysis of the kinetics of the gene 9 reaction in terms of target theory shows that the gene 9 product modifies the particle on the average three times in the rate limiting step of the conversion of the particle from a 9^- to a 9^+ state suggesting that there are at least three sites on the baseplate of the particle at which the gene 9 product can act. Since the baseplate has a six-fold axis of symmetry it is appealing to suppose that there are actually six equivalent sites on the baseplate, but that only three of them need to be modified for the particle to be converted to a 9^+ state. A similar interpretation has been made for the kinetics of the tail fiber addition step in the conversion of fiberless particles to active phage. There are six sites on the baseplate of the

particle onto which tail fibers can be attached, but the attachment of tail fibers to only three of them gives the particle a 50% chance of being infectious (Wood and Henninger, 1968).

In the conversion of fiberless particles to active phage, there is presumably only one requirement that determines if the particle is active or not: whether that particle can adsorb to bacterial cells. But there seem to be three conditions required for a gene 9 defective particle to be 9^+ : stability, release of DNA during infection, and enough sites for the attachment of tail fibers so that the particle can adsorb correctly. Only one action of the gene 9 product might be needed to confer stability to low salt and low temperature conditions to the particle, but three might be needed to provide enough tail fiber attachment sites on the particle. If one action of the gene 9 product was needed on the particle for each tail fiber which could then be added to that particle, then the requirement for three gene 9 product actions on the particle for it to be 9^+ might be a reflection of the requirement for at least three tail fibers in adsorption. If this is correct it could be tested by determining if there is a direct proportionality between the rate of action of the gene 9 product on the particle and the rate of appearance of sites on the particle to which tail fibers could be attached. The number of such sites could be measured by the amount of tail fiber antigen (as determined by serum blocking power) which could be attached to these particles.

The results from stoichiometry experiments, although tentative, indicate that the extent of the gene 9 reaction in vitro can be limited

by the concentration of the gene 9 product in the reaction mixture, implying that the gene 9 product has a stoichiometric rather than a catalytic role in morphogenesis. However, these experiments were not extensive enough to indicate the number of gene products used per particle in the reaction. The mechanism by which the gene 9 product is used up in the reaction is not known; it could be either converted to an inactive state when it modified the baseplate or it could be incorporated into the baseplate as part of its structure.

An indication that the gene 9 product might play a stoichiometric rather than a catalytic role in phage morphogenesis was reached independently by Snustand (1968) on the basis of gene dosage experiments. In these experiments the yield of phage from cells infected with different ratios of amber mutant to wild type phage was measured and was assumed to reflect the concentration dependence of the reaction involving the gene product affected by the amber mutation. This evidence is indirect and valuable therefore only as supporting evidence.

The Role of the Gene 9 Product During Infection

Simon and Anderson (1967b) noted that there is a plug in the center of baseplates derived from extended sheath particles. This plug is absent from baseplates obtained from triggered particles. Simon and Anderson suggested that the disappearance of this plug during infection might be essential to allow release of the phage DNA into the cell. The observations on the structure of the contracted gene 9 defective particles (in which the tail has triggered, but DNA release has not occurred) sug-

gest that a function of the gene 9 product in the assembly of the baseplate is to insure the disappearance of the tail plug from over the core when the baseplate expands from the hexagon configuration (320 Å diameter) to the six pointed star configuration (440 Å diameter). If the tail plug is a solid structure over the end of the core, one method to remove it is to link the expansion of the baseplate to the tail plug so that the tail plug is pulled apart when the baseplate expands in size. A role for the gene 9 product in the structure of the particle might be to connect the tail plug to the baseplate so that the expansion of the baseplate can change the state of the tail plug. Based on the preceding idea a model for the baseplate of normal phage particles and the role of the gene 9 product in determining its structure is illustrated in figure 19a. The important structures in the baseplate that concern this model are the following:

- 1) The baseplate which is in a hexagonal configuration,
- 2) The tail plug at the center of the baseplate blocking the core,
- 3) The gene 9 product which is attached from the baseplate vertex to one sector of the tail plug; six gene 9 products are present in each baseplate,
- 4) The tail fibers which are attached to the junction of the baseplate vertex and the gene 9 product.

