STEPS IN THE ASSEMBLY OF BACTERIOPHAGE T4

Thesis by

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For Rod, Tim, Steve, and Frank

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ABSTRACT

The formation of bacteriophage T4 has been studied by examining the phage components accumulating in cells infected - under restrictive conditions - with mutants blocked at different stages of the assembly process. Structural intermediates in the pathway for tail and tail fiber assembly have been partially characterized by serology, electron microscopy, sedimentation, and in vitro complementation behavior.

Tail assembly: After the baseplate (80S) is completed, the core forms on the baseplate. The functions of genes 19, 48, and 54 are required for this conversion. The gene 18 product, a major sheath structural subunit, polymerizes on the core-baseplate (80S) and then the 3 and 15 gene products fix the sheath subunits in the polymerized form, yielding a sheathed tail (130S).

Tail fiber assembly: The tail fiber (10S) is made up of 8S and 9S components. The 8S component is the product of genes 38, 37, 36 and 35. The 9S component is the product of gene 34. Two precursors to the 8S component, both also 8S, are identified. A pathway for the steps in tail fiber assembly is proposed.

The tail fibers attach to the tail only after the head has joined with the tail. Particles which have not been acted upon by the gene 11 or 12 product adsorb to bacteria but do not kill them.

Electron microscopic observations on the state of phage heads in mutant lysates are also presented. Mutations in three genes result in the accumulation of head membranes empty of DNA.

All the evidence supports the view that phage heads, tails, and tail fibers are formed independently of each other.

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GENERAL INTRODUCTION

The advent of viruses as an experimental tool has stimulated the study of molecular morphogenesis. The early appeal of virus systems was the ease of obtaining in pure form an organism which could be studied by the methods of physical chemistry. The reconstitution of Tobacco Mosaic Virus by Fraenkel-Conrat and Williams (1955) was a particularly dramatic achievement of such studies. At the same time, improvements in the electron microscope, coupled with previous X-ray diffraction studies, permitted detailed analysis of viral architecture.

Structural studies of bacterial viruses were stimulated by the development of the T-even bacteriophages as a high resolution genetic system (Benzer, 1955). The possibility of purifying a phage protein which could be associated with a known gene prompted Brenner et al. (1959) to attempt the purification of phage structural proteins. These investigators chemically degraded T-even bacteriophage and succeeded in purifying some of the larger components, the contracted sheath, core, and tail fibers at the same time examining their preparations with the newly developed technique of negative staining. The picture of T4 which is now so familiar (bottom of Fig. 14) derives from this work.

Investigations of T-even bacteriophage structure were reviewed by Champe in 1963. Since then a number of further studies have been published on the chemical composition of the core and sheath (Sarkar, Sarkar, and Kozloff, 1964a,b) and on the symmetry and morphology of

the sheath and head (Moody, 1965, 1967). Unfortunately none of these studies reveal the structure of the phage in sufficient detail to provide insight into the assembly of the phage.

A few recent structural studies have been directed toward the assembly problem. Eddleman and Champe (1966) characterized a number of polypeptides which are found in the head of T4 and demonstrated that these polypeptides are associated with head formation. Kellenberger and Boy de la Tour (1964, 1965) have investigated the structure of polyhead and polysheath, two structures found in T4 infected cells which appear to be aberrant products of the assembly process. However, detailed knowledge of T4 morphogenesis has been the fruit of extensive genetic investigations.

In early genetic work with T4, the available mutations were either in genes controlling nonessential functions or were sublethals which resulted in only partial loss of gene function. Such mutations were not useful for the identification of essential phage functions (such as the formation of structural proteins). In 1959 R.S. Edgar and R.H. Epstein began isolating on a large scale two kinds of phage mutants - amber and temperature sensitive, both of which were of the class of conditional lethal mutations. Such mutations are lethal for the phage under one set of conditions (restrictive conditions), but do not markedly affect the yield of viable phage under a second set of conditions (permissive conditions). Amber mutants can be propagated on a permissive strain of bacteria, but not on a restrictive strain. Temperature sensitive mutants can be propagated at low temperatures, but not a high temperatures.

Subsequent investigations have shown that the amber mutation results in the formation of a triplet in the DNA of the phage which can be read in a host containing a supressor mutation (su^+) but is nonsense in the restrictive host which lacks the suppressor mutation (su^-). These nonsense codons result in termination of the growing peptide chain so that only a fragment is produced when the phage is grown on the su^- host (Brenner and Stretton, 1964). Thus in most cases amber mutations can be viewed as completely abolishing gene function (Benzer and Champe, 1961; Garen and Siddiqui, 1962).

Temperature sensitive mutations result in a base change leading to an amino acid replacement in the affected protein. The replacement lowers protein stability significantly only at high temperature. Note that it is the growth of the bacteriophage within its host cell, not the phage particle which the mutation renders temperature sensitive.

Such conditional lethal mutations have two important characteristics; 1) they can in principle occur in any gene of the phage which codes for an essential protein, and 2) under restrictive conditions the function of the mutant gene is essentially absent and no viable phage are produced.

Genetic characterization of the amber and temperature mutants identified almost 50 genes of T4 (Epstein et al., 1963). The functions of these genes were probed by investigating the abortive infections by the mutants under restrictive conditions. The mutants fell into two general classes, termed "early" and "late". Cells infected with early mutants (under restrictive conditions) failed to synthesize DNA

or serum blocking protein, and neither lysed nor produced phage parts. Cells infected with late mutants synthesized phage DNA and subsequently lysed, but instead of yielding viable phage, yielded recognizable phage parts - heads, tails, or both - or noninfectious particles. These studies implicated at least 37 genes in the formation of the phage particle; clearly T4 was of a higher order of complexity than TMV.

In 1965 Edgar and Wood (1966) succeeded in assembling viable T4 bacteriophage *in vitro*. These experiments were not analogous to the TMV experiments; Fraenkel-Conrat and Williams reconstituted TMV from precursors which had been obtained from the completed virus. The T4 assembly was an *in vitro* complementation test in which the phage were formed from their presumed normal precursors. For example an extract of cells infected with a mutant blocked in tail formation (and thus containing phage heads) is mixed with an extract of cells infected with a mutant blocked in head formation (and thus containing phage tails) and viable phage are produced. These experiments established that the phage parts which accumulate in mutant infected cells are in general functional phage precursors. By the use of the *in vitro* complementation test any phage protein required for assembly can in principle be assayed for, providing mutations exist in the gene coding for it.

The *in vitro* complementation assay has subsequently been used very effectively by Edgar and Lielausis (1967) in ordering various steps in T4 assembly. The studies reported in this dissertation frequently paralleled related experiments of Edgar and Lielausis (1967; see also Wood and Edgar, 1967) and have profited considerably from the sharing of both experimental data and ideas.

The researches described here follow the tradition of Edgar and Epstein in attempting to understand an organism through its genes. How many instructions are required to program phage structure and assembly? How are the instructions realized? I have tried to identify gene functions in phage assembly by further characterizing the intermediates accumulating in cells infected with the late mutants isolated by Edgar and Epstein. Two advances over previous work have been the use of the ultracentrifuge to separate phage structures from the bulk of the crude lysate or extract, and more effective use of the electron microscope. Serological analysis has been valuable for identifying structural components. The in vitro complementation test was developed in the course of these experiments and has been put to only limited use in the studies reported below.

Experiments on the structure and assembly of the phage tail are reported in Part I. Certain observations on phage heads are also included. In Part II experiments are reported on the structure and assembly of phage tail fibers.

The phage assembly process has now been delineated sufficiently well to permit attempts at generalization to other morphogenetic systems. However, regardless of the generality of the process, its accuracy and efficiency are quite remarkable.

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P A R T I

Tail Assembly

"You're the only one who seems to understand about tails. They don't think — that's what the matter with some of these others. They've no imagination. A tail isn't a tail to <u>them</u>, it's just a Little Bit Extra at the back."

Eeyore

INTRODUCTION

At least seven morphologically discrete components have been identified in bacteriophage T4: tail fibers, baseplate, core, sheath, collar, neck, and head (Brenner et al., 1959). More than forty phage genes control the formation of these structures (Epstein et al., 1963). Recently an in vitro system has been developed for studying the assembly of the phage parts accumulating in mutant infected cells into viable phage particles (Edgar & Wood, 1966). The assembly of T4 thus provides an excellent experimental system for the study of morphogenesis at the molecular level.

The above investigations suggest that T4 is the end product of a branched morphogenetic pathway in which head, tail, and tail fibers are formed independently and then assembled into complete virus. The assignment of phage genes to various branches of the pathway is summarized in Figure 1 of Edgar & Lielausis (1967) and the locations of these genes on the genetic map of T4 are shown in Figure 15 below.

The experiments reported here concern the sequence of assembly of a number of the major morphological components - baseplate, core, sheath, head, and tail fibers - with particular emphasis on the identification of the genes and gene products involved in the formation of the sheath. Phage cores, tail fibers, and contracted sheaths have been isolated and characterized by Brenner et al. (1959), Sarkar, Sarkar & Kozloff (1964a,b), and Poglazov (1966). Since these structures were isolated from disrupted phage particles their status as intermediates in the

assembly of the phage is unknown. A number of workers have studied the morphology of the extended sheath (Moody, 1967b; Kellenberger & Boy de la Tour, 1964; Bradley, 1963) but nothing is known of its assembly.

The approach employed here depends on the characterization of precursors accumulating in cells infected, under restrictive conditions, with various T4 conditional lethal mutants blocked in phage assembly. The use of such mutants permits the isolation of phage structures which are functional intermediates in the assembly process. I have partially characterized three such structures with respect to their antigenic properties, sedimentation behavior, electron microscope morphology, and *in vitro* functional state. The pathway for the formation of the phage tail is inferred from the results of these analyses. Supplementary observations on phage heads are also included.

MATERIALS AND METHODS

(a) *Phage strains*

All mutants are derivatives of T4D from the collection of R. S. Edgar and, excepting a few in genes 3 and 19, have been described previously (Epstein *et al.*, 1963; Edgar & Wood, 1966). Amber mutants are defined by their ability to form plaques on the permissive host *E. coli* CR63 but not on the restrictive host *E. coli* B/5. Temperature sensitive mutants form plaques at 30° but not at 42°. The *r*II mutant, *rdf*41, which has both A and B cistrons deleted, was frequently used as a reference

phage. An rI mutant, r48, was used as a tester phage in serum blocking experiments. ØX174 and MS2 phage stocks for centrifuge markers were kindly provided by Clyde Hutchison. High titer lambda bacteriophage was kindly provided by R. J. Huskey.

(b) Bacterial strains

Escherichia coli host strains for T4 were from the collection of R. S. Edgar. E. coli CR63 is permissive for amber mutants and was used for plating and stock-making. E. coli B/5 and S/6/5 were used as restrictive hosts for amber mutants. F (λ) was used for plating when a host restrictive for both rII and amber mutants was desired. CR (λ) was used as a host permissive for amber mutants but restrictive for rII mutants. E. coli C3000 and C_n^+, both from Clyde Hutchison, were used for plating MS2 and ØX174, respectively.

(c) Media

H broth, used for phage and bacterial growth, and EHA top and bottom agar were prepared as described previously (Steinberg & Edgar, 1962). K medium, also used for phage and bacterial growth, contained 7 g NA_2HPO_4, 3 g KH_2PO_4, 1 g NH_4Cl, 0.5 g NaCl, 0.12 g $MgSO_4$, 2 g glucose and 15 g casamino acids, per 1000 ml. distilled H_2O. NaCl, $MgSO_4$, glucose, and casamino acid solutions were made up and autoclaved separately. Casamino acid solutions were filtered through activated charcoal before use. Tryptone broth, used for growth of E. coli C3000 and C_n^+, contained 10 g Difco bacto-tryptone, 5 g NaCl, and 1000 ml.

distilled H_2O and was adjusted to pH 7.4 before autoclaving. LT top and bottom agar were used for ØX174 and MS2 platings. LT bottom agar contained 10 g Difco bacto-agar, 10 g Difco bacto-tryptone, 5 g NaCl, and 1000 ml. distilled H_2O. LT agar is as above but with only 6.5 g agar. LT top agar was made up to 0.005 M in $CaCl_2$ before plating.

Phosphate buffer (BU) contained 7 g Na_2HPO_4, 3 g KH_2PO_4, 4 g NaCl, and 1000 ml. distilled H_2O; the concentration of $MgSO_4$, added after autoclaving, was as indicated subsequently. Dilution buffer contained 7 g Na_2HPO_4, 3 g KH_2PO_4, 4 g NaCl, 0.5 g gelatin, and 1000 ml. H_2O. 1 ml. of 1 M $MgSO_4$ was added after autoclaving.

(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 frequently used as antifoaming agents. Crystalline DNase I, used in preparing extracts, was obtained from Sigma Chemical Company. D_2O was obtained from Bio-Rad Corporation. All other chemicals were reagent grade.

(e) Phage preparations

Lysates: *E. coli* B/5 cultures were begun as 1000-fold dilutions of stationary phase bacteria in K medium or, occasionally, in H broth, then grown to early logarithmic phase and collected by centrifugation. After resuspension in the appropriate medium at 4×10^8 cells/ml. (cell counts were made with a Petroff-Hauser counting chamber) the cells were

infected with a multiplicity of about 5 phage per cell and incubated with vigorous aeration at 30° or 39.5°C. At 15 minutes after infection they were superinfected with the initial multiplicity. The superinfected cultures were put on ice at about 60 minutes (39.5°C) or 80 minutes (30°C) and lysed with chloroform, or occasionally by quick freezing in dry ice-alcohol and thawing at 30°C. The yield of phage from such wild type or rII lysates was around 10^{11}/ml. The burst size decreased if the bacteria were not infected immediately after concentration, or if the temperature was not carefully controlled at the high temperature; phage yields fell off very sharply above 40°C in liquid culture under these conditions. Using mutant phage under restrictive conditions, the background of viable phage in such lysates was 10^{6}-10^{9}/ml. depending on the mutant used. Occasionally lysates were not superinfected and were allowed to lyse spontaneously; such wild type or rII preparations generally contained about 2×10^{10} phage/ml. Sometimes lysates were centrifuged at low speed to remove unlysed bacteria. In the experiments reported below no differences were observed between lysates prepared in K medium or H broth, or among those subject to spontaneous, chloroform, or freeze and thaw lysis, except that freezing and thawing appeared to disrupt phage heads. Lysates were made in volumes of 3-5 ml.

Experiments were performed with lysates made the same day, except in cases where the lysates had been stored frozen; these were thawed immediately before the experiment.

Extracts, concentrated mutant infected cell preparations used for *in vitro* complementation experiments, were prepared as described in

Edgar and Lielausis (1967). Extracts were prepared only with amber mutants.

(f) Centrifugation

Linear sucrose gradients (4.8-5.0 ml.; 5 to 20%) were made up in BU with 10^{-3} M $MgSO_4$, or in BU/10 with 2 x 10^{-2} M $MgSO_4$ (BUM). Experiments in which the fractions were to be assayed for in vitro complementation activity were done in BUM since the terminal step in phage formation, tail fiber addition, has its optimal rate at 2 x 10^{-2} M Mg^{++}, and the extracts were in BUM. Experiments for measuring serum blocking were generally run in BU. D_2O gradients were 20 to 90% linear gradients made up in BUM. Samples of 0.1-0.3 ml. were layered on the gradient prior to centrifugation in an SW39 rotor in a Spinco Model L ultracentrifuge. The times given for centrifugation include the acceleration time but not deceleration time. Fractions were collected by piercing the bottom of the cellulose nitrate tubes and collecting the resulting drops. When a visible pellet was present, the tube was pierced off center.

(g) Electron microscopy

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

Specimens were prepared by negative staining with uranyl acetate, either 1% or saturated (~pH 4.0) or occasionally with 4% phosphotungstate at pH 7.5. No significant differences were observed between the two

staining methods and all data and photographs presented are from uranyl stained preparations. A drop of sample was applied to a carbon coated grid and after one minute washed off with 2 drops of distilled H_2O. The grid was washed with a drop of stain and then left in contact with a second drop for about 30 seconds. This was washed off with one drop water and the excess liquid blotted with filter paper. The specimens were observed in a Phillips EM200 electron microscope fitted with an anti-contamination device, at 60 or 80 kv. The microscope had double condenser illumination with 300 micron condenser apertures. A 50 micron platinum objective aperature was used for most observations. Micrographs were taken on Kodak fine grain positive film (P426) or Kodak high contrast projector slide plates if measurements were to be made.

Counts were made by selecting a well stained grid square and traversing from one corner to the opposite corner and counting the various structures appearing in the traverse. This was repeated in different squares until sufficient numbers were obtained, usually two or three traverses. I found it convenient to record structures with a multiple hand counter (Clay-Adams, N.Y.). In grids prepared as described above both negatively and positively 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 Å as the length of the core, or from the manufacturer's calibration. Magnification factors are only approximate.

(h) Antisera

Rabbit anti-particle serum was prepared and kindly donated by R. S. Edgar and V. Marinkovich. Because of the strong antigenicity of phage tail fibers, preparation of a neutralizing anti-particle serum requires phage preparations with very low levels of tail fiber antigen. Tail fiberless phage particles were accordingly prepared by purification from a lysate of cells infected under restrictive conditions with a multiple mutant, amx4e, carrying amber mutations in four tail fiber genes, 34, 35, 37 and 38. The three classes of tail fiber antigens are absent from such lysates (Edgar & Lielausis, 1965). The fiberless phage particles were subjected to two cycles of high and low speed centrifugation and then absorbed with sensitive bacteria to further lower the background of particles with tail fibers. A rabbit was injected with this fiberless particle preparation as follows: day 1, 2×10^{11} particles intravenously; day 4, 3×10^{11} particles in Fraund's adjuvant, subcutaneously; day 31, 3×10^{11} particles subcutaneously. The rabbit was bled on day 45, yielding a serum which killed T4D r48 with a first order rate constant of 30, at 48°C. At least 95% of the neutralizing activity of this serum was blocked by fiberless phage particles; less than 5% of its activity was blocked by sucrose gradient fractions comprising the peak of the tail fiber antigen distribution (R. S. Edgar & J. King, unpublished experiments).

Antitail-fiber sera: two absorbed antisera, S34 and S37, specific for the tail fiber antigens A and C, respectively (Edgar & Lielausis, 1965), were used in these experiments. Their preparation and properties are described elsewhere (see Part II).

(i) Serum blocking assays

The serum blocking assay employed is described in Edgar & Lielausis (1965). Samples of interest are incubated with aliquots of antiserum for a period of time sufficient for the reaction to go to completion. The residual neutralizing activity is then measured by determining the rate of inactivation of subsequently added tester phage. The assay only detects those antibodies which kill the phage on complexing with it, and thus can only be used to measure the concentration of those antigens which react with the neutralizing fraction of the antibodies present in the sera.

For the serological analysis of sucrose or D_2O gradients, fractions of between 0.05 and 0.15 ml. were collected directly into 1 ml. of serum in dilution buffer at a k of about 0.025, where k is defined by the equation $P/P_o = e^{-kt}$. These mixtures were then incubated for 16-24 hours at 48^o. At the end of the incubation period 2 drops of r48 tester phage were added to give a final concentration of 4×10^5 per ml. After a further 3½ hour incubation period at 48^o the tubes were titered for survival of the tester phage on B/5 or F(λ). The presence of sucrose in the mixture slows the reaction somewhat, but the effect is small.

Presentation of data: The results of serum blocking assays are presented as percent of serum blocked (1 - k final/k initial) x 100, calculated as described in Edgar and Lielausis (1965). The percent of serum blocked, though not a linear function of antigen concentration, is monotonic with antigen concentration.

(j) In vitro phage formation assays

In a number of experiments samples containing tails were incubated with extracts defective in tail formation and the resulting formation of viable phage used as an assay for tail concentration. The incubation times used were sufficient for the reactions to have gone essentially to completion under conditions where tails were in excess. (Tails are present in 10 to 20-fold excess over heads in undiluted extracts (Edgar, unpublished).) In the concentration region where tails were limiting, the measured yield may have reflected either a rate or an endpoint. Therefore, only qualitative conclusions are drawn from these data.

(k) Terminology

Defective mutant lysates and extracts are designated by the numbers of the gene or genes defective in the infecting phage particles; i.e., 16-defective lysate refers to a lysate of cells infected under restrictive conditions with phage carrying an amber or temperature sensitive mutation in gene 16. Thus mutant lysates and extracts are named for the gene which is nonfunctional. They will also be sometimes described by the major components present or absent; e.g., head$^+$ (H^+), tail fiber$^-$ (TF^-), etc.

Infection of cells with phage mutants always refers to infection under the restrictive conditions. "Lysate," "mutant lysate," and "defective lysate" are used interchangeably.

For multiple mutants, colons are used to separate the numbers designating the mutant genes carried by the strain.

Phage structures are frequently designated by a gene number with a superscript plus or minus after it; in this case the number refers to the gene product. Thus the tails from a 15-defective lysate are 15^-; they have not been acted upon by the 15 gene product. However, such 15^- tails might be 3^+ or 3^-, depending on whether they had or had not been acted upon by the gene 3 product.

3. RESULTS

(a) Electron microscopy of unfractionated mutant lysates

Epstein et al. (1963) found that cells infected with mutants of T4 defective in any of a large number of phage genes accumulated recognizable phage parts. They surveyed lysates made with these mutants for the presence or absence of phage heads, tails, and particles. Under the conditions of their experiments it could not be decided if the tails in a particular mutant lysate had sheath on them, or if the heads contained DNA.

I have resurveyed lysates made with these mutants under conditions in which sheathed tails and full heads are reproducibly present. (This survey was in no sense exhaustive; preparations were examined only with negative staining, and only certain specific structures were looked for and scored for. Polyhead and tail fibers were not scored for, and no effort was made to find structures not clearly visible in the phage particle (for example, precursors of the baseplate).)

(i) Identification of three genes involved in sheath formation

Plate I and Table 1 show that on specimen grids of fresh 23-defective lysate (head$^-$) - prepared as described under Materials and Methods - the majority of the tails were sheathed. Three minority classes were present; tails without sheaths (naked tails), tails on which the sheath extends only part way up the core from the baseplate (partially sheathed tails), and tails with contracted sheaths separated from the baseplate (contracted tails). (This last class (Plate V, J-M) is not included in the table; it represented about 2% of the tails present in lysates containing normal sheathed tails.)

Specimen grids prepared from lysates made with mutants defective in genes 3, 15, or 18 did not exhibit normal sheathed tails. The tails in these lysates were naked (Plate II) and indistinguishable from the naked tails present in head$^-$ control lysates. However, the appearance of the tails from 3- and 15-defective lysates was markedly different if care was taken to prepare the grids within a few minutes after cell lysis. The tails in both lysates were then found to have partial sheaths - as if either partially degraded or partially completed (Plate III) - and were indistinguishable from the minority class of partially sheathed tails present in head$^-$ control lysates. No partially sheathed tails were ever seen in 18-defective lysates. The partial sheath associated with 3$^-$ and 15$^-$ tails had the same distinctive morphology as the complete sheaths seen in control lysates. Counts of the three classes of tails appearing in well stained areas of specimen grids made from the various defective lysates are given in Table 1. The defective phenotypes are

Legend for Table 1

Specimen grids were prepared within 20 minutes after cell lysis. Assignment of tails to the three classes was on the basis of sheath length. Since it was difficult to distinguish between naked tails and those with 1 or 2 sheath annuli, or between sheathed tails and tails with 22 or 23 annuli (complete sheath has 24 annuli), the classes overlap somewhat. The counting procedure is described under Materials and Methods.