The structures in the baseplate after the particle has triggered are pictured as being changed to the following ones:

- 1) The baseplate which is now in the six pointed star configuration,

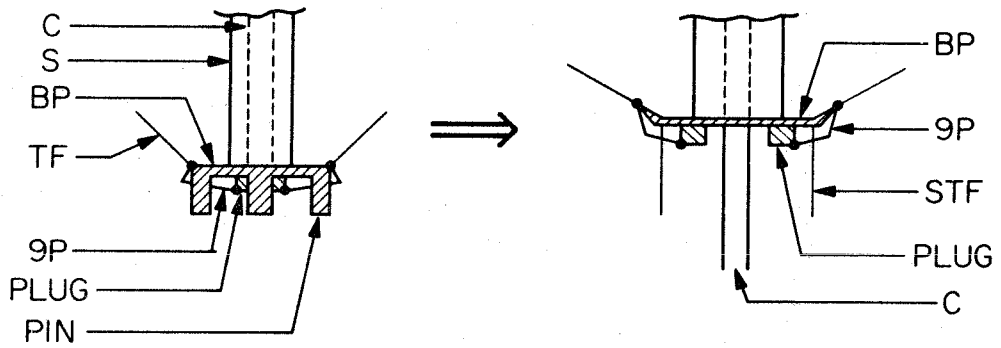
FIGURE 19: A Model for the role of the gene 9 product in the structure of the phage particle. a) Tail triggering in normal phage particles. b) Tail triggering in gene 9 defective particles.

Normal phage particles are pictured as having a hexagonal plug on the bottom of the baseplate which covers the opening at the end of the core and prevents the release of the phage DNA from the head. The gene 9 product is pictured as being attached to one of the baseplate vertices and to one of the triangular portions of the tail plug. When the baseplate changes to the larger configuration during the infection process, the connection provided by the gene 9 product between the baseplate vertex and the section of the tail plug insures that the tail plug will be dissociated and that the end of the core will be open to allow DNA release once the core has penetrated the cell wall of the bacterium. In the gene 9 defective particles the tail plug is not removed from the end of the core when the baseplate changes configuration and the DNA is not released from the head.

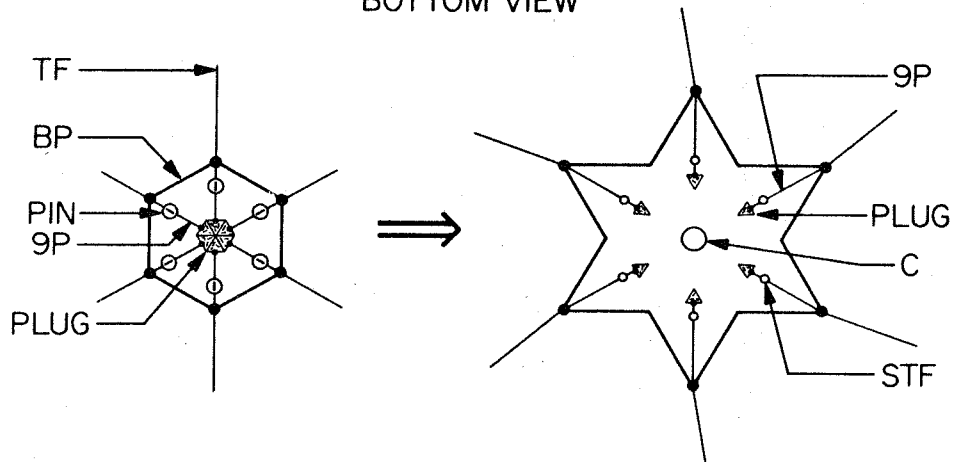
a

TAIL TRIGGERING IN NORMAL PARTICLES

SIDE VIEW



BOTTOM VIEW



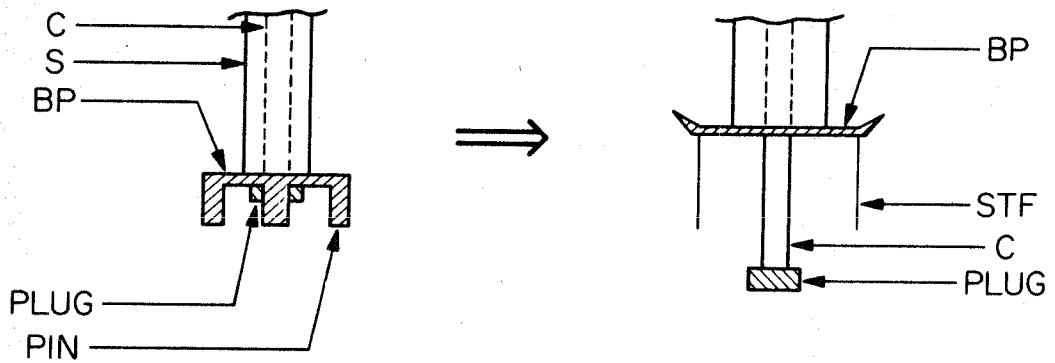
SYMBOLS

- S = SHEATH
- BP = BASEPLATE
- PIN = TAIL PIN
- STF = SHORT TAIL FIBER
- C = CORE
- PLUG = TAIL PLUG
- 9P = GENE 9 PRODUCT
- TF = TAIL FIBER

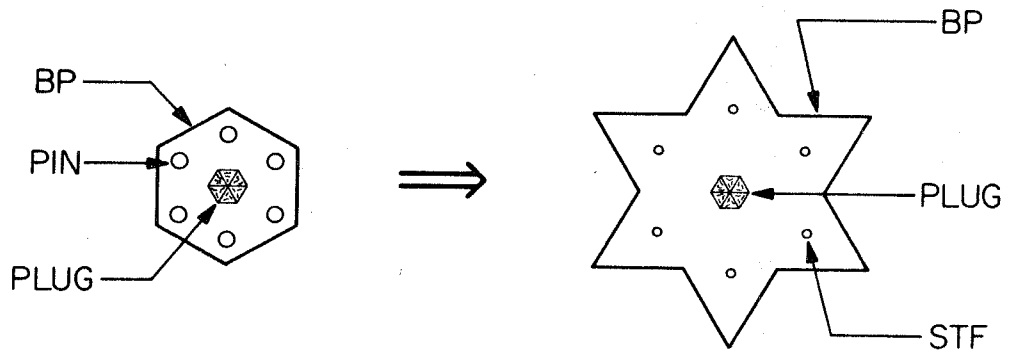
b

TAIL TRIGGERING IN GENE 9 DEFECTIVE PARTICLES

SIDE VIEW



BOTTOM VIEW



SYMBOLS

- S = SHEATH
- BP = BASEPLATE
- PIN = TAIL PIN
- STF = SHORT TAIL FIBER
- C = CORE
- PLUG = TAIL PLUG

- 2) The tail plug which has been split into six pieces and each piece moved from the center of the baseplate,
- 3) The core which has been pushed through the center of the baseplate.

The changes in the baseplates of the gene 9 defective particles (illustrated in figure 19b) are similar to those in normal baseplates except that the absence of the gene 9 product prevents an interaction of the baseplate with the tail plug. When the tail triggers in gene 9 defective particles the core is pushed through the baseplate with the tail plug still attached, thus blocking release of the phage DNA through the core. The tail plug hypothesized to be at the bottom of the core in the contracted 9 particle has not yet been observed in the electron microscope.