TABLE 1

Electron microscope counts of tail structures in fresh unfractionated mutant lysates

Lysate		Normal sheathed tails	Partially sheathed tails	Naked tails
Defective Gene	Mutant			
23	amB17*	406	12	71
	tsN37	98	0	37
	tsA78	123	2	7
	tsL65	124	16	16
18	amE18+	0	0	525
	tsA38	0	0	207
	tsB39	0	0	155
15	amN133+	32	232	17
	tsN26	1	73	121
3	amNG131	23	101	17
	amNG321	26	200	12
	tsA2	9	126	46
	tsC18	7	110	28
	tsCB2+	5	332	58
	tsCB19	0	82	55
	tsCB55	0	136	44
	tsA77	0	147	50
	tsCB69	1	93	20

*Totals from three independent experiments

+Totals from four independent experiments

+Totals from two independent experiments

Plate I. Tails in a 23-defective lysate. (A); x about 80,000. (b); x about 130,000. (C,D); x about 210,000.

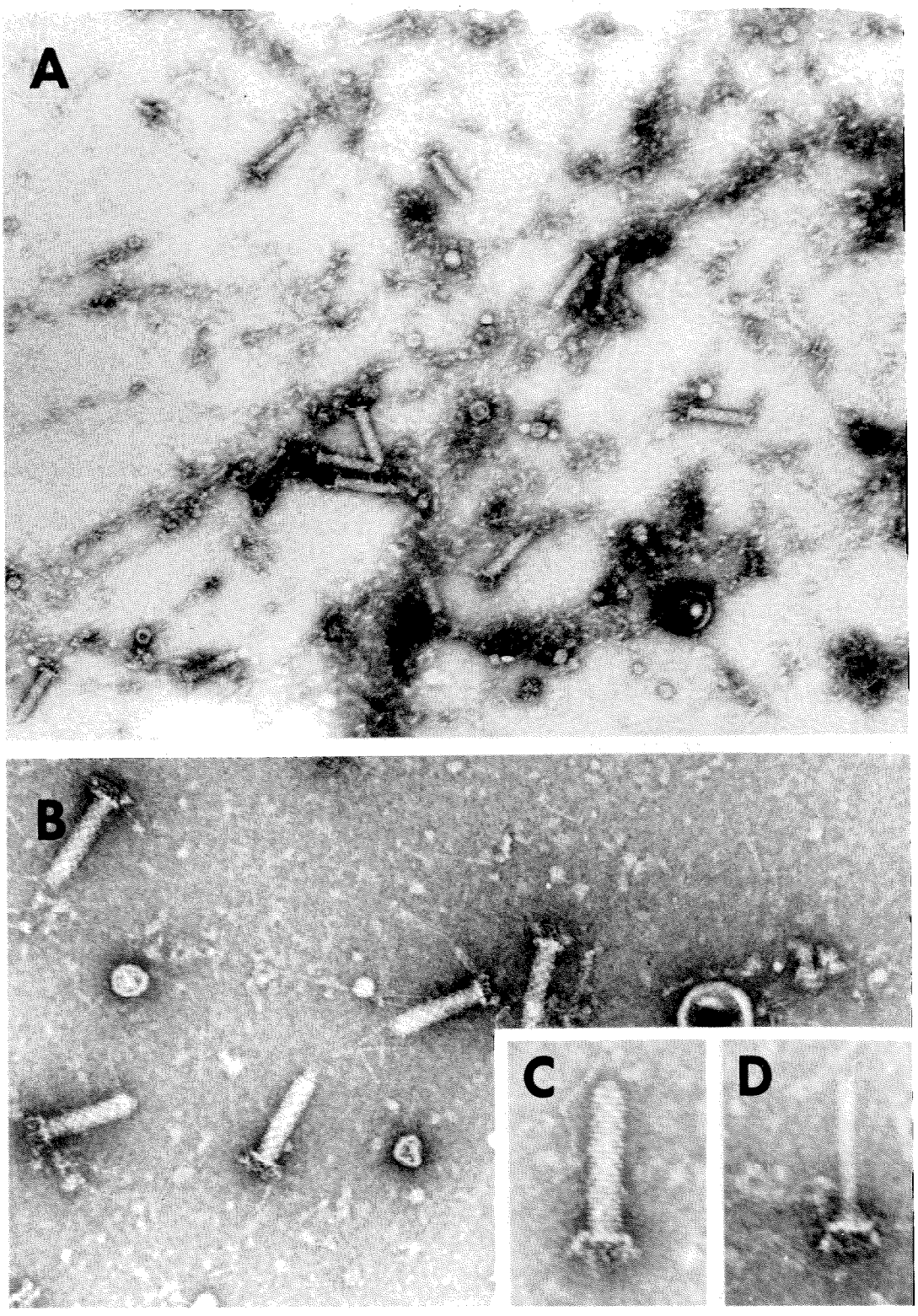

A
B
C
D

Plate II. (A) 15-defective lysate. This specimen grid was prepared about ½ hour after cell lysis. In specimen grids prepared immediately after cell lysis, the appearance of the tails was indistinguishable from those in the 3-defective lysate shown in Plate III. (x about 130,000). (B,C) 18-defective lysate. (B); x about 130,000. (C); x about 210,000.

The "cuffs" above the baseplates on some of these tails were seen frequently on naked tails in unfractionated lysates, including those from 18-defective lysates. Whether they represent specific phage structures or artifacts is not known.

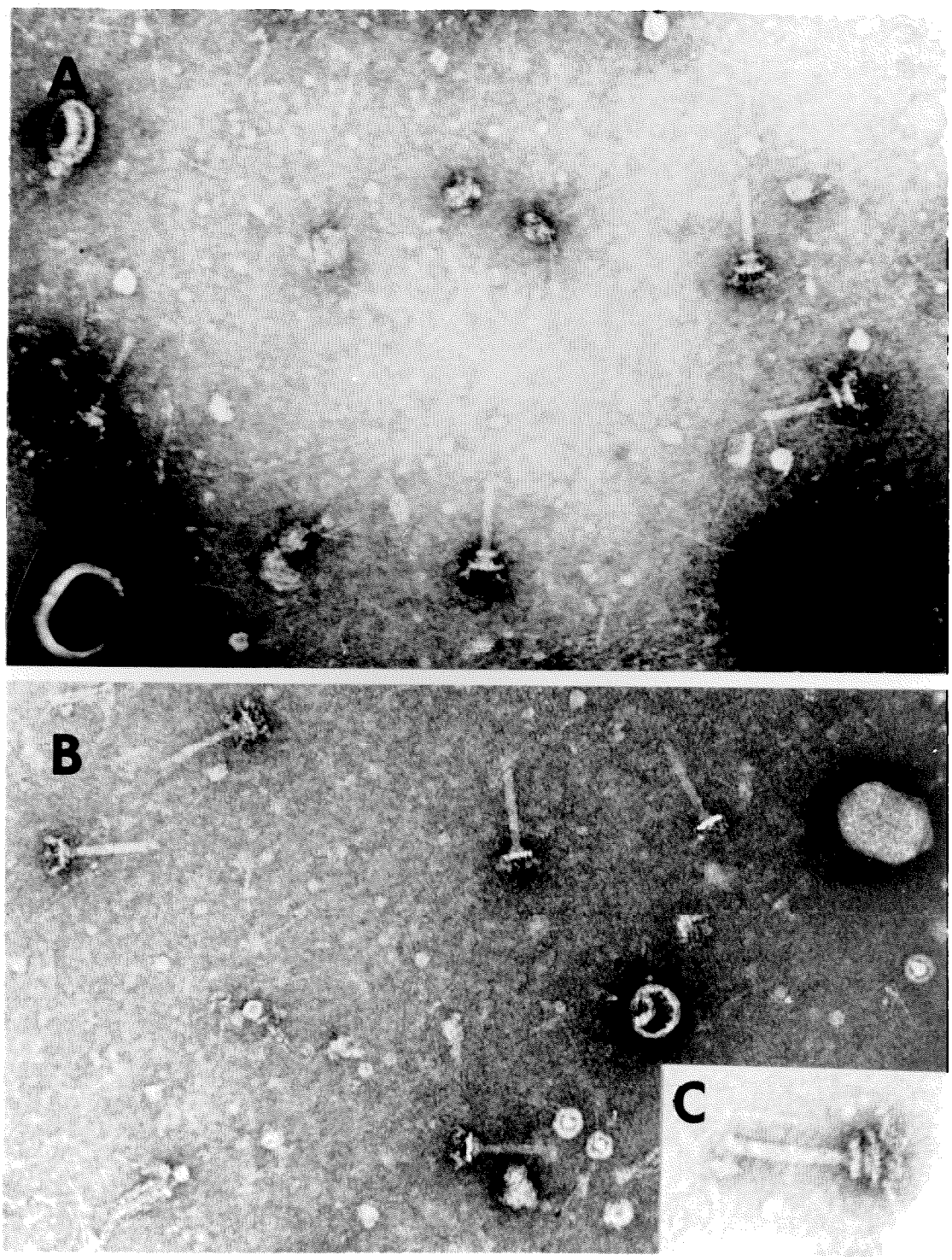
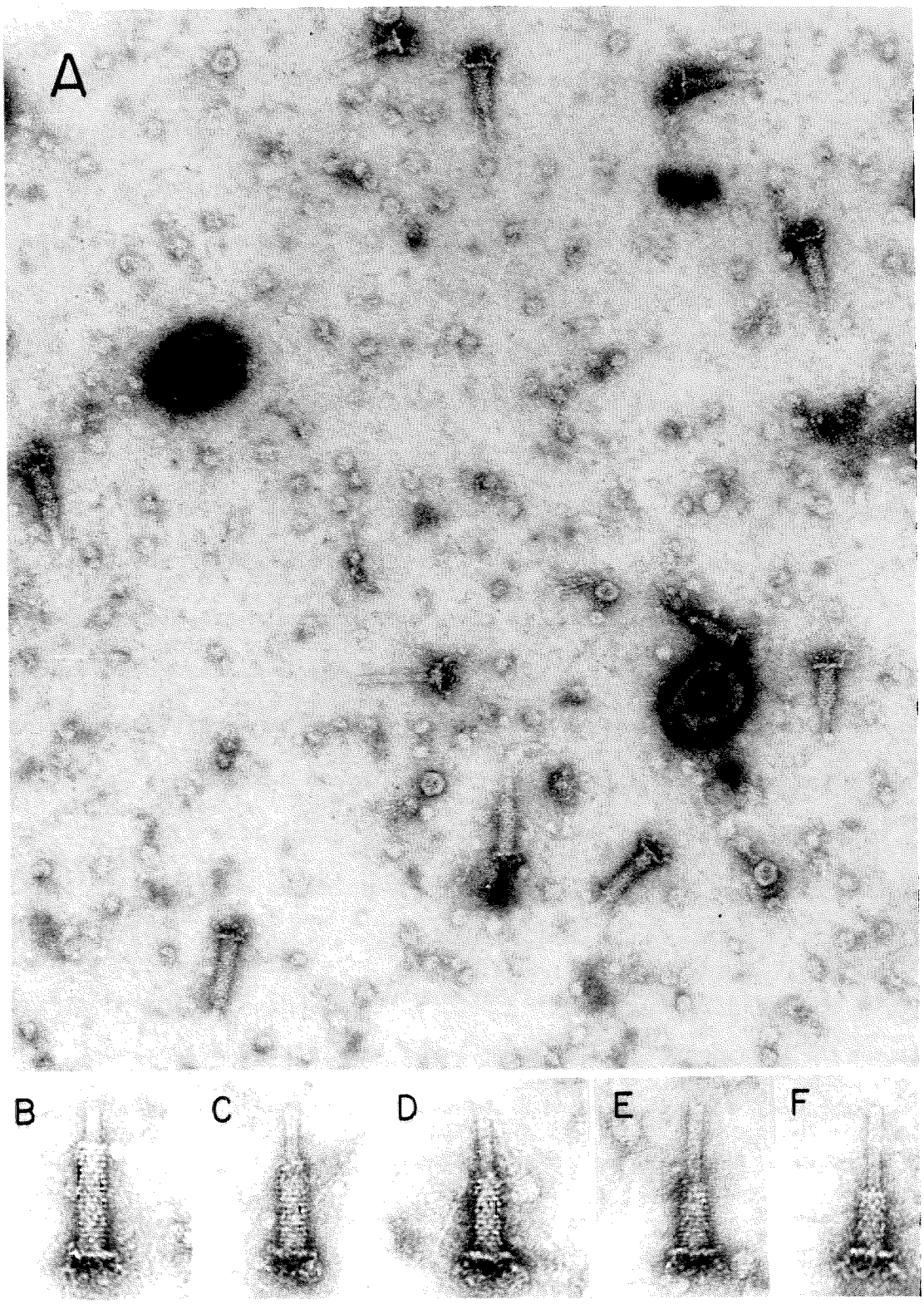

A
B
C

Plate III. Appearance of 3-defective lysate (amNG131) immediately after cell lysis. Note partially sheathed tails. (A); x about 110,000. (B-F); x about 200,000.

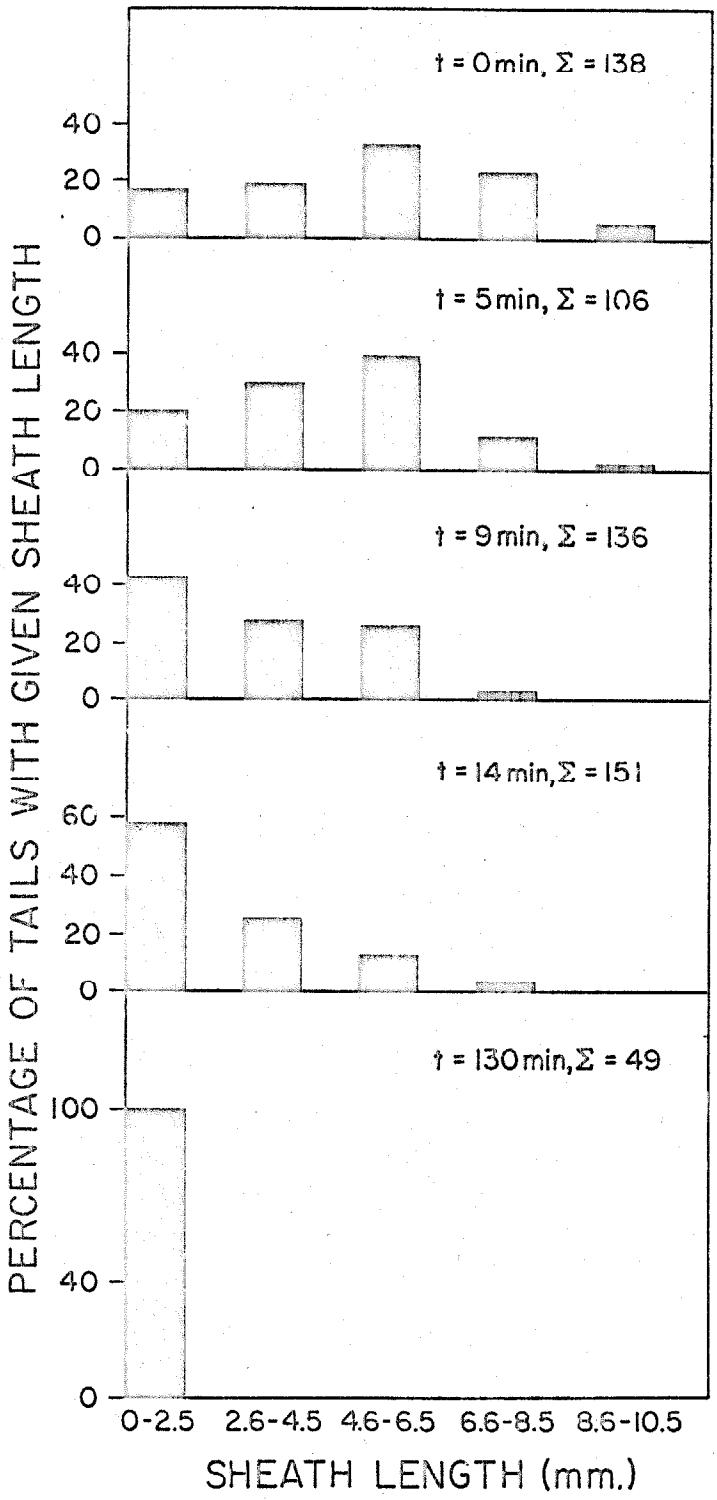

A
B
C
D
E
F

clearly gene specific and not mutant specific. The contracted tails mentioned above were never seen on grids prepared from 3-, 15-, or 18-defective lysates.

The majority of the tails present on specimen grids of all other $tail^+$ lysates (Figure 15) were sheathed and indistinguishable from each other. Thus, of the T4 genes so far identified, 3, 15, and 18 appear to be the only genes affecting sheath assembly.

The partially sheathed tails appearing in fresh 3- and 15-defective lysates lose sheath subunits progressively from the neck end as a function of time after cell lysis. This is shown by the following experiment. A 3-defective lysate was chilled in a water-ice bath immediately after lysis by quick freezing and thawing. At various times thereafter samples were withdrawn and specimen grids prepared for electron microscopy. Photographs of well stained areas of the grids were taken and the lengths of the sheaths on the tails appearing in them measured. The resulting length distribution as a function of time is presented in Figure 1. The time at which the first specimen grid was made was taken at t = 0; by this time the tails had already lost considerable sheath since the length of the fully sheathed tail on this scale would be 11.5mm. In a 23-defective lysate used for a control, the proportion of sheathed tails remained constant over the same time period. The 15-defective tails lose sheath at roughly the same rate; however, these experiments have not yet been done with sufficient accuracy to permit detailed comparison.

Figure 1. Loss of sheath from the tails in a 3-defective lysate. A superinfected 3-defective lysate (tsA2) was lysed by quick freezing and thawing at 55 minutes after initial infection. A sample of 0.5 ml was equilibrated with a water-ice bath and the samples applied to specimen grids at various times thereafter. About three minutes elapsed between application of the sample to the grid and final blotting dry. A series of electron micrographs were taken of these grids at the same magnification and measurements made on prints in which the length of a normal sheath was 11.5 mm. The total number of tails measured for each time point is given by Σ.


t = 0 min, Σ = 138
t = 5 min, Σ = 106
t = 9 min, Σ = 136
t = 14 min, Σ = 151
t = 130 min, Σ = 49
PERCENTAGE OF TAILS WITH GIVEN SHEATH LENGTH
0
20
40
60
100
0-2.5
2.6-4.5
4.6-6.5
6.6-8.5
8.6-10.5
SHEATH LENGTH (mm.)

(ii) Early steps in tail assembly

Cells infected with mutants of T4 defective in any one of the 15 genes listed in Table 2 accumulate phage heads but not phage tails (Epstein et al.,1963, and unpublished). These genes presumably control steps in the formation of the naked tail. Lysates made from mutants defective in these genes have been examined with the electron microscope in an attempt to detect structures which might represent precursors of the naked tail.

(α) Absence of cores in tail$^-$ lysates

No free cores were observed in the tail$^-$ lysates described above (Table 2). I have never seen significant numbers of free cores in any T4 mutant or wild type lysate. They do appear in degraded preparations, and very occasionally in tail$^+$ lysates. Their large dimensions (about 100 x 1000 Å) and tubular structure render them easily identifiable.

(β) Baseplates

Cells infected with mutants defective in any one of genes 48, 54, or 19 accumulate structures which appear to be free baseplates (Table 2, Plate IV, A, B). With the staining conditions used in these experiments the free baseplates were not always easily visible; thus the possibility that some of the other mutant lysates also contained baseplates is not excluded. However, the serological experiments described below indicate that the majority of these mutants are blocked at a stage prior to baseplate completion.

Legend for Table 2

Specimen grids were prepared within ½-hour after cell lysis. Tail fibers, which have about a five-fold smaller diameter than cores, were visible on all these grids. Thus cores, if present, should also have been visible. Most stretches of polysheath were from 2-10 times the length of normal sheath. The polysheath concentration represents a minimal estimate since polysheath is preferentially washed off these grids relative to heads. The relative polysheath concentration on unwashed grids was much higher. In these preparations the majority of the phage heads were full, presumably of DNA.

Baseplates are somewhat difficult to see under these staining conditions. Failure to see them does not preclude their presence. In favorable regions of the grids prepared from 19-, 48-, and 54-defective lysates, baseplates were somewhat more plentiful than heads.