This model for the role of the gene 9 product in the structure of the baseplate can explain the pleiotropic effects which the action of the gene 9 product exerts on the particle. By connecting the tail plug to the baseplate vertex the need for the gene 9 product in DNA release is rationalized. The requirement for the action of the gene 9 product on the particle before tail fibers can be attached is explained by having the tail fiber connected to the baseplate-gene 9 product joint. The increase in the stability of the particle after action of the gene 9 product might be expected from the cross-bracing action which the gene 9 product provides in the baseplate. After the gene 9 product has been added it is no longer possible for the baseplate to switch from the hexagon configuration to the six pointed star configuration, and the internal bonds which hold the baseplate in the hexagon configuration are

thus reinforced leading to an increase in the environmental stability of the particle. This last notion suggests that the baseplate, like the sheath, is built in a high energy form and is converted to a more stable form when the tail triggers. How this model for the baseplate might function in the sequence of stages illustrated in figure 1b which were proposed by Simon and Anderson (1967a) is through the following steps:

- 1) The tail fibers attach to the cell wall.
- 2) The baseplate attaches to the cell wall by means of the tail pins.
- 3) There are changes in the baseplate leading to the breakage of the core-baseplate bonds and the expansion of the baseplate from the hexagon form to the six pointed star form.
- 4) The tail plug is pulled into six pieces by the force exerted on it by the gene 9 product due to the expansion of the baseplate.
- 5) The contraction of the sheath is induced by the change in the baseplate configuration.
- 6) The core is pushed through the baseplate and the cell wall so that the phage DNA is released into the cell.

Protein Interactions Involved in the Gene 9 Assembly Step
and the Stability of the Gene 9 Defective Particles

Since presumably both the gene 9 product and the baseplate are proteins, it would seem reasonable that the stability of the gene 9 defective particles

and the rate of the gene 9 reaction would be determined by the types of interactions which take place between proteins. Conditions affecting these phenomena might thus be explained due to the effects exerted by these conditions on protein-protein interactions. Conditions which modify these phenomena are the salt concentration and temperature; both the stability of the gene 9 defective particles and rate of the gene 9 reaction are inhibited by a reduction in either of these parameters. Since evidence from other systems indicates that reduction in these parameters reduces the strength of some protein-protein interactions, a similar interpretation here would agree with the notion that the stability of the gene 9 defective particles and the rate of the gene 9 reaction are dependent on the interactions between proteins.

Cold lability has been found in two other systems to be due to the dissociation of multimeric protein systems. The activity of pyruvate carboxylase is lost by storage in the cold (2°C) and recovered after preincubation at the normal reaction temperature (37°C); these effects are correlated with the dissociation of the active enzyme into subunits by the cold treatment and the reassociation of these subunits to form the active enzyme by rewarming the solution with the dissociated subunits (Valentine *et al.*, 1966). The polymerized TMV protein (not containing RNA) has also been found to be cold labile in that the state of aggregation of the TMV subunits can be altered by changing the temperature of the solution. At low temperature (4°C) they are present as individual subunits, but if the temperature is increased (to 25°C) they polymerize into long threads with the normal TMV structure (Lauffer *et al.*, 1958).

Similarly the effect of salt (in the concentration range used in this study: 0.01 M to 1 M) is to increase the strength of the interactions between proteins relative to their interactions with the solvent. This effect has been referred to as "salting out" and occurs not only for proteins, but for other molecules as well (Edsall and Wyman, 1958). The salt concentrations which were used to stabilize the gene 9 defective particles and to increase the rate of the gene 9 assembly step are in the range that strongly increases interactions between proteins in other systems.