TABLE 2

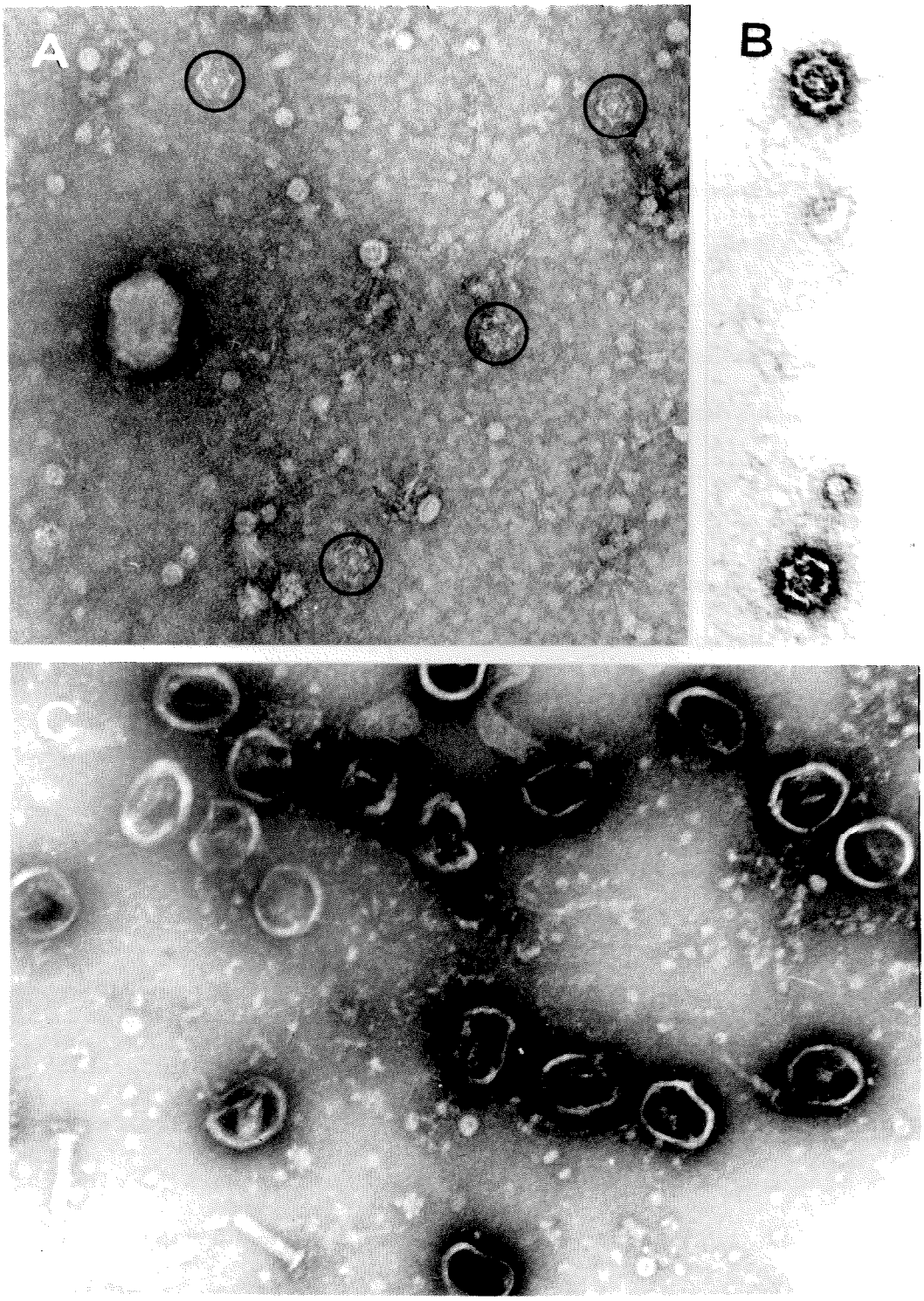
Phage structures appearing in tail⁻ lysates

Lysate: Defective gene	Lysate: am mutant	Full heads	Cores	Normal or contracted sheaths	Stretches of polysheath	Other
53	H28	107	0	0	11	
5	N135	207	0	0	13	
6	N102	176	0	0	56	
	B274	107	0	0	14	
7	B16	100	0	0	27	
8	N132	121	0	0	43	
10	B255	149	0	0	23	
19‡	E275	133	0	0	13	Baseplates
25*	N67	111	0	0	51	
26	N131	148	0	0	11	
51	S29	151	0	0	17	
27	N120	177	0	0	24	
28	A452	116	0	1	10	
29	B7	133	0	0	19	
48	N85	133	0	0	9	Baseplates
	N022	66	0	0	22	Baseplates
54	H21	109	0	0	30	Baseplates
	S6	122	0	0	9	Baseplates

‡Baseplates were also present in a lysate made with a temperature sensitive mutant of gene 19, tsN3.

*Considerable numbers of phage particles were present on this grid due to the high transmission of amN67; the lysate contained 6×10^9 viable phage/ml.

Plate IV. (A) Baseplates from a 19-defective lysate (amE275). (x about 14,000). (B) Baseplates from a 48-defective lysate (amN85). (x about 250,000). Note the similarity between these free endplates and those attached to cores, but lying flat, in Plate IX. (C) Heads from a 49-defective lysate. This field was adjacent to a lysed bacterium. The concentration of heads was much lower in regions not adjacent to such bacteria. (x about 110,000).


A
B

(γ) Absence of normal sheath formation in tail⁻ lysates

No normal or contracted sheath was seen in the tail⁻ lysates examined (Table 2); polysheath, however, was plentiful. (Polysheaths are long, tubular structures of irregular length whose diameter and surface lattice resemble those of contracted sheath (Moody, 1967a), and which are found in T4 infected cells under conditions of lysis inhibition. Kellenberger & Boy de la Tour (1964) concluded that these structures were aberrant polymerization products of sheath subunits.)

In agreement with Kellenberger & Boy de la Tour (1964), I did not see normal extended sheaths not associated with the core and baseplate. Since polysheath, sheathed tails, and contracted sheath (from disrupted phage) all stick to specimen grids it seems unlikely that the absence of free normal or contracted sheath from tail⁻ lysates was due to their not sticking to the grid surface.

Thus the absence of normal or contracted sheaths from these lysates suggests that normal sheath polymerization requires prior formation of the naked tail.

(iii) Polysheath and the functions of genes 3, 15, and 18

To determine if polysheath accumulated in cells infected with mutants defective in genes 3, 15, and 18, experiments were performed as in Table 2, except that the specimen grids were washed more sparingly to permit detection of lower quantities of polysheath. Polysheath was found in 3- and 15-defective lysates, but never in 18-defective lysates. Polysheath is much less plentiful in tail⁺ and 3- and 15-defective lysates than in tail⁻ lysates, suggesting that, when possible,

normal sheath is formed preferentially to polysheath. The absence of polysheath in 18-defective lysates might then be due to the preferential formation of an unstable form of normal sheath. To examine this possibility, the double mutants 3:54, 15:27, and 18:27 (blocked early in the tail pathway) were constructed, since the results of Table 2 suggested that polysheath was the only sheath structure formed in cells infected with such mutants. Table 3 shows that in lysates made with the 18:27 double mutant no polysheath was detected.

Examples of polysheath from two tail$^-$ lysates and from the 3:54- and 15:27-defective lysates are shown in Plate V; they appear to be equivalent structures.

The absence from 18-defective lysates of both normal sheath and polysheath suggests that gene 18 codes for a major sheath structural protein. The products of genes 3 and 15 appear to be required for the stabilization of normal sheath, but not for the formation of polysheath.

(iv) The "connector"

In favorable electron micrographs of sheathed tails, a small protrusion which looks like an extension of the core is clearly visible at the neck end (Plate V, I): this structure will be referred to as the connector. Is this segment a part of the core, or a separate structure formed after the sheath has been completed? The two possibilities imply different mechanisms for the termination of sheath polymerization. In an attempt to answer this question I measured the lengths of the tails from 18-, 3-, and 15-defective lysates (lacking complete sheaths) and from head$^-$ control lysates (with complete sheaths). Electron

TABLE 3

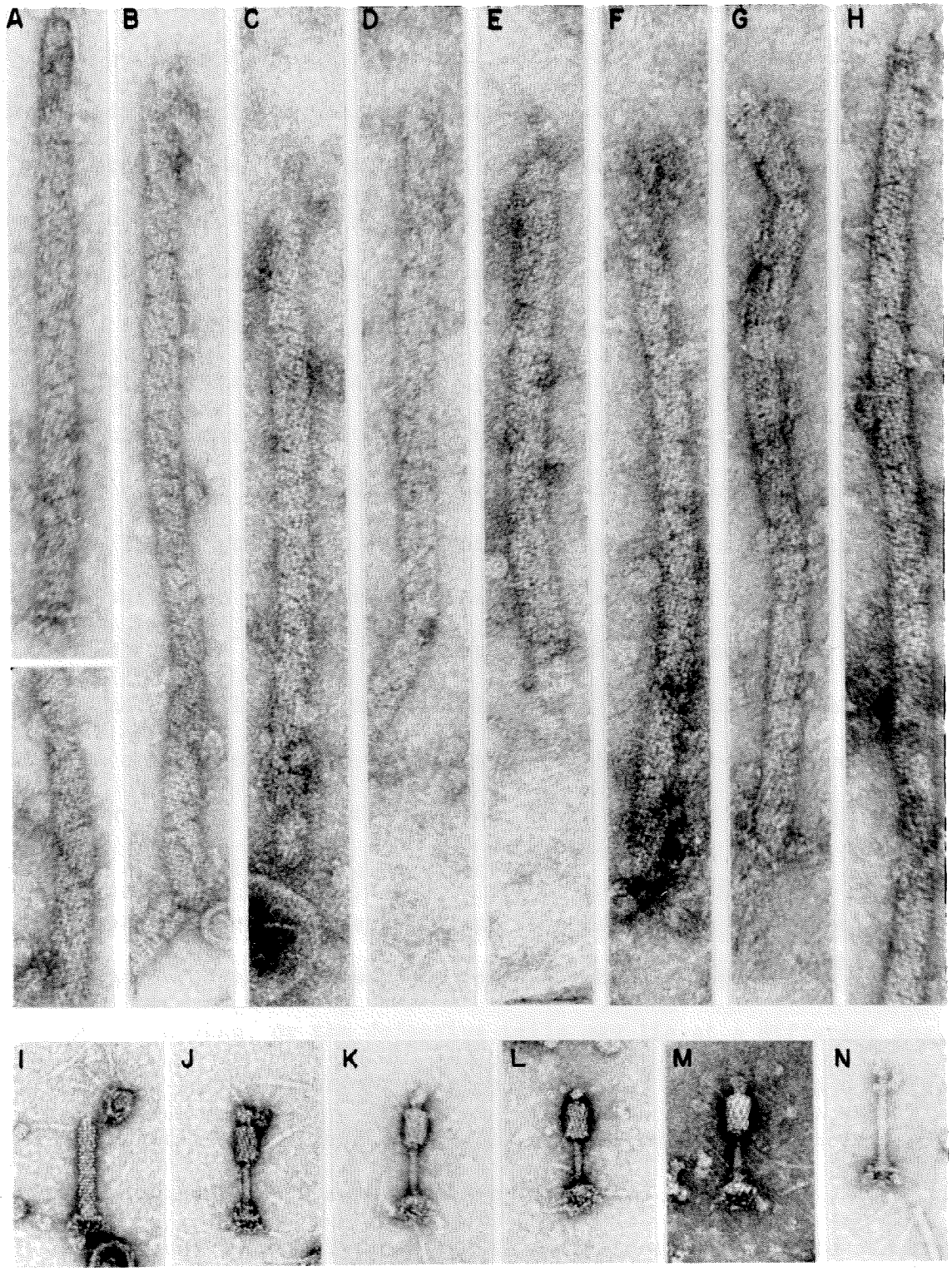
Polysheath formation

Lysate Defective Gene(s)	Mutant(s)	Stretches of polysheath/full phage heads
54	amH21	71/462
27	amN120	84/147
18	amE18	0/496
18:27	amE18:amN120	0/447
3:54	tsA2:amH21	54/480
15:27	amN133:amN120	62/276

These lysates were all prepared under conditions of lysis inhibition. Specimen grids were washed sparingly to avoid washing off polysheath. Most stretches of polysheath were from 2-10 times the length of normal sheaths.

Plate V. (A-H) Typical examples of polysheaths from mutant lysates. (A,B); from 27-defective lysate. (C,D); from 15:27-defective lysate. (E,F); from 3:54-defective lysate. (G,H); from 54-defective lysate. (All x about 200,000). The "loose" form of polysheath described by Kellenberger & Boy de la Tour (1965) and Moody (1967a) was rare in fresh lysates.

(I-N) Tails from a 23-defective lysate, showing examples of normal sheathed (I) and normal naked (N) tails, and of relatively infrequent tails with contracted sheath (J-M). Tails with such contracted sheaths were never seen in 3-, 15-, or 18-defective lysates. (All x about 140,000).


A
B
C
D
E
F
G
H
I
J
K
L
M
N

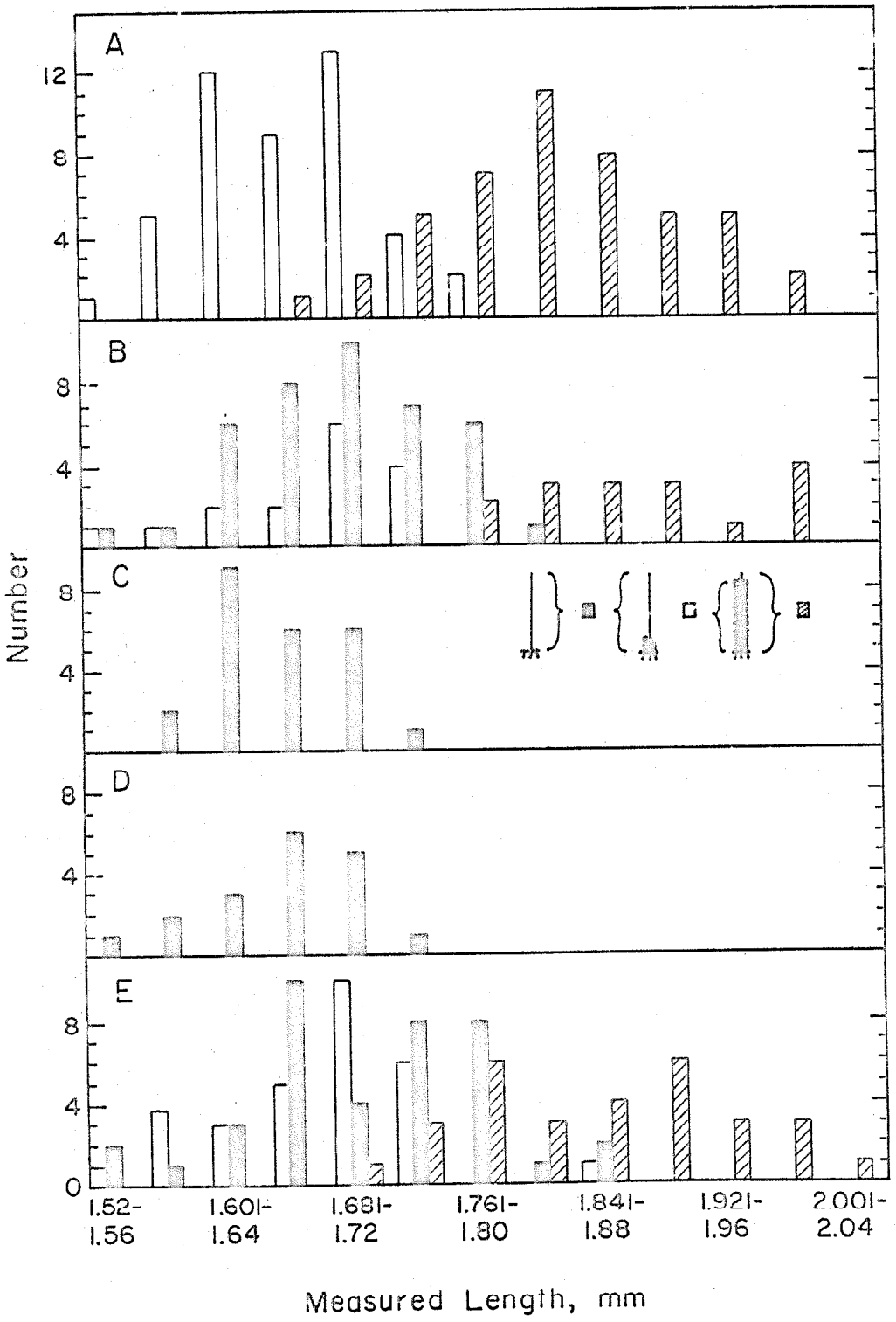
micrographs of mutant lysates and mixtures of lysates were taken on glass photographic plates, and tail and sheath length measurements were made directly on the plates with an optical comparator.

Three classes of measurements are graphed in Figure 2: the lengths of the cores of naked or partially sheathed tails (solid bars), the lengths of the cores of sheathed tails (crosshatched bars), and the lengths of the sheath of sheathed tails (open bars). Two head$^-$ lysates serve as controls. In the head$^-$ lysate illustrated in (b), a large number of the tails were naked. Their measured lengths were about 10% shorter than the lengths of the sheathed tails present on the same grids. The length of the naked tails was about the same as the length of the sheath on the sheathed tails. The lengths of the 3$^-$ (partially sheathed) and 18$^-$ (naked) tails appear to be the same as the length of the naked tails from the control lysate. The results of measurements made on a grid prepared from a mixture of 15- and 23-defective lysates are shown in (3). Again there appear to be at least two length classes, but the distinction is less clear-cut.

These measurements were subject to considerable experimental artifact; the cores of tails without complete sheaths may broaden and shorten on drying, or the defective tails may lie at a different angle to the grid surface than normal sheathed tails, resulting in a difference in projected length. Furthermore, there was considerable uncertainty in identifying the end of any tail being measured.

It is unlikely that these length differences were due to variations in the absolute magnification of the electron microscope since they occurred among different classes of tails appearing in the same micrograph.

Figure 2. Tail lengths. Specimen grids were prepared from lysates or mixtures of lysates. Electron micrographs were taken at constant instrumental magnification on glass photographic plates. The measurements - from the inside edge of the baseplate to either the end of the core or the end of the sheath - were made with a Nikon Shadograph fitted with a micrometer stage. Data from different plates taken of the same grid at the same time were pooled. Filled bars represent the lengths of the cores of naked or partially sheathed tails. Crosshatched bars give the lengths of the cores of sheathed tails present in the 23-defective control lysates. The open bars give the lengths of the sheaths on these tails. For clarity, the lengths of the sheath on the 3- and 15-defective tails are omitted. In these experiments the mean lengths of these partial sheaths were about ½ the normal length. Since the sheath length distribution on tails in the mixed grids was distinctly bimodal, there was no problem in deciding if a tail was from the 23-defective lysate or 15-defective lysate. About 60-80% of the tails appearing on the plates were measured. Tails which were bent, broken, or very poorly defined were not measured. (a) 23-defective lysate; (b) 23-defective lysate in which a large number of tails were naked; (c) 18-defective lysate; (d) 3-defective lysate; (e) mixture between 15-defective and 23-defective lysates.


A
B
C
D
E
12
8
4
0
Number
1.52-
1.56
1.601-
1.64
1.681-
1.72
1.761-
1.80
1.841-
1.88
1.921-
1.96
2.001-
2.04
Measured Length, mm

These results suggest the possibility that the connector is a separate structure from the core, and is either formed after sheath completion, or is required for sheath completion.

(b) Isolation and characterization of precursor tail structures

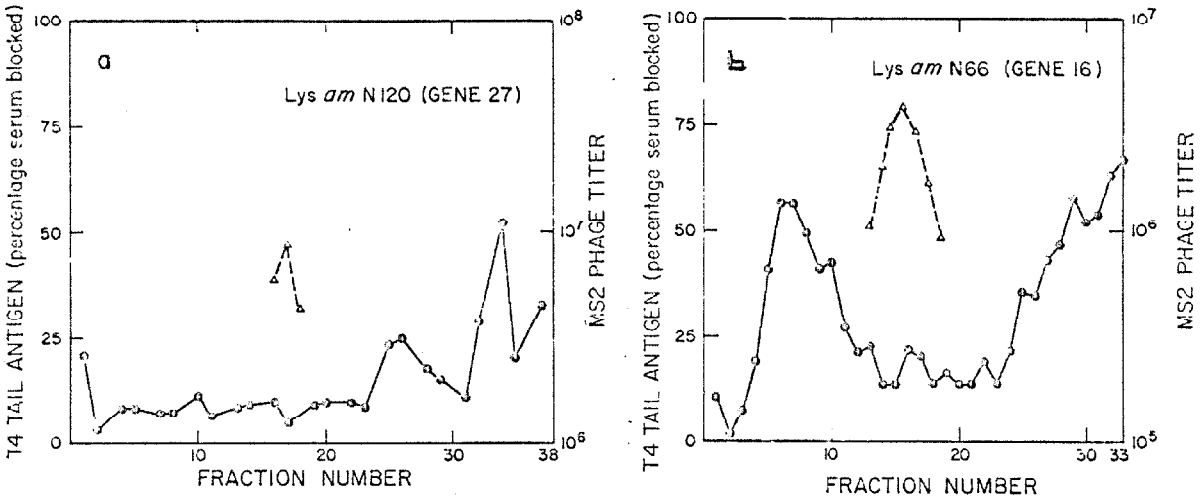
Many stages of assembly of phage structures may not be distinguishable with the electron microscope. Characterization of these intermediates by other methods requires their isolation from the rest of the phage and bacterial material present in crude lysates and extracts. (For example, consider an assembly sequence A + b + c → D, where A and D represent large structures, such as phage tails, and b and c represent small structures such as single proteins. Suppose b is eliminated by mutation; the determination of whether A still associates with c will generally require separation of A from free c.) Therefore the attempt was made to separate sheathed and naked tails, and possibly baseplates, from the crude lysates or extracts. Because of the relatively large size of tails, centrifugation through sucrose or D_2O gradients appeared to be an effective isolation method.

(i) Serological assay

To identify phage tail structures in such gradients, the fractions were assayed for phage particle antigens (distinct from tail fiber antigens; see Materials and Methods) using a serum blocking test.

Typical antigen patterns from experiments with lysates of mutants representative of the classes H^+T^+, H^+T^- (baseplate$^-$), and H^-T^+ are presented in Figures 3 and 9. Bacteriophages ØX174, $S_{20,w}$ = 114, or MS2, $S_{20,w}$ = 81, were used as centrifuge markers. In all cases slowly-

Figure 3. Serological analysis of sucrose gradient fractions from T4 mutant lysates. (a) H^+T^- (baseplate$^-$) lysate. (b) H^+T^+ lysate. Samples were layered onto sucrose gradients and centrifuged 50-60 minutes at 37,000 rev./min, 20-24°C. Fractions were collected directly into anti-particle serum and assayed for serum blocking activity. Centrifugation is from right to left in this and subsequent figures. ○ = anti-particle serum blocking activity; Δ = MS2.

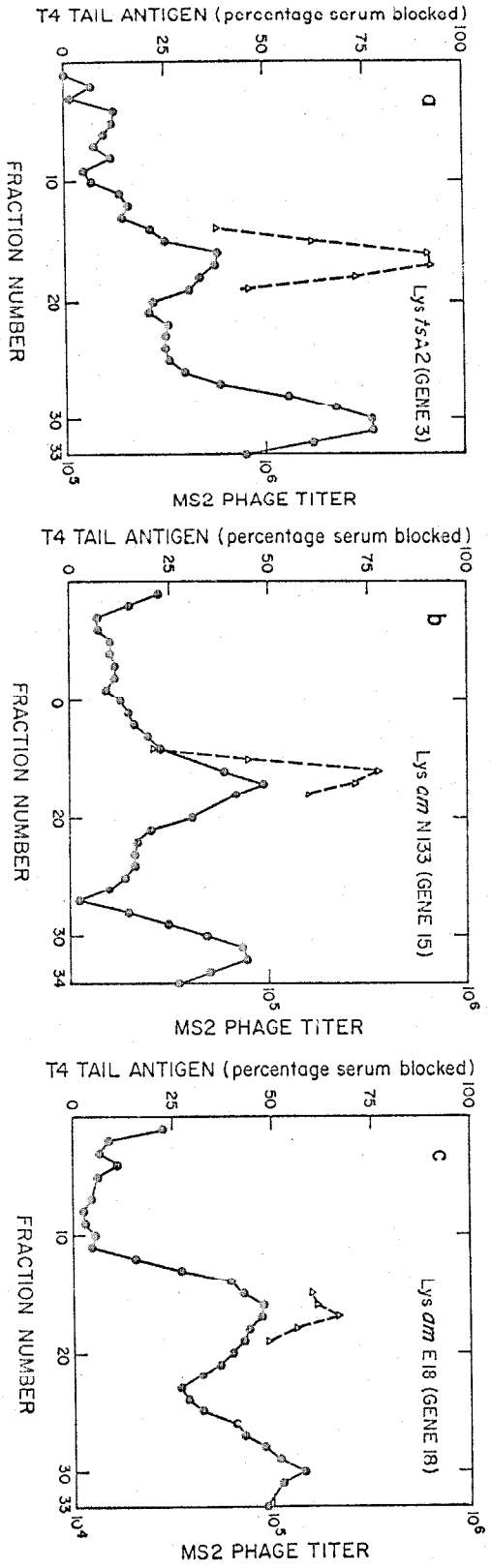

a
Lys am N120 (GENE 27)
T4 TAIL ANTIGEN (percentage serum blocked)
MS2 PHAGE TITER
FRACTION NUMBER
b
Lys am N66 (GENE 16)
T4 TAIL ANTIGEN (percentage serum blocked)
MS2 PHAGE TITER
FRACTION NUMBER

sedimenting antigenic material was present at the top of the centrifuge tube, presumably representing smaller tail precursors or precursor proteins. In addition, a rapidly sedimenting peak of serum blocking activity with sedimentation coefficient of about 130S was found with control rdf41 lysates and with $tail^+$ lysates: this peak was absent from both classes ($baseplate^+$ and $baseplate^-$) of $tail^-$ lysates. A minor peak of serum blocking activity with sedimentation coefficient of about 80S was also frequently found with $tail^+$ lysates (Fig. 9), but was not seen with the $baseplate^-$ class of $tail^-$ lysates. The difference between the two $tail^+$ lysates shown - in the amount of 130S antigen and the presence or absence of 80S antigen - is probably not gene specific, since such differences were also found with lysates made from the same mutant under slightly different conditions.