The considerations suggest that an association between certain proteins in the gene 9 defective particle is critical in maintaining the particle in a non-triggered state. The suggestion of Simon and Anderson (1967b) that the critical factor in whether the tail triggers or not is the state of the baseplate implies that the critical aggregate in the gene 9 defective particle whose dissociation causes triggering is in the baseplate; when these proteins dissociate the baseplate can change configuration and the sheath contract, producing a triggered gene 9 defective particle. This idea agrees with the notion mentioned earlier that the baseplate is constructed in a high energy form which is unstable and that it can spontaneously change to the larger, more stable form. The forces holding it in the unstable form in the normal particle would be the interactions among certain proteins in the baseplate and the cross bracing action of the gene 9 product.

Similar considerations also suggest that protein interactions are important in the rate limiting step of the gene 9 reaction. The conditions

that affect protein association might either be directly involved in the interaction between the gene 9 product and the baseplate or in modifying one of the substrates in the reaction so as to affect its association with the other one. At present it is not possible to decide which of these alternatives might be correct.

Speculation Concerning the Origin of the Gene 9 Product
in Relation to the Evolution of the Phage Tail

The study of conditional lethal mutants of T4 defective in morphogenesis has resulted in the discovery of at least fifteen gene products concerned with the assembly of the baseplate of the phage particle (Wood et al., 1968). This group of fifteen gene products can be divided into two classes by the effect their absence has on phage morphogenesis. The baseplate is the structure on which the other components of the tail are assembled. One class of twelve gene products is necessary for the formation of a baseplate component which serves as a precursor structure for the later steps in tail assembly. Infected cells missing one of these gene products lack not only baseplates, but cores and sheaths as well, since both core and sheath are assembled on the baseplate. However, these gene products are not required for core or sheath assembly per se since both structures can be assembled on baseplates added to extracts missing one of the twelve baseplate gene products. The other class of three gene products (from genes 9, 11 and 12) act on the baseplate, but their function is not needed for core or sheath assembly on the baseplate. Phage morphogenesis in the absence of any one of these gene products

results in the production of non-infective, defective particles rather than just component parts of the particle. The gene 9 defective particles lack tail fibers, the gene 11 and 12 defective particles have tail fibers and appear normal but are defective in adsorption (King, 1968). Thus while these gene products are essential for the baseplate to be active in infection, they are not needed for the early assembly steps in particle morphogenesis.

A possible explanation for the existence of these two classes of baseplate gene products might involve steric considerations. The first class of gene products form the basic "skeleton" of the baseplate including the surface onto which the core and the sheath are assembled; all these gene products would thus be essential for the formation of the sites at which the assembly of the core and the sheath would be initiated. The second class of gene products perhaps determines the other surface of the baseplate, and does not affect the sites needed for starting core and sheath assembly. This explanation would be consistent with their role being mainly in the infection process, a process in which the outer surface of the baseplate is clearly involved. Modifications of the surface perhaps could occur at any time during morphogenesis and would not per se affect the morphogenesis of the sheath and core or attachment of the head to the tail.

If, however, steric restrictions do not solely determine the pathway of particle morphogenesis, then the evolutionary history of the phage might be reflected in the sequence of assembly steps that now exist. Conceivably evolutionarily recent acquisitions to the structure of the phage particle

might be less likely to be integrated into the older assembly patterns.

Possibly the ancestor of the present T4 phage was an infections particle which lacked tail fibers. Following this line of speculation the tail fibers are a relatively recent acquisition, evolutionarily speaking, which could not be added directly to the particle without modification of the baseplate. The origin and function of the gene 9, 11, and 12 products as modifiers of the baseplate would cause them to appear as a special class of baseplate gene products in terms of assembly sequence.

If the pattern of assembly depends to some extent at least on considerations such as the evolutionary origin of the gene products, then a comparison of the patterns of assembly between different phages might provide the basis for a phylogenetic classification of phage and even indicate the order in which different structural features of the phage were acquired.