When 3-, 15-, and 18-defective lysates were subjected to similar analyses, all the rapidly sedimenting serum blocking activity was found in the 80S peak (Fig. 4). In these experiments the tails from 3- and 15-defective preparations would have lost their sheaths in the time between cell lysis and layering of the sample onto the gradients. When care was taken to centrifuge preparations immediately after cell lysis, the resulting distribution of 3^- and 15^- tails was not homogeneous, but exhibited a much broader leading edge than the distribution of 18^- tails.

The antigen distributions in the sucrose gradients of 19- and 54-defective lysates were similar to the patterns obtained with 3, 15, and 18 mutants; a single peak of rapidly sedimenting serum blocking activity was present at about 80S (Table 4). Preliminary experiments with 48-defective lysate indicated a second smaller peak of serum blocking activity sed-

Figure 4. Serological assay of sucrose gradient fractions from 3-, 15-, and 18-defective lysates. Experimental conditions were the same as in Figure 3. ● = Anti-particle serum blocking activity; Δ = MS2.


T4 TAIL ANTIGEN (percentage serum blocked)
0
25
50
75
100
a
Lys tsA2 (GENE 3)
FRACTION NUMBER
10
20
30
33
10^5
10^6
MS2 PHAGE TITER
T4 TAIL ANTIGEN (percentage serum blocked)
b
Lys am N133 (GENE 15)
FRACTION NUMBER
0
20
30
34
10^5
10^6
MS2 PHAGE TITER
T4 TAIL ANTIGEN (percentage serum blocked)
c
Lys am E18 (GENE 18)
FRACTION NUMBER
10
20
30
33
10^4
10^5
10^6
MS2 PHAGE TITER

Legend for Table 4

Serological experiments were performed with lysates, as described in Figures 3 and 9. In many of the experiments in which a 130S peak of serum blocking activity was found, a smaller 80S peak was also present; these have not been included in the table. For the serological experiments with gene 11 and 12 mutants, double mutants containing a mutant blocked in head formation were employed (amN93:amB17 and amN69:amB17). This prevented ghosted whole phage particles from confusing the serological assay; ghosts sediment with much slower than normal phage particles. On specimen grids prepared from the rdf41 lysate used above, free tails and phage were present in about equal numbers.

Extracts were used in experiments in which fractions were examined in the electron microscope and tested for in vitro complementation activity. These experiments were similar to the experiments of Figure 6, except that fractions comprising the top third of the gradient were not assayed, and centrifugation was at 20-24°C.

The variation in the measured S value of the peaks from different lysates of the same mutant was about 10% for the 80S peak and somewhat less for the 130S peak. The best experiments indicate that the actual values are closer to 75S and 125S.

The 0 values for serological or in vitro assay of fractions means no peak was present and the maximum values were not significantly above background.

TABLE 4

Isolation and characterization of tail structures by sedimentation

Electron microscope phenotype	Extract or lysate: Defective gene	Mutant	Rapidly sedimenting tail antigen	Rapidly sedimenting tail fiber antigen	Presence of sheathed tails in electron micrographs of 130S fractions	Functional tails: Peak of activity with T extract	Functional tails: Max. activity with T TF extract
Baseplate$^-$	5	amB256	0	0			
	6	amB251	0	0			
	7	amB16	0	0			
	8	amN132	0	0			
	10	amB255	0	0			
	25	amS52	0	0			
	51	amS29	0	0			
Baseplate$^+$	19	tsN3	80S				
	48	amN85	80S*				
	54	amH21	80S				
Sheath$^-$	3	tsA2	80S	0	0	80S	0
	15	amN133	80S	0	0	80S	0
	18	amE18	80S	0	0	80S	0
Sheath$^+$	2	amN51			+	130S	0
	64	amE1102			+	130S	0
	11	amN93	130S	0	+	130S	0
	12	amN69	130S	0	+	130S	0
	13	amE609			+	130S	0
	14	amB20			+	130S	0
	16	amN66	130S	0	+	130S	0
	17	amN56	130S	0	+	130S	0
Head$^-$	20	amN50	130S	0	+	130S	0
	22	amB270	130S	0			
	23	amB17	130S	0	+	130S	0
	24	amN65	130S	0			
	rII	rdf41	130S	0			

*A second, smaller peak was present at ~105S.

imenting 30% faster than the 80S peak. These serological experiments with baseplate$^+$ lysates are still in progress.

Since rapidly sedimenting phage antigen was present in all lysates blocked subsequent to baseplate formation (including those lacking heads and lacking sheath), and was absent from all lysates blocked prior to baseplate formation, the antigens measured appear to be related to the baseplate. The correlation of the centrifuge pattern of these baseplate antigens with the electron microscope phenotypes of the mutants suggests that the 130S components are sheathed tails, and the 80S components from the baseplate$^+$ lysates are baseplates. The 80S components from the sheath$^-$ lysates may be baseplates, core-baseplates (naked tails), or both.

(ii) Electron microscopy of gradient fractions

To further define the nature of the 80S and 130S components, extracts of mutant-infected cells (Edgar & Wood, 1966), in which phage components are 10 to 100-fold more concentrated than in lysates, were centrifuged through sucrose gradients, and the resulting fractions examined with the electron microscope. Plates VI and VII show that fractions from the 130S region of the gradient of tail$^+$ extracts contained sheathed tails.

Fractions from the 80S region of the gradient in such experiments contained very low concentrations of identifiable phage structures, in agreement with the serological indications. To increase the concentration of the 80S component a concentrated wild type lysate which had been subjected to degradation at low pH was used as a source of tails. In this preparation all the rapidly sedimenting tail antigen was 80S. Plate VIII shows that fractions from this region of the gradient contained naked tails.

Plate VI. Sheathed tails from 130S sucrose gradient fractions of 2-defective extract. Ribosomes are prominent in the background of this and some of the subsequent photographs. (x about 90,000).

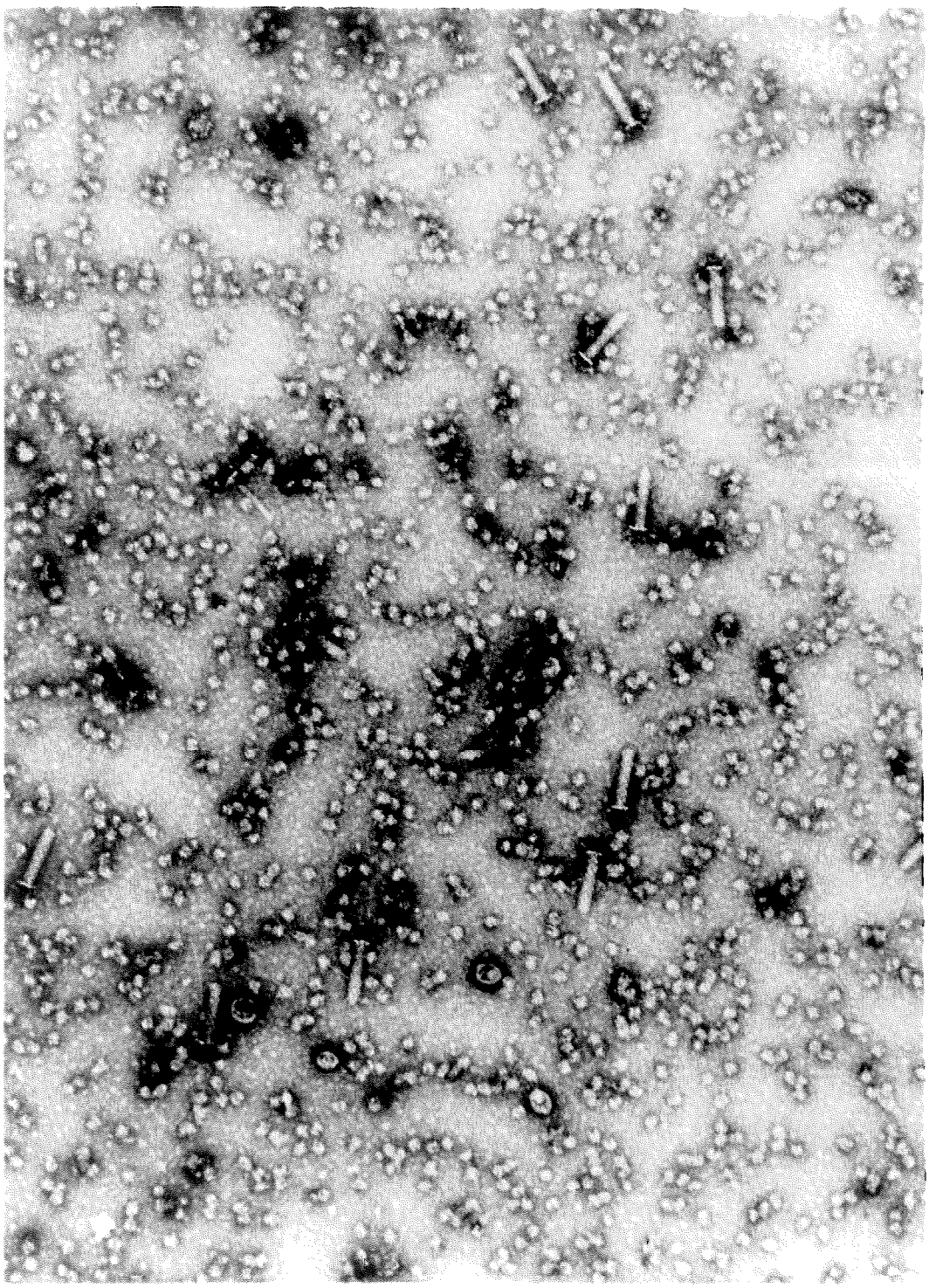


Plate VII. Sheathed tails at higher magnification. These are from 130S sucrose gradient fractions of 64-defective extract. The sheathed tails isolated by centrifugation from the various tail^{+} extracts were indistinguishable in appearance. (x about 200,000).

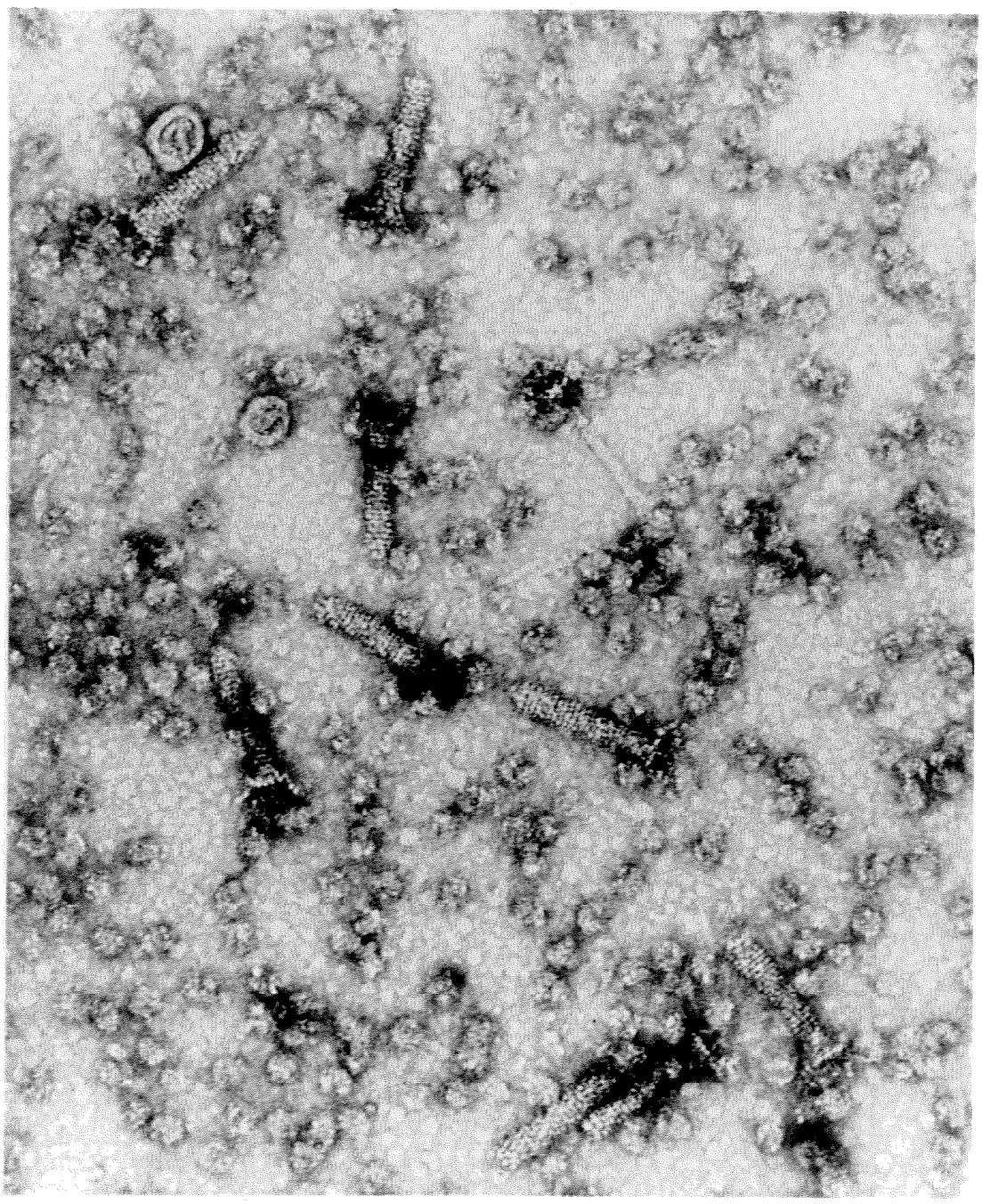
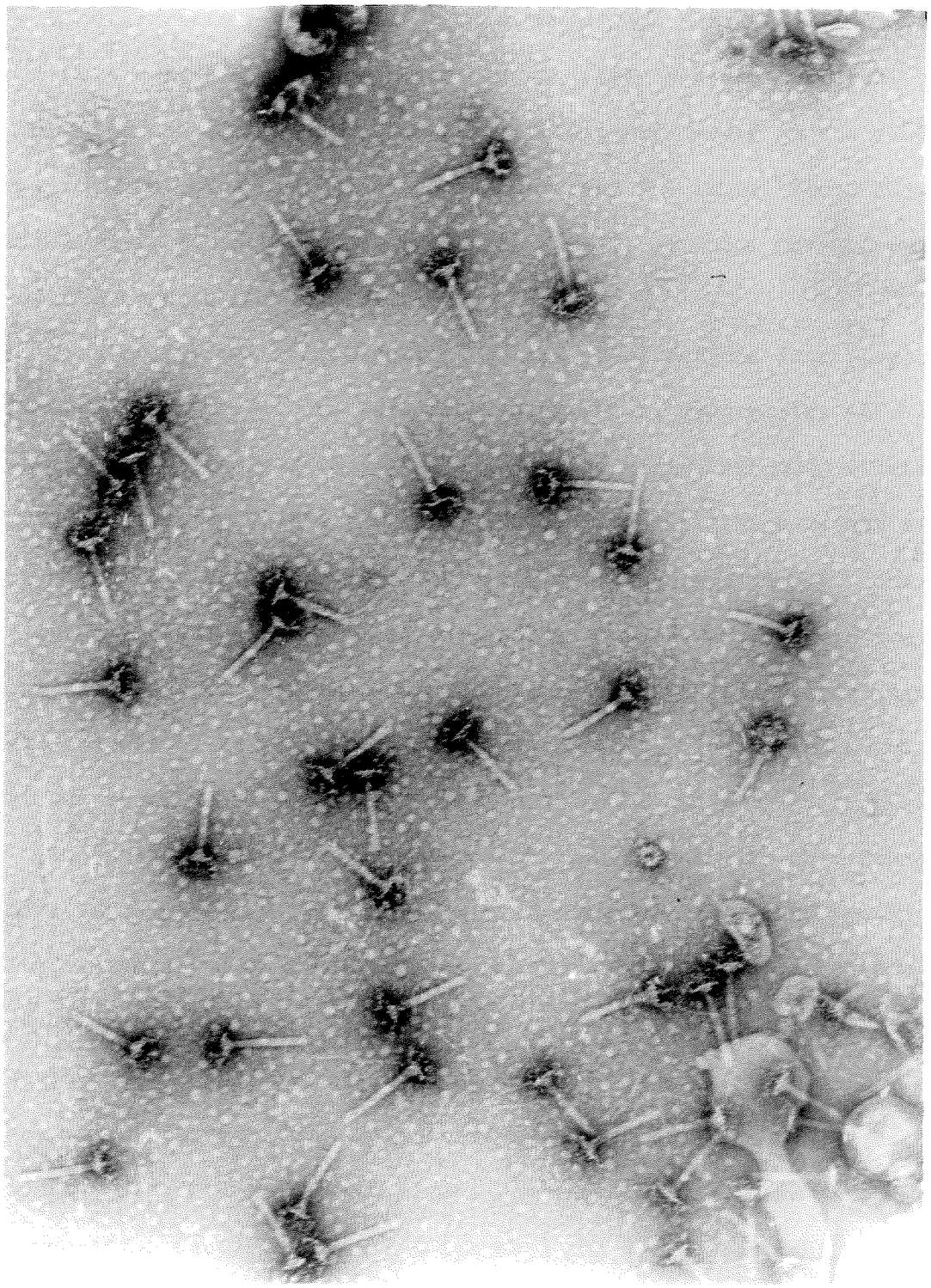


Plate VIII. Naked tails from 80S sucrose gradient fractions of a degraded T4D wild type lysate. Tail fibers can be seen on some of the tails. (x about 120,000).

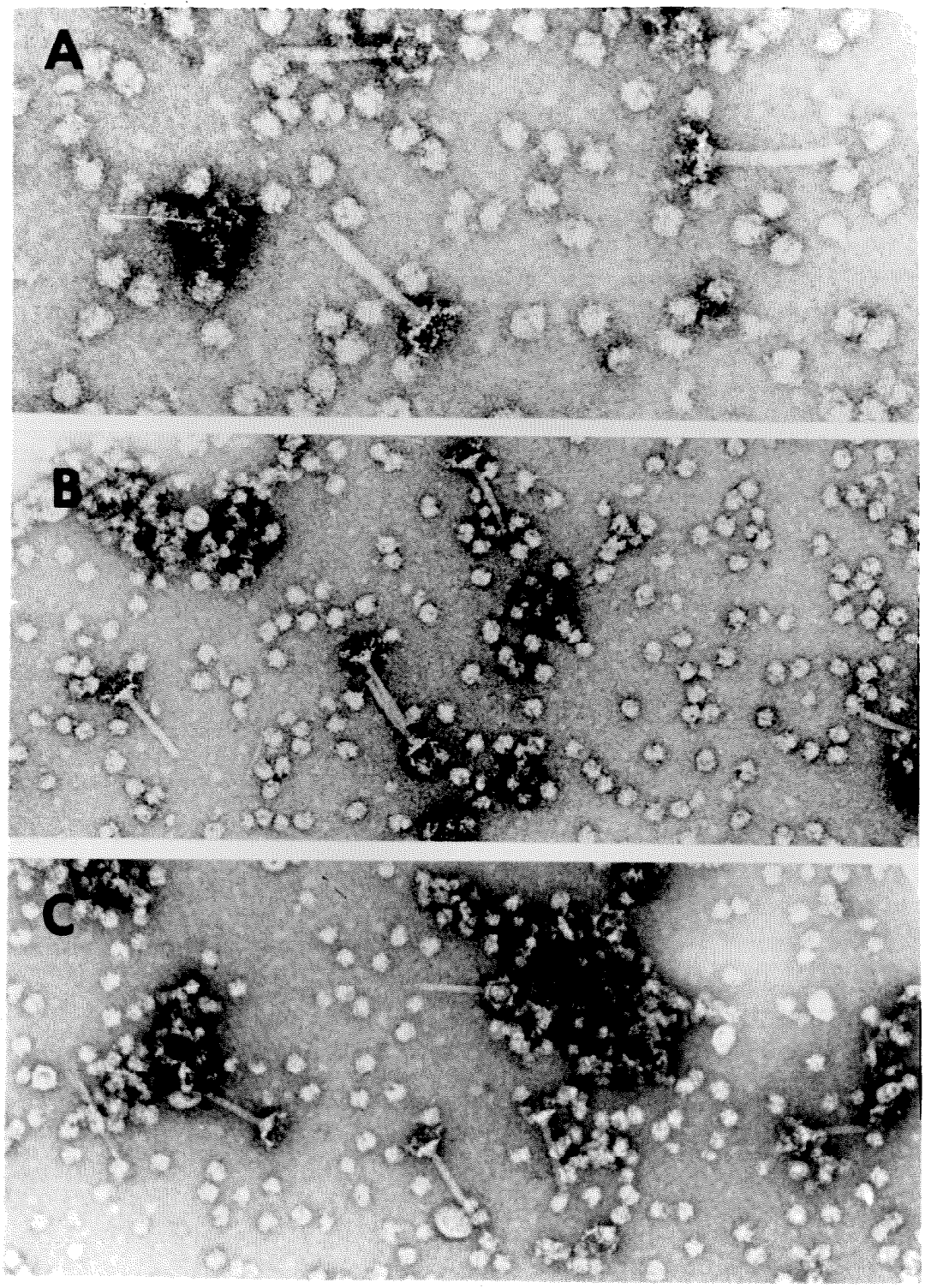


When extracts of cells infected with mutants defective in genes 15 or 18 were centrifuged through sucrose gradients and the fractions examined with the electron microscope, no tails were found in the 130S fractions but naked tails were seen in the 80S fractions (Plate IX). (This experiment was not performed with gene 3; no amber mutants were available in this gene at the time.)

Free baseplates were plentiful in the 80S fractions from sucrose gradients of 19-, 48-, and 54-defective extracts. Baseplates were also present in the 80S fractions of 15- and 18-defective extracts (Plate IX, A and C, top, right of center). However, baseplates were not seen reproducibly on different grids prepared from the same sample. This was in part due to the high concentration of ribosomes in these fractions, and in part to the necessity of the negative stain being just the right thickness for the baseplates to be sufficiently contrasted with the background.

Though the preceding experiments are consistent with the 130S component being sheathed tails and the 80S component in sheath$^-$ lysate being, in part, naked tails, the possibility remains that this correspondence is fortuitous. The antigenic activity might be due, for example, to the presence of ribosome-bound antigen. If the tail interpretation is correct, the sedimentation pattern of sheathed and naked tails assayed for by electron microscopy should be the same as the sedimentation patterns shown in Figures 3 and 4. To test this prediction the following experiment was performed: A 22-defective extract (exhibiting both 130S and 80S antigenic peaks) and a 15-defective extract

Plate IX. Tails from 80S sucrose gradient fractions of 15-defective extract. Free baseplates are also present. (A,C; top, right of center). (A); x about 210,000. (B,C); x about 120,000.

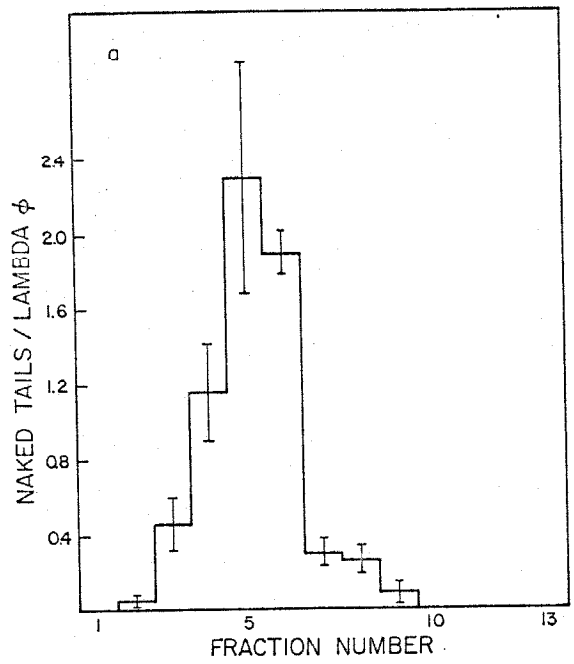

A
B
C

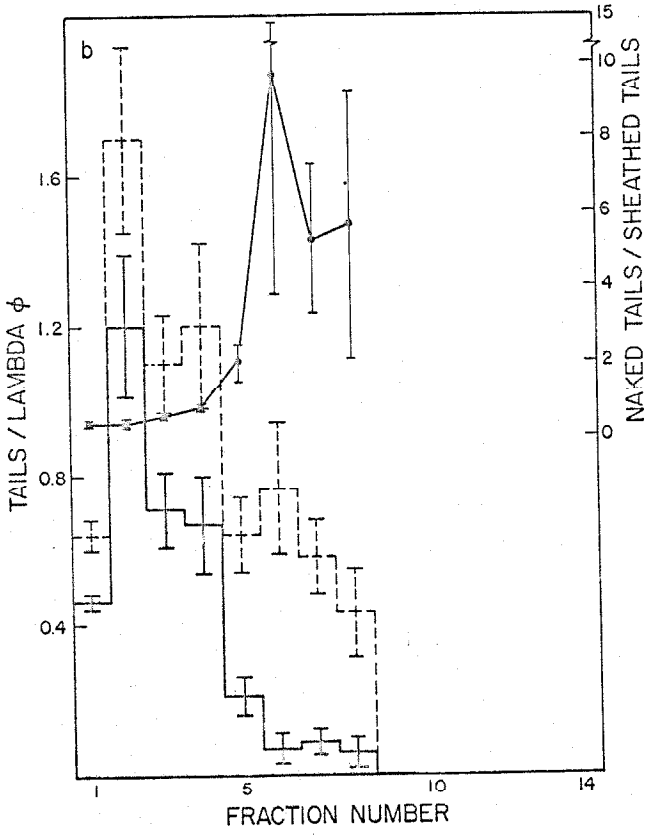
(exhibiting only the 80S antigenic peak) were centrifuged through sucrose gradients. (The extracts were dialyzed for a number of hours in the cold, so that 15$^-$ tails would lose their incomplete sheath.) Following drop collection an aliquot of high titer λ phage was added to each fraction. Specimen grids for electron microscopy were prepared from each fraction and the T4 phage tails and λ phage were counted in electron micrographs of well stained areas of the sample grids. Since the λ phage concentration in each fraction was the same, the number appearing on the specimen grids provided a rough base line for determining phage tail concentration despite the variation in amount of material adhering to the grid surface. The results of this experiment are presented in Figure 5. The tails from the gradient of the 15-defective extract were all naked and sedimented as a single band with sedimentation coefficient of about 80S. The sheathed tails from the 22-defective extract sedimented appropriately faster (~130S) and also as a single band. The correspondence of the morphological tail sedimentation pattern with the antigen sedimentation patterns indicates that the 130S antigen does represent sheathed tails, and the 80S antigen (from sheath$^-$ preparation) represents naked tails (core-baseplates) + free baseplates.

The complete interpretation of the tail sedimentation pattern of the 22-defective extract is complicated by the presence of naked tails in fractions farther down the gradient than 80S. This was probably due to breakdown of the sheathed tails to naked tails during specimen grid preparation and not to the existence of a class of naked tails sedimenting faster than the 15$^-$ tails. However, these naked tails might be the

Figure 5. Determination of tail sedimentation patterns by electron microscopy. (a) 15-defective extract (amN133). (b) 22-defective extract (amB270). Open bars = naked tails. Shaded bars = sheathed tails. Dotted bars = naked tails + sheathed tails. The extracts were thawed at 30°C and centrifuged at 3000 x g for 20 minutes to remove large debris. The supernatants were then dialyzed for three hours in the cold against 1000 volumes of BU/10, 5 x 10^{-4} M in $MgSO_4$. Samples of 0.3 ml. were layered on D_2O gradients (made up in the above buffer) and were centrifuged for 30 minutes at 37,000 rev./min, 20-24°C. Bacteriophage lambda was added to each fraction collected to give a final concentration of about 10^{11} phage/ml. Specimen grids of these fractions were then prepared and electron micrographs taken of well stained fields. The ratios are based on counts of 25-400 lambda bacteriophage per fraction. The increase in the ratio of naked tails to sheathed tails from the 130S region to the 80S region in (b) makes it unlikely that all the naked tails seen were due to breakdown of sheathed tails after fraction collection; a considerable fraction of the naked tails were probably initially present and sedimenting at 80S in keeping with the patterns shown in Fig. 6a and Fig. 9 and the counts in Table 1.

The error bars were calculated by substituting the standard errors of the counts into the formula given on page 498 of Margenau & Murphy (1943).


a
NAKED TAILS / LAMEDA ϕ
2.4
2.0
1.6
1.2
0.8
0.4
1
5
10
13
FRACTION NUMBER


b
TAILS / LAMBDA ϕ
1.6
1.2
0.8
0.4
NAKED TAILS / SHEATHED TAILS
15
10
8
6
4
2
0
1
5
10
14
FRACTION NUMBER

products of breakdown of sheathed tails which occurred during centrifugation.

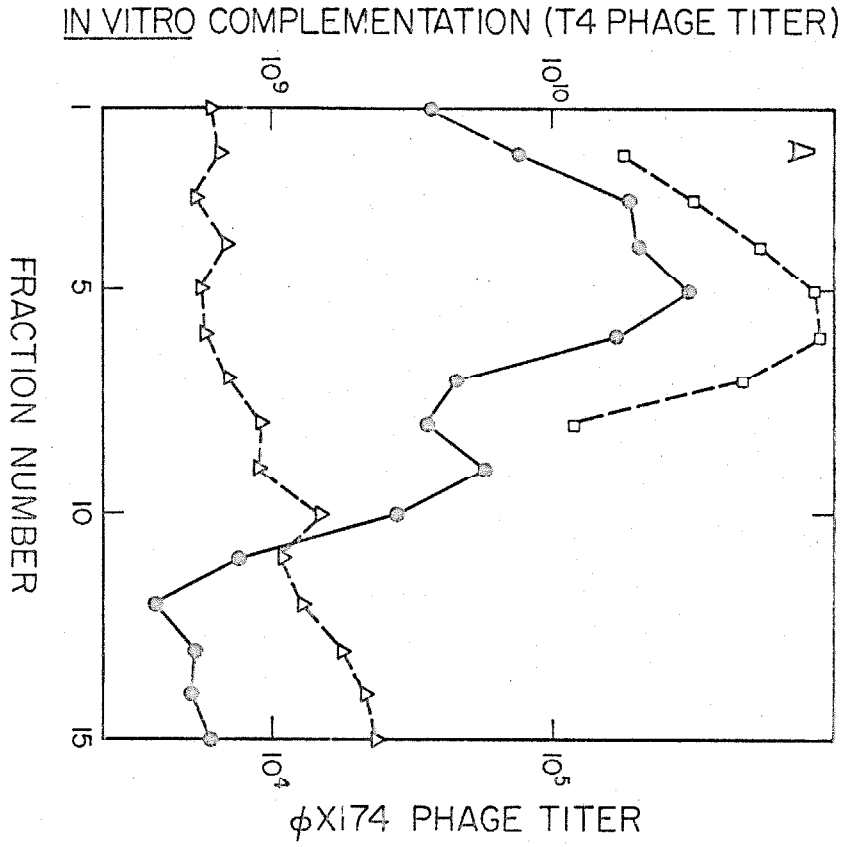
Because of the difficulties mentioned above, I did not attempt to quantitate the baseplate distribution in sucrose gradients using the electron microscope.

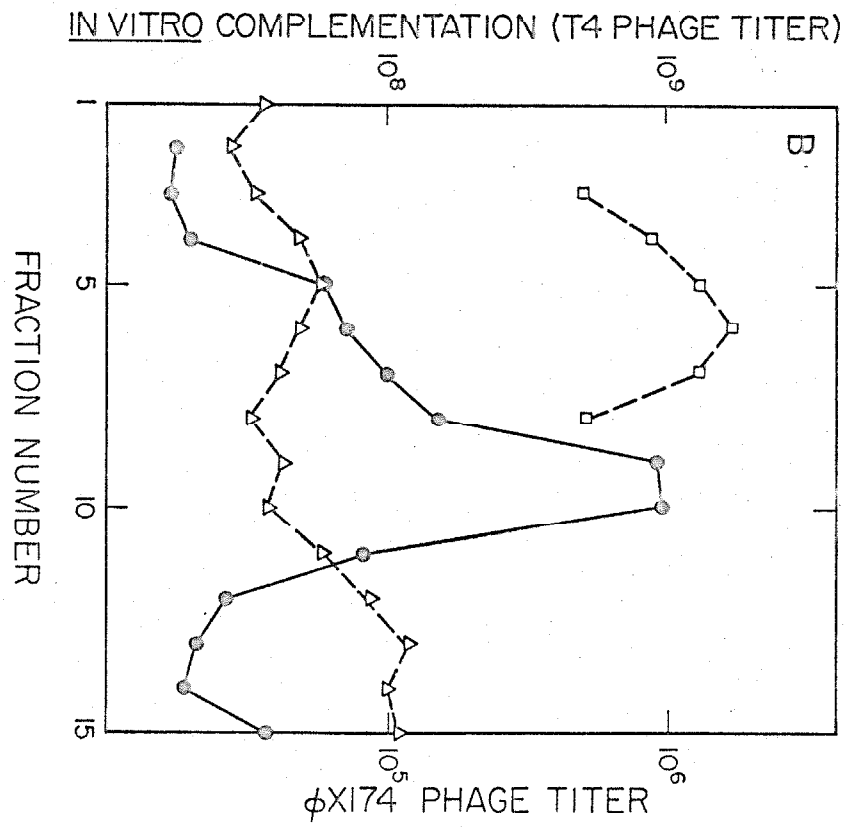
(iii) In vitro complementation activity

Both the sheathed and naked tails isolated by centrifugation from mutant extracts are functional intermediates in phage assembly. This was demonstrated by testing gradient fractions with the in vitro complementation assay of Edgar & Wood (1966). If functional tails were present in these fractions, they should result in the formation of viable phage upon incubation with a $tail^-$ extract (which contains everything required for phage formation but tails). As shown in Figure 6 (filled circles), assay of the gradient fractions for the ability to donate functional tails to a T^- extract gave a series of centrifuge patterns essentially identical with those obtained by electron microscope assay for morphological tails and serological assay for tail antigens. Edgar & Lielausis (1967) characterize in detail the functional stage of the tails accumulating in various mutant infected cells.

Edgar & Wood (1966) did not find complementation in vitro between extracts which are $baseplate^+$ and $baseplate^-$. Tail formation from baseplates apparently requires too many subsequent steps to be efficiently carried out in vitro under the conditions used in these experiments. Recently, however, Edgar & Lielausis (unpublished experiments) have found that if the mutant infected cell extracts are prepared under

Figure 6. In vitro complementation activity of tails from gradient fractions. (a) 17-defective extract (amN56); (b) 18-defective extract (amE18). After thawing, 18- and 17-defective extracts were centrifuged at 2000 x g for 15 minutes to remove large debris. Samples of 0.25 ml. were layered onto sucrose gradients made up in RUM and centrifuged for 70 minutes at 37,000 rev./min, 5°C. Fractions were tested for their ability to complement a 27-defective extract ($tail^-$) or an x4e:27-defective extract ($tail^-$, $tail\ fiber^-$). (The latter test will be discussed in section (c) below.) One drop of fraction was incubated with one drop of test extract for 2½ hours at 30°C. The reaction was stopped by 40-fold dilution with dilution buffer and the tubes subsequently titered for viable phage. Activity of the x4e:27-defective extract was confirmed by incubation with a TF^+T^+ extract. ○ = in vitro complementation activity with $tail^-$ test extract (phage/ml.); Δ = in vitro complementation activity with $tail^-$, $tail\ fiber^-$ test extract (phage/ml.). □ = ØX174.


IN VITRO COMPLEMENTATION (T4 PHAGE TITER)
10^9
10^{10}
A
FRACTION NUMBER
1
5
10
15
10^4
10^5
φX174 PHAGE TITER


IN VITRO COMPLEMENTATION (T4 PHAGE TITER)
10^8
10^9
B
FRACTION NUMBER
1
5
10
15
10^5
10^6
φX174 PHAGE TITER

more favorable conditions and manyfold concentrated compared to the extracts used in these experiments, complementation between baseplate$^+$ and baseplate$^-$ extracts does proceed *in vitro*. Furthermore, the baseplates isolated from 54-defective extracts complemented baseplate$^-$ extracts indicating that these baseplates are functional phage precursors.

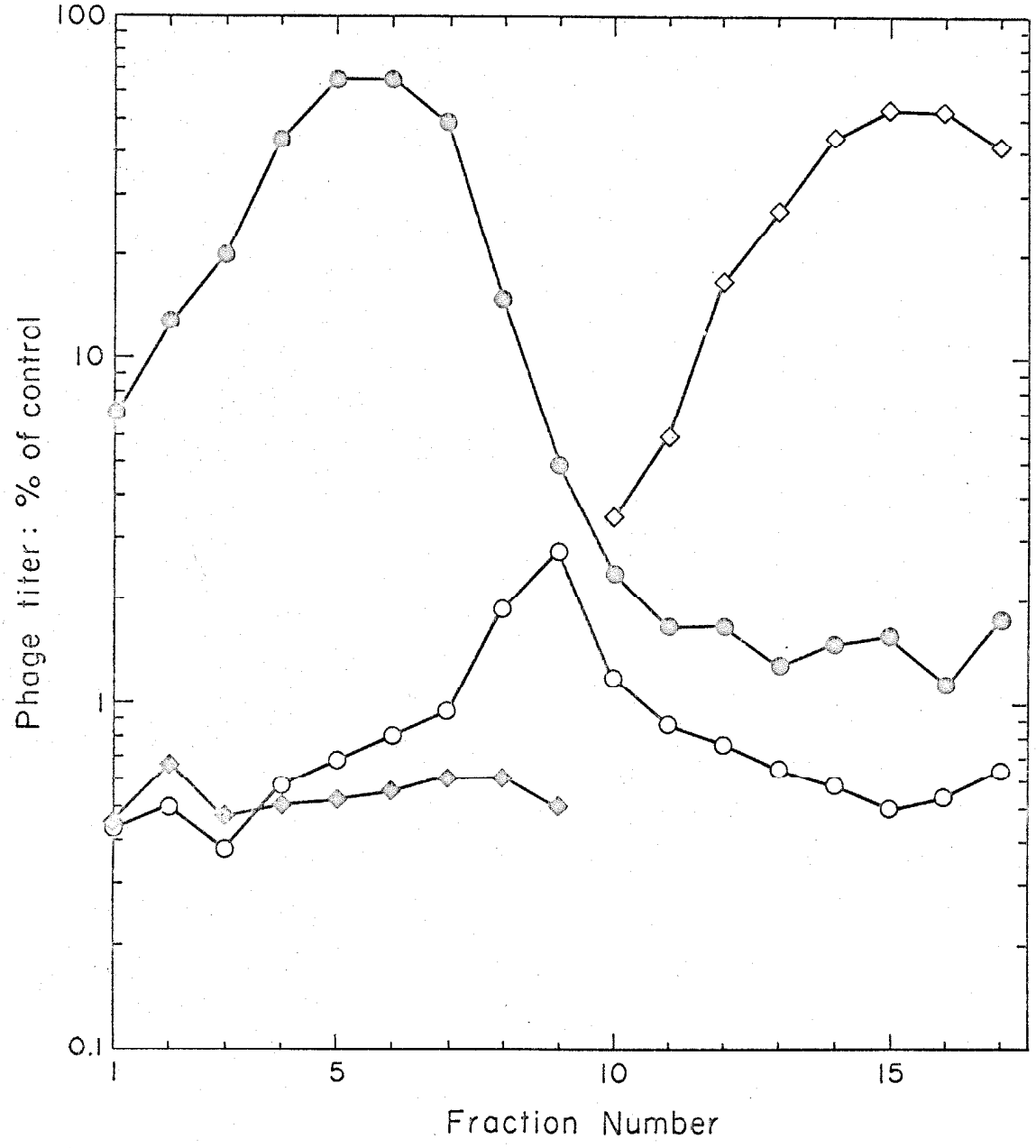
However, on the basis of the above considerations it appears unlikely that phage formation from baseplates occurred in mixtures of extracts prepared as described in Materials and Methods. The subsequent experiments on the functional state of the 80S components therefore reflect the state of the tails, and are not likely to be affected by the possible presence of free baseplates.

(iv) *Partial sequencing of the steps controlled by genes 3, 15, and 18*

The sequencing of steps in the assembly of the bacteriophage by the use of an *in vitro* complementation test is described by Edgar & Lielausis (1967). To sequence steps in tail completion, one isolates tails from the extract - in this case by sedimentation - and determines which gene products have acted upon them prior to isolation, by assay for the ability to complement various mutant extracts. Such an experiment, with the tails from a 3-defective extract, is illustrated in Figure 7. The results indicate that 3$^-$ tails isolated by sedimentation fail to complement both 15- and 18-defective extracts.

The fractions from sucrose gradients of extracts can also be assayed for the presence of gene product alone; the assay for the 15 gene product shown in Figure 7 established that it was present and functional, but was sedimenting independently of the 3$^-$ tails. The

Figure 7. Characterization of 3-defective tails. Immediately after thawing, a 0.15 ml. sample of a 3-defective extract (amNG131) was layered on a sucrose gradient made up in BUM and centrifuged for 63 minutes at 37,000 rev./min, at 5°C. One drop of fraction was added to one drop of tester extract and incubated for 2 hours at 30°C at which time the mixtures were diluted and titered for viable phage. Results are expressed as phage formed in the fraction + tester extract mixture as percentage of phage formed in the uncentrifuged extract + tester extract mixture. The 3^- tails were presumably sheathed at the beginning of centrifugation (note broadness of tail peak), but would have lost their sheath during the course of centrifugation and fraction collection. ○ = total tails (activity of fractions when tested with 27-defective extract); ● = 15^+ tails (activity of fractions when tested with 15:27-defective extract); ◇ = 15 gene product (activity of fractions when tested with 15-defective extract); ◆ = 18^+ tails (activity of fractions when tested with 18:27-defective extract).


100
10
1
0.1
Phage titer: % of control
1
5
10
15
Fraction Number

small peak of activity with the 15:27-defective extract is due to overlap between the trailing edge of the tail distribution and the leading edge of the free 15 product distribution.

The reverse experiments - assaying the tails isolated from 15- and 18-defective extracts with 3-defective extract - were not feasible because of the high background of phage and gene 3 product in 3-defective extracts.

If gene 18 codes for the morphological sheath subunits, the failure of 15^- tails (Edgar & Lielausis, 1967) and 3^- tails to complement 18-defective extract could be due to the loss of the 18 subunits during isolation of the tails, and not to the 18 step being subsequent to or simultaneous with the 3 and 15 steps. One might then predict that 3^- and 15^- tails, if tested immediately after cell lysis, would complement 18-defective extract. This prediction can be tested because the reaction between the 18 product and tails exhibits a very high concentration dependence on the 18 product; since dilution stops the reaction between 18 product and tails, dilution of any extract into an 18:27-defective extract (18^-, $tail^-$) gives an estimate of the number of tails present in the donor extract, at the time of dilution, which have a full complement of gene 18 subunits.