BIBLIOGRAPHY

- Anderson, T.F. (1953) "The Morphology and Osmotic Properties of Bacteriophage Systems" Cold Spring Harbor Symposia on Quantitative Biology 18: 197-203.
- Bernstein, H., R.S. Edgar, and G.H. Denhardt (1965) "Intragenic Complementation among Temperature Sensitive Mutants of Bacteriophage T4D" Genetics 51: 987-1002.
- Brenner, S., G. Streisinger, R.W. Horne, S.P. Champe, L. Barnett, S. Benzer, and M.W. Rees (1959) "Structural Components of Bacteriophage" Journal of Molecular Biology 1: 281-292.
- Crick, F.H.C. and L.E. Orgel (1964) "The Theory of Inter-allelic Complementation" Journal of Molecular Biology 8: 161-165.
- Davison, P.F., D. Friefelder, R. Helde, and C. Levinthal (1961) "The Structural Unity of the DNA of Bacteriophage T2" Proceedings of the National Academy of Sciences, Washington 47: 1123-1129.
- Edgar, R.S., G.H. Denhardt, and R.H. Epstein (1964) "A Comparative Genetic Study of Conditional Lethal Mutations of Bacteriophage T4D" Genetics 49: 635-648.
- Edgar, R.S. and I. Lielausis (1964) "Temperature Sensitive Mutants of Bacteriophage T4D: Their Isolation and Genetic Characterization" Genetics 49: 649-662.

- Edgar, R.S. and I. Lielausis (1968) "Some Steps in the Assembly of Bacteriophage T4" *Journal of Molecular Biology* 32: 263-276
- Edgar, R.S. and W.B. Wood (1966) "Morphogenesis of Bacteriophage T4 in Extracts of Mutant-Infected Cells" *Proceedings of the National Academy of Sciences, Washington* 55: 498-505.
- Edsall, J.T. and J. Wyman. "Thermodynamics, Electrostatics, and the Biological Significance of the Properties of Matter", *Biophysical Chemistry, Volume I.* (Academic Press: New York) 1958, pp. 263-282.
- Epstein, R.H., A. Bolle, C.M. Steinberg, E. Kellenberger, E. Boy de la Tour, and R. Chevalley, R.S. Edgar, M. Susman, G.H. Denhardt, and I. Lielausis (1963) "Physiological Studies of Conditional Lethal Mutants of Bacteriophage T4D" *Cold Spring Harbor Symposia on Quantitative Biology* 28: 375-394.
- Hershey, A.D. and M. Chase (1953) "Independent Functions of Viral Protein and Nucleic Acid in Growth of Bacteriophage" *Journal of General Physiology* 36: 39-56.
- Imamoto, F., J. Ito, and C. Yanofsky (1966) "Polarity in the Tryptophan Operon of *E. coli*" *Cold Spring Harbor Symposia on Quantitative Biology* 31: 235-249.
- Kellenberger, E., A. Bolle, E. Boy de la Tour, R.H. Epstein, N.C. Franklin, N.K. Herne, A. Reale-Scafati, J. Scchaud, I. Bendet, D. Goldstein, and M.A. Lauffer (1965) "Functions and Properties Related to the Tail Fibers of Bacteriophage T4" *Virology* 26: 419-440.

- King, J. (1968) "Assembly of the Tail of Bacteriophage T4" *Journal of Molecular Biology* 32: 231-262.
- King, J.A. (1968a) "Steps in the Assembly of Bacteriophage T4" Thesis, California Institute of Technology.
- King, J. and W.B. Wood (in press) "Assembly of Bacteriophage T4 Tail Fibers: The Sequence of Gene Product Interaction" *Journal of Molecular Biology*.
- Kozloff, L.M. "A Biochemistry of the T-even Bacteriophages of Escherichia coli", *Molecular Bases of Virology* (H. Fraenkel-Conrat, editor) (Reinhold: New York) 1968, pp. 435-496. (ACS Monograph No. 164).
- Lauffer, M.A., A.T. Ansevin, T.E. Cartwright, and C.C. Brinton, Jr. (1958) "Polymerization-Depolymerization of Tobacco Mosaic Virus Protein" *Nature* 181: 1338-1339.
- Moody, M.F. (1965) "The Shape of the T-even Bacteriophage Head" *Virology* 26: 567-576.
- Moody, M.F. (1967a) "Structure of the Sheath of Bacteriophage T4. I. Structure of the Contracted Sheath and Polysheath" *Journal of Molecular Biology* 25: 167-200.
- Moody, M.F. (1967b) "Structure of the Sheath of Bacteriophage T4. II. Rearrangement of the Sheath Subunits During Contraction" *Journal of Molecular Biology* 25: 201-208.