The results of such experiments indicate that 15^- tails do complement 18-defective extracts and that this ability depends critically on the concentration of the extract and on temperature. Thus 15^- tails appear to have a full complement of sheath subunits prior to isolation. Tails from 3-defective extracts did not, however, complement 18-defective

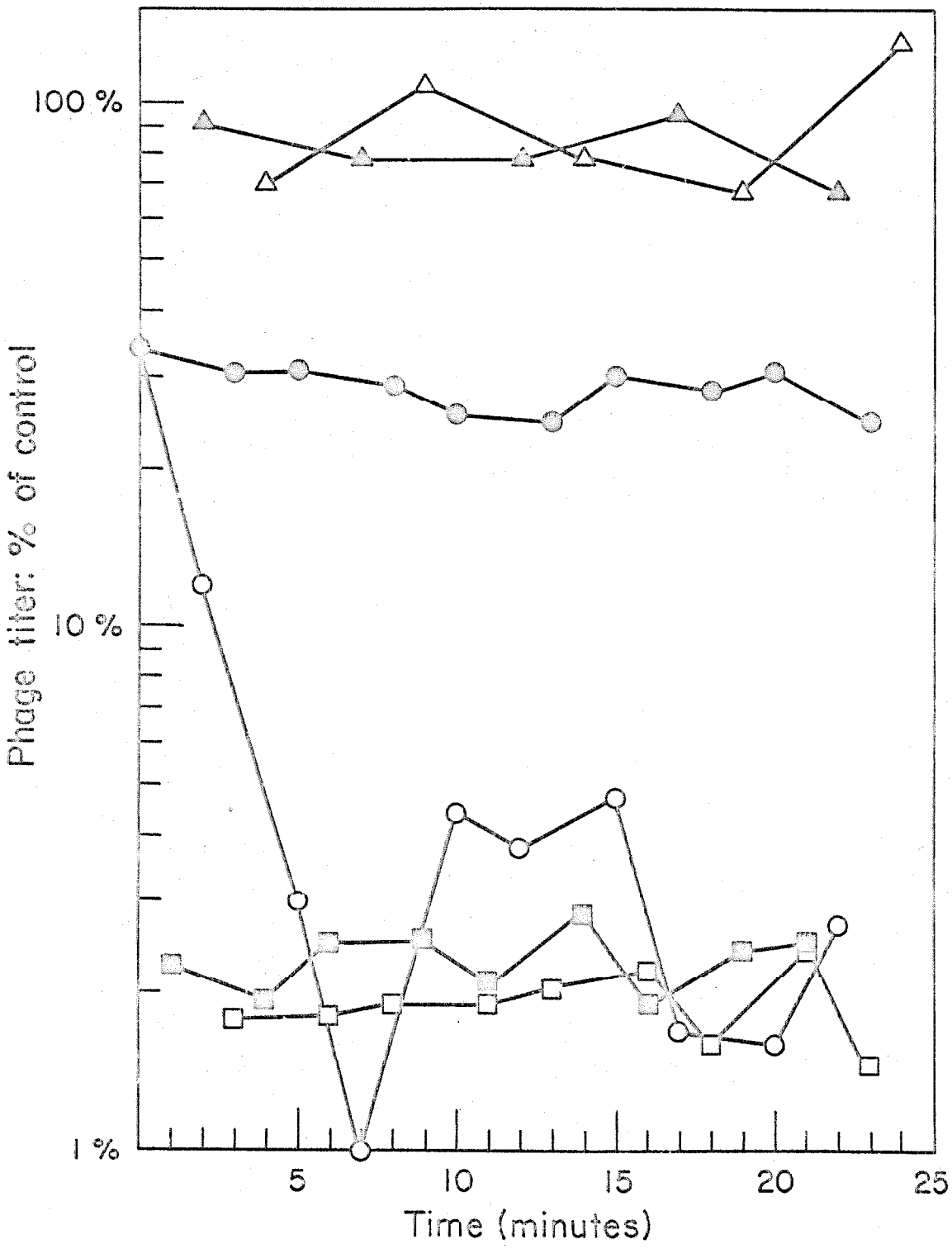
extracts, even at the earliest times tested. Either they have a non-functional form of sheath, or a full complement of sheath subunits which is less stable than the sheath on 15$^-$ tails and is consequently lost too rapidly for detection. Figure 8 presents the results of such a dilution experiment as a function of temperature and time after thawing of the donor extracts. The rate limiting step in the loss of the ability of 15$^-$ tails to complement 18:27-defective extract appears to proceed faster at low temperature. Use of a head$^-$ extract as a source of normal sheathed tails established that the ability of these tails to complement the 18:27-defective extract was stable over the course of the experiment and independent of temperature; dilution of the 3- and 15-defective extracts into 27-defective extract (tail$^-$) (data not shown) established that the number of total tails in these extracts was similar to the head$^-$ control extract and stable during the experiment, and was also independent of temperature.

Because conditions were somewhat different, these experiments were not strictly comparable with the experiment of Figure 1. Direct electron microscopy of extracts was not practicable due to the high concentration of bacterial debris.

In order to unambiguously sequence the 3, 15, and 18 steps, the complementary experiments - diluting 15$^-$ and 18$^-$ tails into 3-defective extract, and diluting 3$^-$ and 18$^-$ tails into 15-defective extract - would have to be performed. However, the high background of 3 product in 3-defective extract prevented the former, and the fact that 15 product is made in great excess prevented the latter (Edgar & Lielausis, 1967).

Figure 8. Differentiation between 3-defective and 15-defective extracts. Samples (0.5 ml.) of 3-, 15- and 23-defective extracts were thawed at 30°C and half of each extract placed in a water-ice bath, the other half remaining at 30°C. Samples were diluted 15-fold into 18:27-defective extract at various times thereafter. The first sample was taken within 4 minutes after placing the extracts at 30°C and titered for viable phage. Incubation of equal volumes of 3-, 15-, or 23-defective extract with 18:27-defective extract yielded $\sim 10^{11}$ phage/ml. This represented a 500-fold increase over background for the 15 + 18:27 and 23 + 18:27 mixtures, and a 20-fold increase over the high background of the 3 + 18:27 mixture. Phage heads are limiting in these control mixtures. Therefore, in calculating the percentage yield, the titer of the 23 + 18:27 mixture was multiplied by 2 to correct for the approximately 2-fold dilution of heads in this mixture (equal volumes of $head^-$ and $head^+$ extracts). The same results were obtained when 18-defective extract was used in place of 18:27-defective extract.

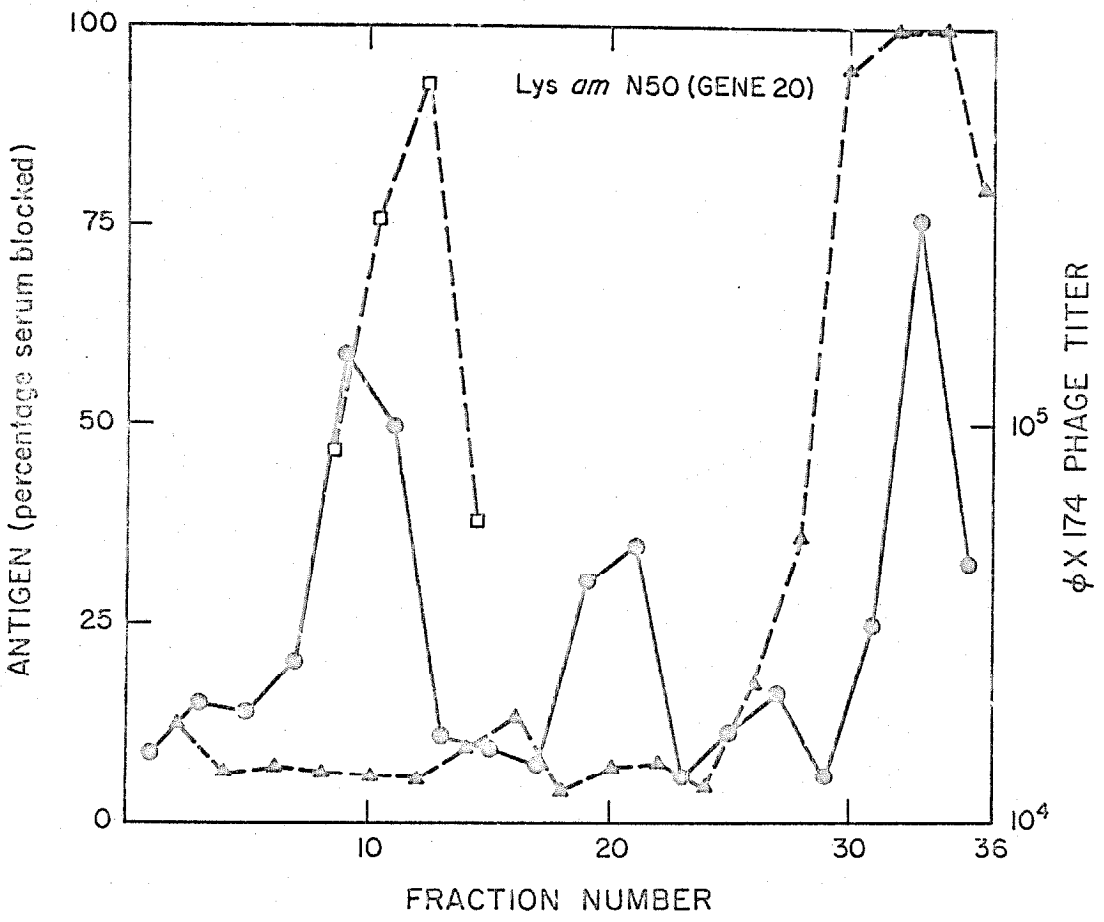
The difference between the level of 18^+ tail activity in the 15- and 23-defective extracts at 30° may have been due to inactivation of the 15-tails as they passed from 0° up to 30° during thawing of the extracts. Note that the absence of phage formation on incubating diluted 3-defective extract with 18:27-defective extract shows that 15-fold dilution of the gene 18 product was sufficient to prevent the formation of viable phage. ▲ = 23-defective extract, 30°C; △ = same extract, 0°C; ● = 15-defective extract, 30°C; ○ = same extract, 0°C; ■ = 3-defective extract, 30°C; □ = same extract, 0°C.


100 %
10 %
1 %
Phage titer: % of control
5
10
15
20
25
Time (minutes)

(c) The substrate for tail fiber attachment

Phage tail fibers are formed independently of the phage head and phage tail (see Part II). Are the tail fibers attached to the tail before or after attachment of the head to the tail? The free tails present in unfractionated lysates did not have visible tail fibers attached to them (Plates I, II, III). Three kinds of experiments demonstrate that tail fibers are not attached to the free tails isolated by velocity centrifugation as described above: 1) Electron micrographs of both sheathed and naked tails from gradient fractions of various mutant extracts reveal few, if any, attached tail fibers (Plates VI, VII, IX); 2) When similar fractions were assayed with antitail-fiber serum for cosedimenting tail fiber antigen (Fig. 9), the results were negative for all mutants tested; the tail fiber antigen was found only in the fractions at the top of the tube where the free fibers and fiber components sediment (Part II, this thesis). These experiments are summarized in Table 4. Since no phage particles are made in these defective lysates, the samples assayed should represent the whole pool of precursor tails and not a selected fraction. Similar experiments performed on lysates containing phage with tail fibers (see Fig. 10 below), establish that the assay is sensitive enough to detect tail fiber antigen if the number of tails with fibers is in the range of the normal burst size of viable phage. 3) Though gradient fractions containing sheathed or naked tails complement in vitro with a T^- extract (containing heads and tail fibers, but lacking tails) they do not complement a 27:34:35:37:38-defective extract (containing heads,

Figure 9. Absence of tail fiber antigen from precursor tails. Experimental conditions were the same as in Figure 3. Alternate fractions were collected into anti-particle serum and S37 serum. Similar experiments on naked tails with fibers, from degraded phage preparations, indicated that they sedimented similarly to the naked tails from mutant lysates. ○ = anti-particle serum blocking activity; △ = S37 serum blocking activity; □ = ØX174.


Lys am N50 (GENE 20)
ANTIGEN (percentage serum blocked)
100
75
50
25
0
10
20
30
36
FRACTION NUMBER
10^5
10^4
φX 174 PHAGE TITER

but lacking tails and tail fibers) as shown in Figure 6 (open triangles) and summarized in the right-hand columns of Table 4 . These results suggest that the isolated tails require a source of tail fibers as well as heads for conversion to active phage. They argue against the possibility that fibers are attached to free tails, but are folded up against the sheath in such a way that their antigenic sites are masked and their presence made difficult to recognize in electron micrographs.

The above findings suggest that tail fiber attachment can occur only after the completed tail has united with the head to form a fiberless particle. I was curious to determine whether further steps subsequent to head-tail union might also be required for tail fiber attachment. If mutants existed in a gene controlling such a step, the expected phenotype would be noninfectious particles, since tail fiberless phage particles do not adsorb to bacteria (Kellenberger et al., 1965)(Fig. 13 below). Mutants of T4 defective in genes 9, 11, or 12 exhibit such a phenotype upon infection of the restrictive host (Epstein et al., 1963). Furthermore, experiments of Jeffrey Flatgaard in this laboratory had suggested that 9^- particles were not complemented in vitro by tail fiber$^-$ extracts. These noninfectious phage particles accumulating in 9-, 11-, and 12-defective lysates were assayed serologically for associated tail fiber antigen after sedimentation through sucrose gradients. The 11^- and 12^- particles had normal levels of tail fiber antigen associated with them, but no tail fiber antigen was detected associated with the 9^-particles (Fig. 10). Tail fiber counts on electron micrographs of the three classes of particles confirmed the absence of tail fibers on 9^- particles and their presence on 11^- and 12^- particles (Fig. 11). As previously observed

Figure 10. Assay of phage particles from 9-, 11-, and 12-defective lysates for associated tail fiber antigens. (a) 9-defective lysate; (b) 11-defective lysate; (c) 12-defective lysate. Experimental conditions as in Figures 3 and 9 but centrifugation for just 5 minutes at speed. Alternate fractions were collected into S34 serum and S37 serum, but one drop every two fractions in the region of the peak was used for viable phage assay. The viable phage used as a marker represent the background phage present in the lysate due to leakage and reversion. Their concentration is too low to be detected by the serological assay. ○ = S34 serum blocking activity; △ = S37 serum blocking activity; □ = viable T4 phage.

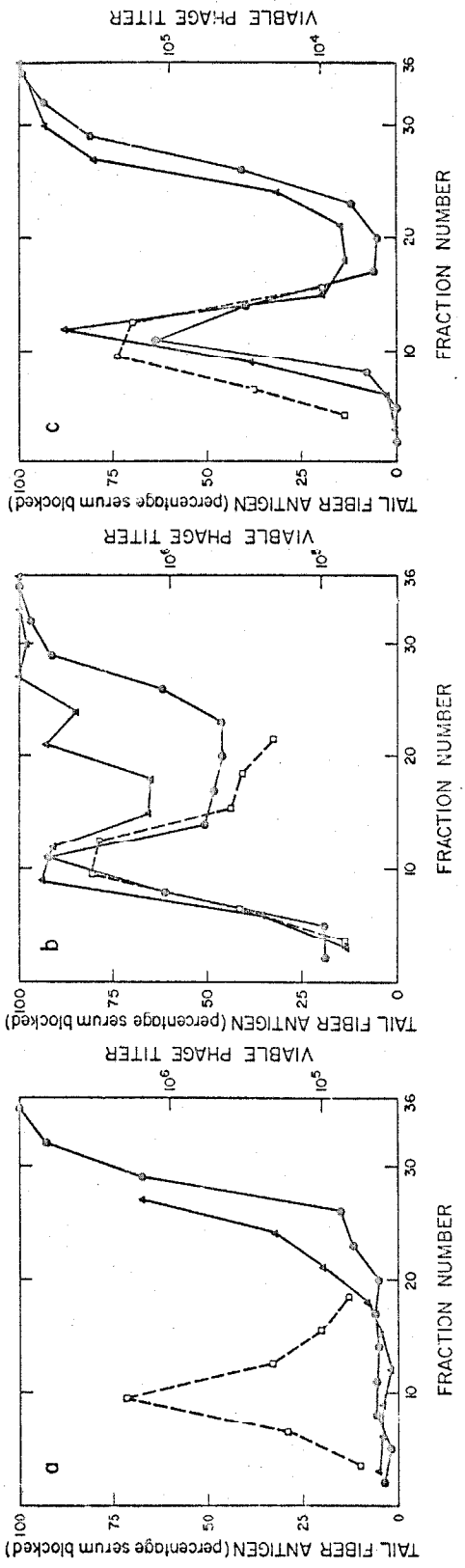
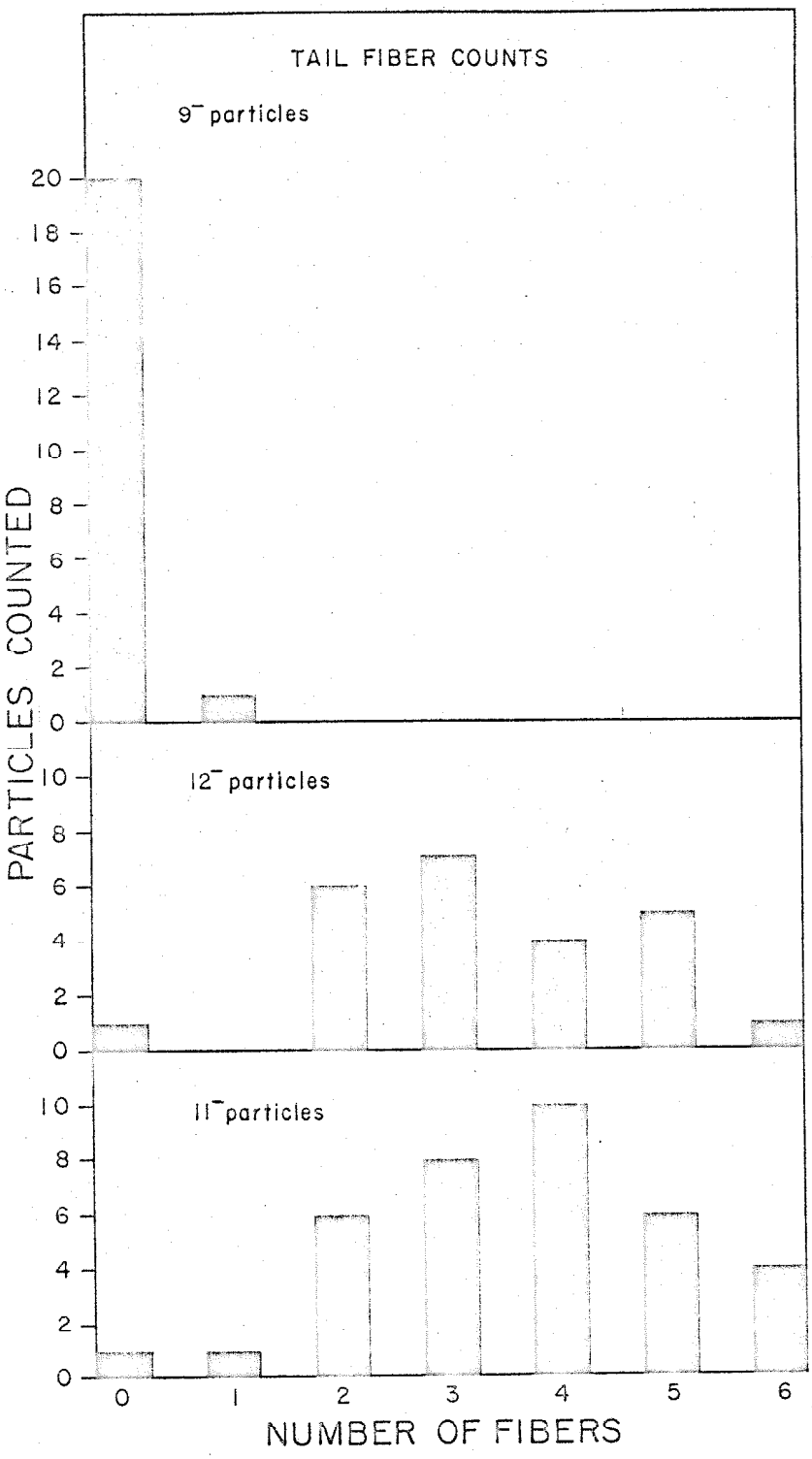

a
b
c
TAIL FIBER ANTIGEN (percentage serum blocked)
VIABLE PHAGE TITER
FRACTION NUMBER
0
25
50
75
100
10
20
30
36
10^6
10^5
10^4

Figure 11. Tail fiber counts on particles from 9-, 11-, and 12-defective extracts. Particles from the above extracts were purified away from bacterial and phage debris by banding in sucrose gradients as in Figure 9. Specimen grids were prepared and the fibers counted on the particles appearing in the resulting electron micrographs. The tail fiber distribution over the 11- and 12-defective particles is essentially identical to the tail fiber distribution found with wild type phage (Kellenberger et al., 1965).


TAIL FIBER COUNTS
9⁻ particles
12⁻ particles
11⁻ particles
PARTICLES COUNTED
NUMBER OF FIBERS
0
1
2
3
4
5
6

(Epstein et al., 1963), many of the 9^- particles exhibited contracted sheaths. The 11^- and 12^- particles were indistinguishable from normal phage.

(d) Properties of the defective gene 11 and 12 particles

In spite of the apparently normal tail fiber complement of the 11^- and 12^- particles, these particles are unable to kill host bacteria, as shown in Figure 12. Further experiments revealed, however, that the 12^- particles, and to a lesser extent the 11^- particles do adsorb to the host. This was initially determined by electron microscopic counts of particles before and after incubation with the host bacteria, but a more sensitive experiment is presented in Figure 13. It makes use of the observation of Edgar & Lielausis (1967) that the 11^- and 12^- particles can be converted to viable phage in vitro by incubation with phage infected cell extracts containing the missing gene products. 11^- and 12^- particles were incubated with host bacteria and at various times samples diluted into 11^+ and 12^+ extract in order to convert the unadsorbed or reversibly adsorbed particles to active phage. (Since the bacteria used for adsorption were restrictive for amber mutants, any particles converted to active phage while still attached to the bacterium would not be able to form a plaque.) Such experiments demonstrate that the 12^- particles adsorb with essentially normal kinetics, while the 11^- particles exhibit a decreased rate of adsorption. These results imply that at least the 12^- particles are defective in some step required for successful infection which is subsequent to initial adsorption.

Figure 12. Absence of bacterial killing by $1\overline{1}$ and $1\overline{2}$ particles. B/5 bacteria growing exponentially were concentrated by centrifugation and resuspended in buffer. Equal volumes of bacteria and lysate were mixed giving a final bacterial concentration of 3×10^9 bacteria/ml. The mixtures were incubated at $37^{\circ}C$ and titered at various times for surviving bacteria. The multiplicity of infection with the rdf41 lysate was about 5. Electron microscopic examination indicated that the multiplicities with the defective particles were about 5-20. Δ = 12-defective lysate (amN69); O = 11-defective lysate (amN93); □ = 34:35:37:38-defective lysate (amx43); Δ = rdf41 lysate.