- Nakata, A. and F.W. Stahl (1967) "Further Evidence for Polarity Mutations in Bacteriophage T4" *Genetics* 55: 585-590.
- Rubenstein, I., C.A. Thomas, Jr., and A.D. Hershey (1961) "The Molecular Weights of T2 Bacteriophage DNA and Its First and Second Breakage Products" *Proceedings of the National Academy of Sciences, Washington* 47: 1113-1122.
- Russell, R.L. (1967) "Speciation Among the T-even Bacteriophages" Thesis, California Institute of Technology.
- Rutberg, L. and B. Rutberg (1964) "On the Expression of the rII Mutation of T-even Bacteriophages in Escherichia coli Strain B" *Virology* 22: 280-283.
- Schlesinger, M.J. and C. Levinthal (1963) "Hybrid Protein Formation of E. coli Alkaline Phosphatase Leading to in vitro Complementation" *Journal of Molecular Biology* 7: 1-12.
- Simon, L.D. and T.F. Anderson (1967a) "The Infection of Escherichia coli by T2 and T4 Bacteriophages as Seen in the Electron Microscope I. Attachment and Penetration" *Virology* 32: 279-297.
- Simon, L.D. and T.F. Anderson (1967b) "The Infection of Escherichia coli by T2 and T4 Bacteriophages as Seen in the Electron Microscopy II. Structure and Function of the Baseplate" *Virology* 32: 298-305.
- Snustand, D.P. (1968) "Dominance Interactions in Escherichia coli Cells Mixedly Infected with Bacteriophage T4D Wild-Type and Amber Mutants

- and Their Possible Implications as to Type of Gene-Product Function: Catalytic vs. Stoichiometric" *Virology* 35: 550-563.
- Stahl, R.W. and N.E. Murray (1966) "The Evolution of Gene Clusters and Genetic Circularity in Microorganisms" *Genetics* 53: 569-576.
- Stahl, F.W., N.E. Murray, A. Nakata, and J.M. Crasemann (1966) "Intragenic cis-trans Position Effects in Bacteriophage T4" *Genetics* 54: 223-232.
- Stretton, A.O.W., S. Kaplan, and S. Brenner (1966) "Non Sense Codons" *Cold Spring Harbor Symposia for Quantitative Biology* 31: 173-179.
- Towe, K.M. (1965) "Carbon Films for Electron Microscopy: A Reliable Method for Stripping from Glass Surfaces" *The Review of Scientific Instruments* 36: 1247.
- Valentine, R.C., N.G. Wrigley, M.C. Scrutton, J.J. Irias, M.F. Utter. (1966) "Pyruvate Carboxylase VIII. The Subunit Structure as Examined by Electron Microscopy" *Biochemistry* 5: 3111-3115.
- Winkler, U., H.E. Johns, and E. Kellenberger (1962) "Comparative Study of Some Properties of Bacteriophage T4D Irradiated with Monochromatic Ultraviolet Light" *Virology* 18: 343-358.
- Wood, W.B., R.S. Edgar, J. King, I. Lielausis and M. Henninger (1968) "Bacteriophage Assembly" *Federation Proceedings* 27: 1160-1166
- Wood, W.B. and M. Henninger (in press) "The Attachment of Tail Fibers in Bacteriophage T4 Assembly: Some Properties of the Reaction in vitro and its Genetic Control" *Journal of Molecular Biology*.