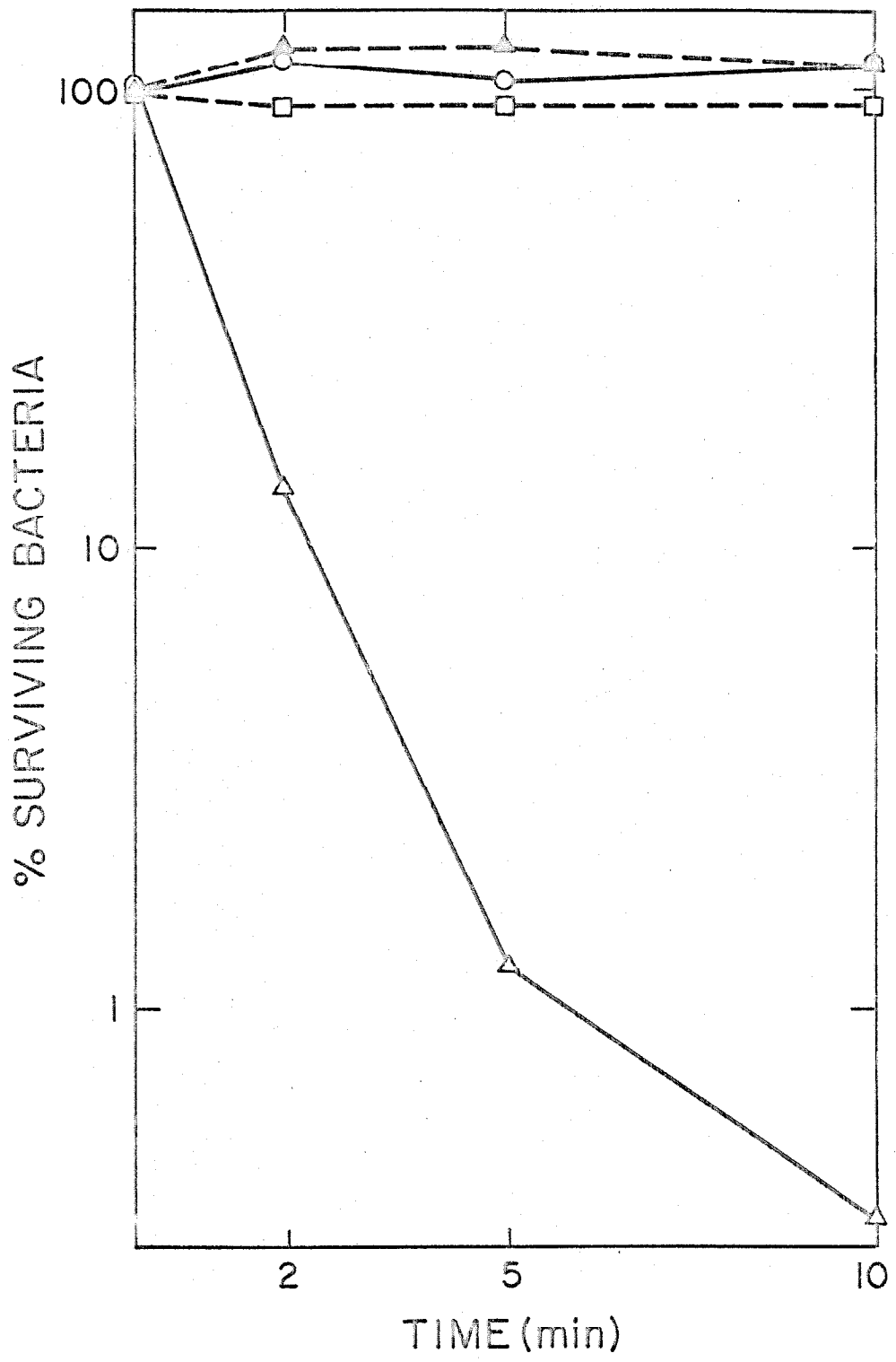
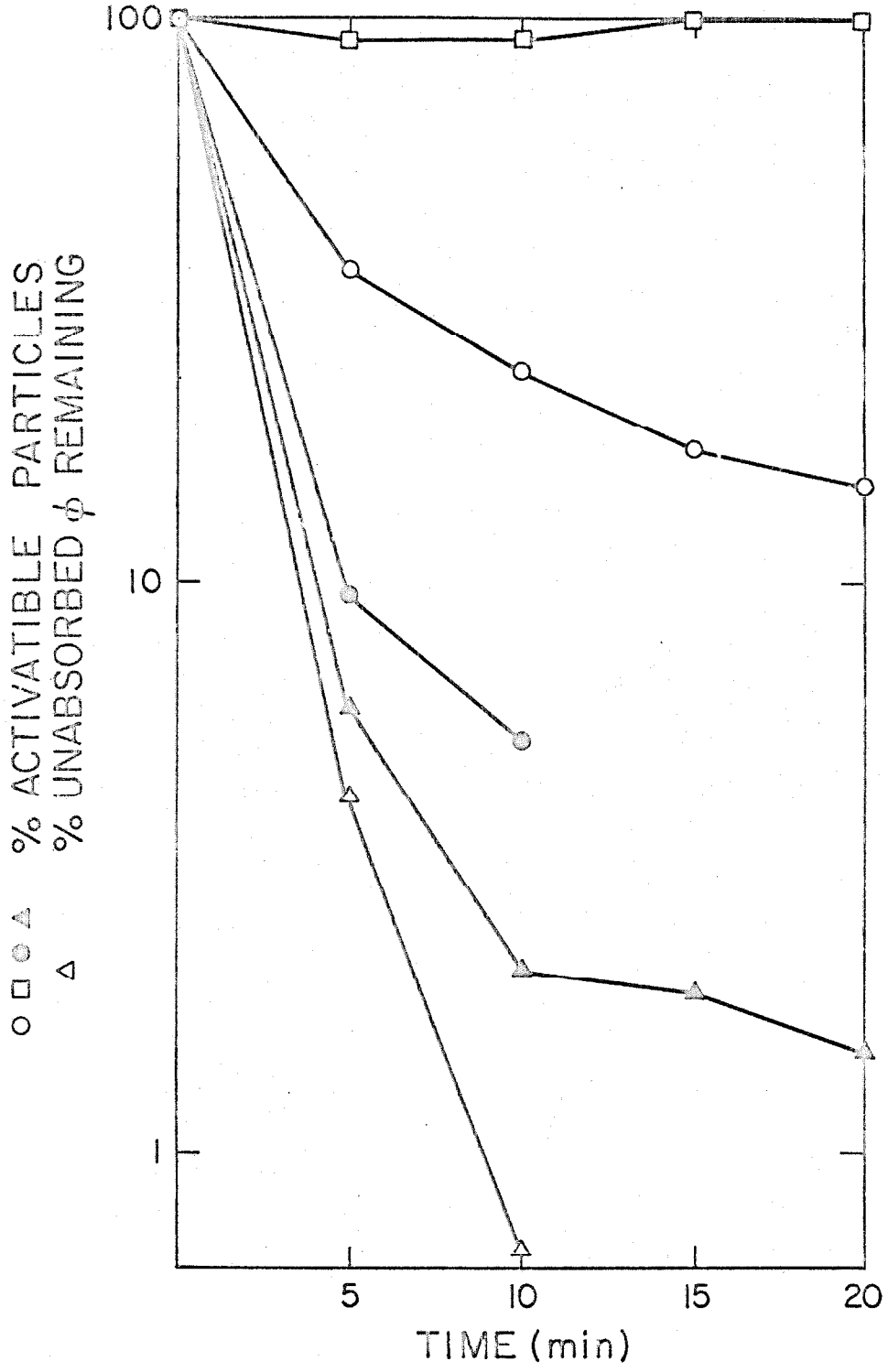

100
10
1
% SURVIVING BACTERIA
2
5
10
TIME (min)

Figure 13. Adsorption of 11^- and 12^- particles. Exponentially growing B/5 bacteria were concentrated by centrifugation and resuspended in H broth to 5 x 10^8 cells/ml. Equal volumes of bacteria and a five-fold dilution of lysate (in H broth) were mixed and incubated at 30°C. At various times adsorption was stopped by 40-fold dilution into 15: rII-defective extract. (The wild type samples were chloroformed before diluting through extract to lyse infected cells.) Since this extract was 11^+ and 12^+, and tail fiber$^+$, any defective particles which had not adsorbed irreversibly would be converted to active phage. These mixtures were incubated for 1½ hours at 30°C and then titered for viable phage using Cr(λ) to prevent the background phage in the extract from forming plaques. Besides diluting through extract, the wild type phage was titered directly for unadsorbed phage. The difference between the wild type curves suggests that about 5% of the wild type population was inactive but could be activated by dilution through extract. □ = tail fiber-particles (amx4e); 0 = 11^- particles (amN93); 0 = wild type diluted through extract; Δ = wild type plated directly; Δ = 12^- particles (amN69).


100
10
1
5
10
15
20
TIME (min)
O □ ◎ △ % ACTIVATIBLE PARTICLES
△ % UNABSORBED ϕ REMAINING

(e) Head maturation

The functions of at least eight genes (20-24, 31, 40, and 66) are required for the formation of the protein coat of the phage head (Kellenberger, 1966). However, the functions of at least ten more genes are required if the heads formed are to be complete and functional (Edgar & Wood, 1966; Edgar & Lielausis, 1967). The role of these genes in the assembly process is unclear, but some of them might be involved in making the head a substrate for tail attachment.

(i) Normal phage heads

Phage heads are plentiful on grids prepared from the various sheath$^-$ and tail$^-$ lysates (Tables 2 and 3). With fresh lysates the majority of these appear intact and full of DNA, and generally sit on the grid surface in the same sense that phage do, with the long axis parallel to the grid (Plates II,B, III,A, and IV,A). The two apices of the long axis were usually indistinguishable; I have not seen structures clearly recognizable as necks or collars attached to the head or free. However, G. Kellenberger & Boy de la Tour (personal communication) have seen heads with clearly defined necks and collars in tail$^-$ preparations. In my experiments they may have been degraded during specimen preparation or obscured by the stain.

(The relative rarity of full heads observed in the studies of Epstein et al. (1963) was probably due to their using lysates which were not fresh; the heads in lysates made in K medium or H broth lose their DNA upon storage.)

(ii) Incomplete phage heads

Cells infected with mutants defective in genes 13 or 14 accumulate incomplete phage heads which can be completed in vitro if the missing gene product is supplied (Edgar & Lielausis, 1967). Cells infected with mutants defective in genes 2, 64, 50, 65, 4, 16, 17, or 49, accumulate defective heads which cannot be made functional in vitro (Edgar & Wood, 1966). The defective phenotypes of all these mutants were originally reported as unconnected heads and tails (Epstein et al., 1963). However, as shown in Table 5, the phenotype of 2-defective lysates made with two different amber mutants appears to be noninfectious but normal appearing particles. These results suggest that gene 2, and, as shown below, some of the others listed above, are concerned with aspects of head maturation other than the attachment of the tails.

Recently Minagawa (personal communication) has found that the DNA concatenates (Frankel, 1967) in cells infected with mutants defective in genes 16, 17, and 49 do not give rise to phage-size pieces of DNA. Following his lead I examined lysates made with these mutants to determine if the heads have DNA in them. In accord with Minagawa's results, the heads from 16, 17, and 49-defective lysates were almost all empty (Table 5 and Plate IV,C), whereas the majority of the heads from tail⁻ control lysates were full. These mutants were also examined by preparing specimen grids of the mutant infected cells before lysis. In such preparations one finds among the unlysed population occasional cells which have lysed on the grid. With 50- and 65-defective lysates, and

Legend for Table 5

Lysates are listed in the order in which the grids were prepared. At 75 minutes after initial infection (30°) 0.1 ml. of chloroform was blown into superinfected lysates prepared in K medium. After addition of chloroform aeration was continued for 1-2 minutes, at which time samples were applied to specimen grids and stained with saturated uranyl acetate. Major classes of structures observed are given. Where not listed, less than two polysheaths and less than five empty or full headed phage particles were observed. Small numbers of baseplates were present in all the $tail^{+}$ lysates. The number of viable phage in the 2-defective lysate was 4×10^{7} phage/ml. The number of particles seen was roughly equivalent to a titer of 10^{11} ml. A similar result was obtained with amN140 (gene 2).

TABLE 5

Electron microscopy of lysates containing defective or incomplete heads

Lysate							
	Defective gene	*am* Mutant	% Full heads	(Total heads counted)	% Sheathed tails	(Total tails counted)	Other
Control	54	H21	78	(142)			69 baseplates, 22 stretches of polysheath
	16	N66	1.6	(123)	80	(158)	
	17	N56	0	(138)	48	(70)	
	49	E727	0	(114)	61	(144)	41 empty-headed phage particles
	2	N51	80	(70)	83	(90)	134 normal appearing phage particles
	64	E1102	70	(178)	84	(198)	
	50	A458	45	(49)	76	(155)	
	65	E348	46	(76)	87	(156)	
	4	N112	38	(88)	84	(104)	
	13	E609	17	(168)	65	(117)	
	14	B20	23	(229)	66	(125)	
Control	29	B7	90	(125)			34 stretches of polysheath

with a 25-defective control lysate, such lysed cells were surrounded by full heads, whereas with 16, 17, and 49-defective lysates these cells were surrounded by empty heads.

Though full heads were present in the five other head-defective lysates, the results of Table 5 and similar experiments indicate that the 65^-, 50^-, 64^-, and 4^- heads were less stable than the heads from $tail^-$ lysates and lost their DNA either prior to or during grid preparation much more readily. This also appeared to be true for heads from 13- and 14-defective lysates. Since under the conditions of these experiments necks and collars were not clearly visible on normal heads (from $tail^-$ lysates), it was not possible to determine if the incomplete heads lacked these structures.

(f) Independence of the pathways for head and tail formation

In the course of the experiments reported above, the defective phenotypes resulting from mutations in all of the known morphogenetic genes have been reexamined. The centrifugation experiments on phage tails are summarized in Table 4. Lysates of all mutants not characterized by centrifugation were examined directly in the electron microscope for the presence of sheathed tails. These data are summarized in Figure 15. All mutant preparations lacking phage heads, or with nonfunctional phage heads, contained sheathed tails. These sheathed tails were indistinguishable from each other by any of the criteria used. All the results indicate that the formation of the sheathed tail proceeds independently of the formation of the head, and that the tails accumu-

lating in cells infected with all mutants affecting head formation are complete and equivalent.

Edgar & Wood (1966) demonstrated that the heads accumulating in tail$^-$ and sheath$^-$ extracts were precursors to viable phage. The heads from all tail$^-$ and sheath$^-$ lysates (Tables 2 and 3, summarized in Fig. 15) were full and morphologically indistinguishable in the electron microscope. These observations are consistent with the hypothesis that formation of complete, functional phage heads proceeds independently of the formation of the phage tail, and that the heads accumulating in cells infected with all mutants affecting tail formation are complete and equivalent.

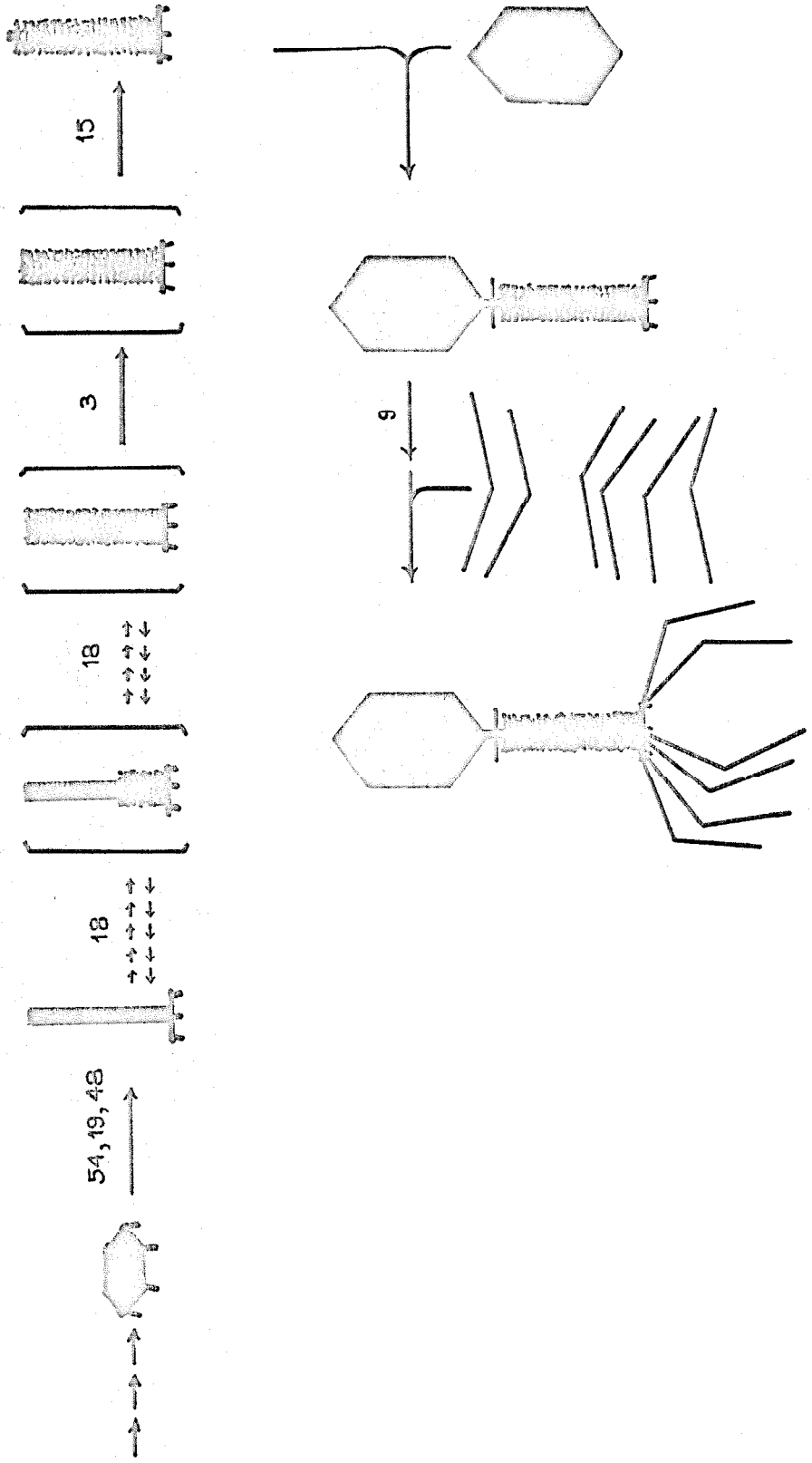
DISCUSSION

Tail Assembly

The picture of phage tail assembly emerging from these experiments is summarized in Figure 14. The baseplate is completed first, and the core forms only on the completed baseplate. This core-baseplate structure, the naked tail, provides a scaffolding for the polymerization of the gene 18 product, a major sheath structural subunit. The products of genes 3 and 15 are then required for the stabilizing and finishing of the sheath, and for the formation of the connector. The completed head attaches to the connector, and, after the gene 9 product has acted, the tail fibers are attached. All these steps appear to be strictly sequenced. These conclusions will now be discussed in further detail.

The presence of free baseplates in 19-, 48- and 54-defective lysates in numbers equivalent to a normal burst of phage suggests that these structures are intermediates in phage assembly. Since baseplates were not detected with the electron microscope or serologically (where tested) in lysates of mutants defective in genes 53, 5, 6, 7, 8, 10, 25, 26, 51, 27, 28, or 29, the functions of these genes are presumably required for baseplate formation. The baseplates found in 19-, 48-, and 54-defective lysates were regular hexagons with a plug in the center and with a barely visible internal ring also exhibiting hexagonal symmetry. The apices of the outer hexagon were frequently prominent and may represent the spikes of the baseplate. These baseplates were

Figure 14. Tentative pathway for the assembly of the phage tail. Numbers over the arrows refer to the genes controlling that step. Brackets around a structure indicate an intermediate unstable outside the cell. See text for further discussion.


54,19,48
18
18
3
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indistinguishable from the structure shown by Bradley (1965, Fig. 23), who identified it as a phage collar. However, the studies by Simon & Anderson (1967b), and those reported here indicate that the hexagons are baseplates and not collars.

If cores were formed independently of baseplates and subsequently joined to them, free cores should be present in baseplate⁻ lysates. These would be expected to be identifiable as such, since the cores isolated from disrupted phage are stable structures and are recognizable in the electron microscope (Brenner et al., 1959; Sarkar, Sarkar & Kozloff, 1964b). Their absence from lysates suggests that the free core is not an intermediate in phage assembly but is formed only on the baseplate. (Epstein et al. (1963) reported seeing cores in a lysate of amB274. These structures were many times the length of normal cores (R.S. Edgar, personal communication) and therefore might have been bacterial fimbriae. Such structures were not seen in the lysate of amB274 examined in these experiments.)

Genes 19, 48, and 54 may be directly involved in core formation, or with steps in the completion of the baseplate required prior to core formation. Though the baseplates from 19-, 48-, and 54-defective lysates were morphologically similar, experiments of Edgar & Lielausis (unpublished) indicate that baseplates isolated by sedimentation from 54-defective extracts are functional in vitro, while baseplates isolated from 48-defective extracts are not.

The absence of normal extended or contracted sheaths from tail⁻ lysates, including those which accumulate baseplates, indicates that

the sheath is not formed unless complete naked tails are present. The high concentration of polysheath in $tail^-$ lysates relative to $tail^+$ lysates supports this conclusion; in the absence of the substrate for normal sheath polymerization the concentration of free subunits builds up until the relatively unfavored aggregation into polysheath occurs.

The absence of both normal sheath and polysheath from 18-defective lysates suggests that gene 18 codes for a major structural subunit of the sheath. In mixed infection experiments between T4 wild type and gene 18 amber mutants (J. King, unpublished), the burst size decreased proportionally to the wild type/mutant input ratio, indicating that gene 18 codes for a structural protein which is limiting in phage formation. Furthermore, as noted in Results, the reaction between tails and 18 product is very sensitive to dilution of the 18 product (Edgar & Lielausis, 1967, and Fig. 8) suggesting that a large number of 18 gene products must interact with the tail for successful phage assembly.

The studies of Moody (1967a,b) indicate that two morphological subunits are present in the sheath, but might represent two parts of a single polypeptide chain. At present the question of whether the sheath is initially formed of one or two species of structural subunit remains open.

The specific functions of genes 3 and 15 in sheath formation are unclear. However, the appearance of the 3^- and 15^- tails just released from infected cells, and the observation that having lost their sheath

these tails remain functional intermediates in phage formation, suggest a model of sheath formation (illustrated in Fig. 14) consistent with all the experimental observations: Within T4 infected cells there is an equilibrium between the free sheath subunits and the polymerized form on the tail. This equilibrium is such that within the cell essentially all of the tails present have a full complement of sheath subunits. The 3, and then the 15 gene products act on these tails to fix the sheath in the polymerized form. The action of the 3 product alone is not sufficient to completely stabilize the sheath, but does shift the equilibrium further toward the polymerized form. However, upon sufficient dilution of the pool of free subunits, both $3^{+}15^{-}$ and $3^{-}15^{-}$ tails lose their sheath and revert to naked tails ($18^{-}15^{-}3^{-}$). The interactions which partially stabilize $3^{+}15^{-}$ tails are presumably hydrophobic in nature, accounting for the decreased stability of these tails at low temperature (Kauzmann, 1959).

The 3 and 15 gene products might be analogous to lockwashers and nuts, fastening the sheath to the end of the core, or alternately the 3 gene product might interact with each of the sheath subunits individually. This cannot be the case with the 15 product since Edgar (unpublished experiments) has found that tail formation <u>in vitro</u> is first order with respect to the concentration of 15 product, and the 15 product, though present in large excess, is used up in the reaction. Temperature sensitive mutants of gene 15 exhibit intragenic complementation (J. King, unpublished experiments) indicating that the 15 gene product is composed of at least two polypeptide chains.

The defective phenotype of all three sheath mutants is unconnected heads and tails. From electron micrographs of complete phage it is clear that the sheath does not extend all the way to the collar but that a stretch of core, which I have termed the connector, intervenes. If this structure exists prior to sheath formation it is difficult to understand why these tails are not connected to heads in the absence of sheath formation. Furthermore, if the connector is just the terminal end of the core and not a separate structure, one clearly must postulate a singularity in the core 9/10ths of the way up from the baseplate, since at this point the sheath terminates, and firmly attaches to the core. During sheath contraction the majority of the subunits slide along the core - it is only the end of the sheath that remains fixed to the core. The existence of a separate connector explains these phenomena; the core determines the length of the sheath, its end signaling the end of the sheath. After the full complement of sheath subunits has polymerized the connector is formed, clearly providing for a singularity at the joint. If the sheath is not completed, the connector cannot be put on, thus preventing attachment of the head. The measurements given in Figure 2 are compatible with this interpretation. Genes 3 and 15 would then be involved in the finishing of the sheath-core joint prior to connector attachment, or might be involved in the formation of the connector itself.

Tail fiber attachment

The experiments reported above establish that the tail is not a substrate for tail fiber attachment until it has joined with the head

and been acted upon by the gene 9 product. Experiments of J. Flatgaard (personal communication) indicate that the gene 9 product acts subsequently to head-tail union. Why the tail must be incorporated into the whole phage particle before it can be a substrate for fiber attachment is not clear. Perhaps attachment of the head induces an allosteric change in the tail which is a prerequisite for fiber attachment, or perhaps the whole phage must serve as a jig for the fiber alignment.

The absence of tail fibers from free tails explains the finding of Kellenberger & Sechaud (1957) that free T4 tails (which they called rods) did not adsorb to bacteria.

Functions of genes 11 and 12

Edgar & Lielausis (1967) show that the gene 11 and 12 products act on the tail of the phage, and probably on the baseplate (unpublished experiments). Both the lack of infectivity of the 11^- and 12^- particles and their inability to kill the host bacteria suggest that they are unable to inject their DNA. The observation of Edgar & Lielausis (1967) that the 11 and 12 gene products can activate the defective phage particles formed in their absence suggests that these products act on the surface of the baseplate. Recently Simon & Anderson (1967a,b) have demonstrated that the attachment of T4 to bacteria is a complex stepwise process; the 11 and 12 gene products may be involved in intermediate steps in this attachment process.

Head formation

The results of Minegawa and those reported above suggest that the functions of genes 16, 17, and 49 are necessary for the cutting out or packaging of phage of phage size pieces of DNA. The products of these three genes might affect the structure of the head, or might interact with the phage DNA directly, independently of the head membrane. Piechowski and Susman (1967) have presented evidence that mutations of T4 which permit phage maturation in the presence of 9-aminoacridine map in gene 17.

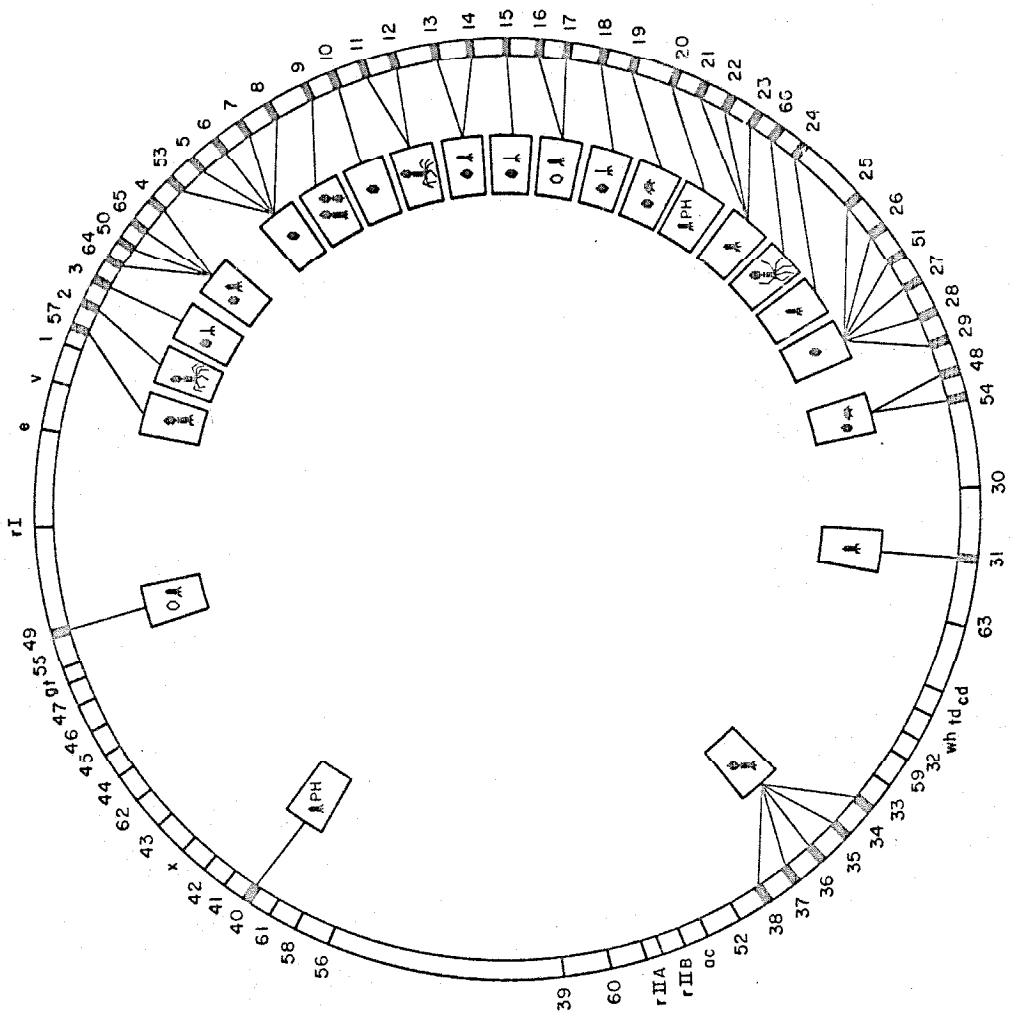
The results of Edgar and Lielausis (1967) and those reported in Part I and Part II of this thesis indicate that phage assembly is rather strictly sequenced,so that in the presence of a mutational block in the pathway, the last intermediate before the blocked step accumulates. There is no a priori reason to think that head assembly differs from this pattern. Therefore the accumulation in cells infected with mutants of gene 16,17, and 49, of normal appearing but empty phage heads indicates that these structures are normal intermediates in phage assembly. This implies that the head is not formed on a DNA condensate, but rather the heads coil the DNA into them, as with a spinning reel and spinning line.

Phage morphogenesis

Sufficient knowledge of T4 morphogenesis has now accumulated to permit two generalizations: 1) Phage assembly is a branched assembly line process; the head, tail, and tail fibers are formed independently of each other. 2) The steps within each subassembly pathway, and the

joining of the finished products appear to be strictly sequenced. This sequencing is not due to the temporal ordering of gene product formation; the experiments of Hosada and Levinthal (personal communication) indicate that late proteins of T4 are made simultaneously. Therefore the sequencing of the steps must be inherent in the structure of the proteins and in the changes they undergo during successive interactions in the assembly process.

Figure 15. Defective phenotypes and map location of the morphogenetic genes of T4. This diagram summarizes previous work, primarily of Edgar, Epstein, and their coworkers, and includes the results reported here. Genes affecting phage assembly are shown as solid bars. Map distances are drawn in accordance with the results of Mosig (1966 and unpublished). The boxes show the major structure formed upon infection of E. coli with conditional lethal mutants of the various T4 genes, under restrictive conditions. (Thus, in most cases, the function of a particular gene is necessary for the formation of the structure absent from the box.) For clarity, tail fibers are in general not shown. Genes 34-38, and 57 are required for tail fiber formation. The defective phenotypes of the early genes can be found in Edgar & Wood (1966). Gene 66 mutants result in the formation of phage particles with small heads. PH refers to polyhead. Further details on the defective phenotypes of genes controlling head formation are given by Kellenberger (1966).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P A R T II

Tail Fiber Assembly

INTRODUCTION

Six long slender tail fibers are a conspicuous feature of the T-even bacteriophages. These fibers are required for the adsorption of the phage to its host bacterium (Kellenberger et al., 1965) and determine host range specificity (Williams and Fraser, 1956; Wildy and Anderson, 1964). The functions of at least six phage genes are required for the formation of the tail fibers (Epstein et al., 1963; Edgar and Lielausis, 1965; Russell, 1967); five of these genes (genes 34, 35, 36, 37 and 38) are located in a cluster on the genetic map and the sixth (gene 57) is unlinked (Fig. 15). Lysates made with T4 mutants defective in these genes contain inactive particles which lack tail fibers, but are otherwise complete (Epstein et al., 1963; Edgar and Wood, 1966; Eiserling, Bolle, and Epstein, 1967).

Tail fibers can exist in at least two configurations relative to the phage particle; extended, or folded up against the phage sheath, in which case the phage is unable to adsorb (Kellenberger et al., 1965). Cofactor requiring mutants, described early in phage research (Anderson, 1948; Wollman and Stent, 1950), require tryptophan for fiber extension; some of these mutants map in one of the structural genes (gene 34) for the tail fiber (Edgar, Denhardt, and Epstein, 1964; Herskowitz, unpublished results). Recent experiments of Simon and Anderson (1967) suggest that the adsorption of the fibers to the bacterial surface triggers configurational changes of the baseplate which are required for subsequent steps in the attachment and injection process. The tail fibers

thus appear to be functionally complex organelles.

The neutralizing activity of rabbit anti-T4 serum is directed primarily against phage tail fibers (Franklin, 1961). Edgar and Lielausis (1965), exploiting this fact, adsorbed anti-T4 serum with defective lysates made from cells infected with mutants of the tail fiber genes; by characterizing the residual neutralizing activity remaining after absorption of the whole serum with a given mutant lysate, they were able to identify three phage antigens, and to relate their synthesis to the function of the six genes required for tail fiber formation. All three antigens appear in the completed phage particle. The results of Edgar and Lielausis (1965) are summarized in Table 6. Antigen A appears to be under the control of gene 34. Antigens B and C appear to be under multigenic control; the production of antigen C required the functions of genes 37 and 38 (though mutants defective in gene 38 do make low levels of C and B antigen). The function of a third gene, 36, is required for the production of antigen B. Mutations in gene 35 do not affect the levels of any of the antigens. Amber mutations at the single site which defines gene 57 result in the production of low levels of all three antigens in the mutant infected cells; this gene was tentatively assigned a regulator role by Edgar and Lielausis (1965). Since there is little, if any, non-nucleic acid associated carbohydrate in the T-even bacteriophages (Taylor, 1946), these antigens probably represent structural proteins of the phage.

Though no fine structure has been reported in electron micrographs of tail fibers, Brenner et al. (1959) found that fingerprints of purified

TABLE 6

Antigens present in lysates made with mutants of the tail fiber genes

Defective gene	Antigens		
	A	B	C
rII	+	+	+
34	0	+	+
35	+	+	+
36	+	0	+
37	+	0	0
38	+	$\cong$ 10%	$\cong$ 10%
57	$\cong$ 10%	$\cong$ 10%	$\cong$ 10%

This table summarizes results of Edgar and Lielausis (1965). + = antigen levels comparable to the rII control. 0 = no detectable antigen. Intermediate levels are given as % of rII control.

tail fibers were exceedingly complex. Thus the results of both peptide and antigen analysis indicate that phage tail fibers are not only functionally, but also structurally complex organelles.

In the experiments reported below, the tail fiber antigens from cells infected with mutants blocked in fiber assembly have been characterized by centrifugation. These experiments indicate that such antigens represent structural intermediates in tail fiber assembly. Furthermore, these intermediates have been shown to be functional in phage assembly by the criterion of the in vitro complementation test developed by Edgar and Wood (1966). From the results of these analyses and the previous work of Edgar, a pathway for the formation of the phage tail fiber is presented.

MATERIALS AND METHODS

Materials and methods not specifically described below are as described in Part I.

Preparation of whole anti-T4 serum.

Whole anti-T4 serum was generously donated by Jeffrey Flatgaard and was prepared as follows; T4D grown in E. coli B/5 was purified by three cycles of low speed and two cycles of high speed centrifugation. The final preparation was suspended in Tris buffer to a titer of 3×10^{12} phage/ml. Two young New Zealand white rabbits were given a series of about ten injections over a period of about 1½ months. Each

injection was 0.2 - 0.5 ml. of the above phage stock administered subcutaneously. At the end of this injection series the first order rate constants for the killing of T4 at 37°C were k = 300 for rabbit 1 and k = 130 for rabbit 2. Six months later both rabbits were given a series of booster injections, as described above, every other day for one week. The sera obtained at this time had titers of k = 2000 for rabbit 1 and k = 1300 for rabbit 2. These sera killed T4 exponentially down to less than 10^{-3} survival before the rate began to decrease (The tester phage titers in all serum titering experiments were less than 10^{8}/ml., to avoid killing of phage in aggregates (Hershey, Kalmanson, and Bronfenbrenner, 1943).) Before being used further these sera were absorbed with E. coli B/5.

In all subsequent experiments antiserum titering and absorption was performed at 48°C - to inhibit bacterial growth and to speed the reactions.

Preparation of antisera specific for the A,B, and C antigens of T4

These sera were prepared as described in Edgar and Lielausis (1965). Before describing the details of the preparation it may be useful to review the general procedure. As noted on page 18, we are only discussing those antibodies in the serum which kill the phage on complexing with it. Such neutralizing antibodies react with T4 irreversibly (Hershey, 1943). To prepare a specific serum the whole serum is incubated with a mutant lysate which presumably contains all phage proteins except those under the control of the mutant gene. The neutralizing

antibodies from the serum will complex irreversibly with the complementary antigens present; if a fraction of the neutralizing antibody is directed against the missing gene product(s), it will remain free in the mixture. This mixture can be used as a serum specific for the gene product(s) without further purification.

Mutant lysates for absorption of whole sera were prepared with E. coli B/5 in H broth, at 30°C, in volumes of 250-1000 ml. The lysates were centrifuged at low speed to remove large bacterial debris. Serum blocking curves for both whole sera were determined and were similar to the results reported by Edgar and Lielausis (1965). Lysates for the preparation of anti-A were made with a double mutant containing amB25 and amA455, both in gene 34. Lysates for the preparation of anti-B were made with amE1, in gene 36. Lysates for the preparation of anti-BC were made with a double mutant containing amN52 and amB270, both in gene 37. The locations of these mutations on the genetic map is given in Edgar and Lielausis (1965).

A lysate prepared with an amber mutant of, for example, gene 34, will contain some very low level of A antigen. This is due primarily to input unadsorbed phage and to the "leakiness" of the mutant. At very high lysate concentrations, therefore, the whole serum will be entirely blocked by the mutant lysate. At a lower lysate to serum ratio, the amount of A antigen present will not be sufficient to block the anti-A antibody. However, all other antigens will be present in sufficient amount to block the corresponding antibodies. Thus all species of antibody will be blocked except the anti-A antibody. The

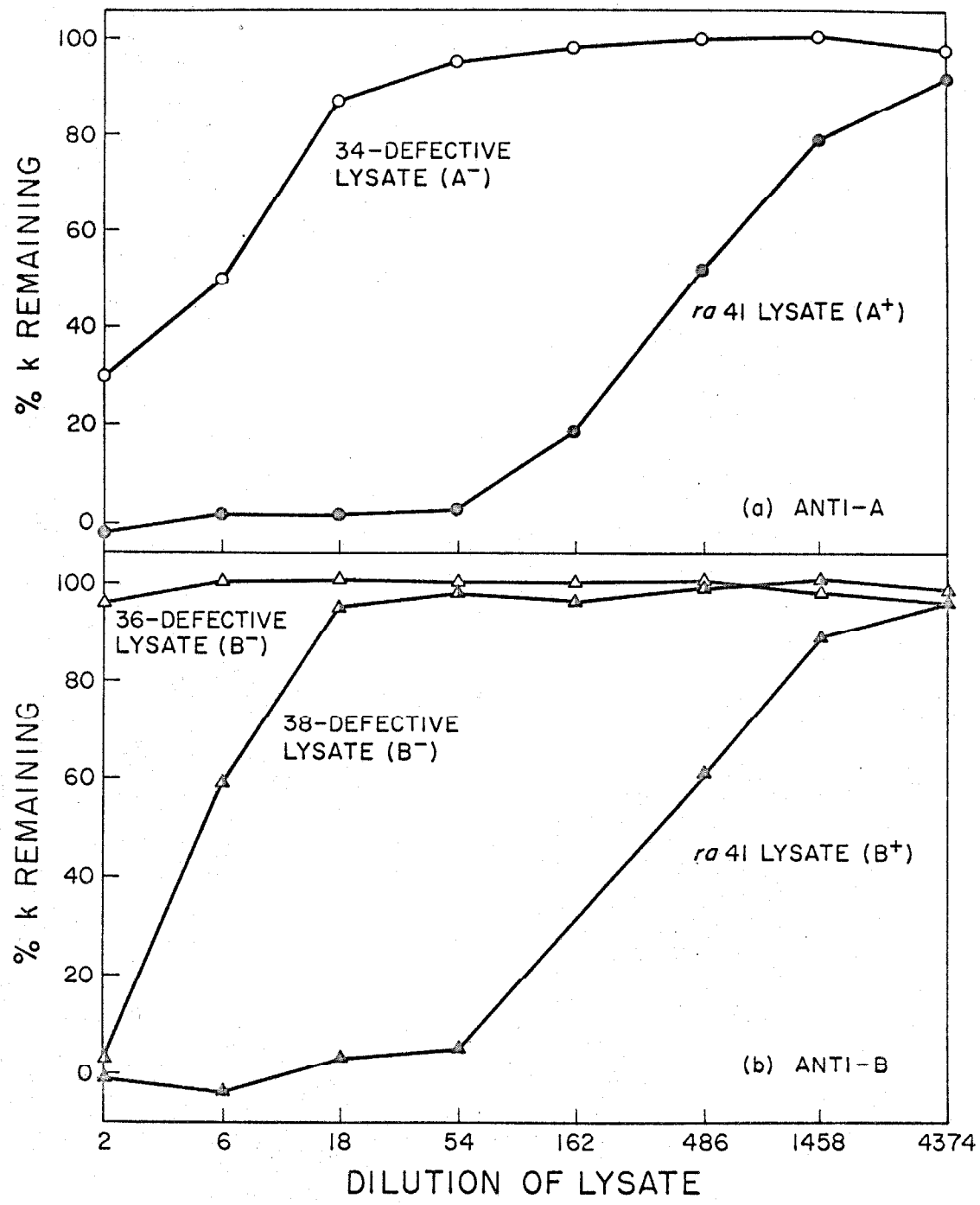
sera in these experiments were prepared by mixing lysate and whole serum in a ratio intermediate between the above two ratios. This insures that those antibodies which one is attempting to remove from the serum, will be adsorbed under conditions of antigen excess.

The properties of the specific sera prepared from the two whole sera did not differ. In most of the experiments specific sera prepared from the higher titer whole serum were employed.

The kinetics of the killing of T4 by anti-A serum were exponential down to less than 10^{-2} survival; between 10^{-2} and 10^{-3} survival the rate began to decrease. The curves for the killing of T4 by anti-B and anti-C exhibited shallow shoulders (when plotted as log survival vs. time), but then also killed exponentially down to less than 10^{-2} survival before the rates began to decrease. These curves extrapolated back to about 2. Edgar and Lielausis (1965) reported back extrapolates of about 5 for anti-B and anti-BC killing curves. The nature of this difference is not clear. With regard to blocking experiments, no significant error was introduced by assuming that killing was exponential and calculating serum titers accordingly.

The specificities of the sera were checked by determining serum blocking curves with appropriate lysates. Such curves for anti-A and anti-B sera are shown in Figure 16. There is a considerable range of concentration over which the sera are blocked by the ra41 lysate but not blocked at all by the relevant mutant lysate. Thus both these sera can be regarded as completely specific. As noted above, there is always some low level of background antigen present in mutant lysates (though

Figure 16. Specificity of anti-A (S34) and anti-B (S36) sera. Lysates were prepared in parallel; the ra41 lysate contained 2×10^{10} phage /ml. Lysates and serial 3-fold dilutions of lysates were incubated with antisera in equal volumes (0.5 ml. + 0.5 ml), to yield a final k of 0.025. The mixtures were incubated overnight at 48°C, and then 4×10^5 r48 tester phage added to each 1 ml. mixture. After three hours subsequent incubation at 48°C the mixtures were titered for tester phage survival. The titer of the residual serum was calculated assuming exponential kinetics for the neutralizing reaction. The abscissa gives the lysate dilution in the final serum + lysate incubation mixture.


100
80
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20
0
% k REMAINING
34-DEFECTIVE
LYSATE (A⁻)
ra 41 LYSATE (A⁺)
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80
60
40
20
0
% k REMAINING
36-DEFECTIVE
LYSATE (B⁻)
38-DEFECTIVE
LYSATE (B⁻)
ra 41 LYSATE (B⁺)
(b) ANTI-B
2
6
18
54
162
486
1458
4374
DILUTION OF LYSATE

note that in (a) the background B antigen in the 36-defective lysate was too low to be detected). In all the experiments to be described subsequently, the concentration of lysate in the final lysate-serum incubation mixture was sufficiently low so that background levels of antigen were undetectable. The specificity of the anti-BC serum can be seen from the control curves included in Figure 18 (E,F).

Though the anti-BC serum would be expected to contain antibodies directed against both B and C antigens, it is in fact effectively specific for antigen C. In the whole serum from which anti-BC was derived, anti-B antibody accounted for less than 20% of the total neutralizing activity, whereas anti-C antibody accounted for about 50%. When this whole serum was absorbed with 37-defective lysate (B^-C^-) in relatively high concentration (as described above), there was sufficient background B and C antigen to absorb out essentially all the B antibody but only some of the C antibody. Since all the anti-A antibody was of course adsorbed by the very high concentration of A antigen, the final serum is essentially anti-C. This can be seen from Figure 18,D; all of the neutralizing activity of the anti-BC serum was blocked by the C antigen from a 36-defective lysate (B^-). Thus this serum will be referred to subsequently as anti-C.

For clarity, the terms anti-A, anti-B, and anti-C are used in the text. However, the more accurate names for the sera - employed by Edgar and Lielausis (1965) - have been inserted in the figures. In this terminology the specific sera are named according to the lysates used to adsorb the whole serum. Thus, a specific serum resulting from the

adsorption of whole serum with 34-defective lysate is termed S34(=anti-A), serum resulting from adsorption with 36-defective lysate is S36 (=anti-B), and serum resulting from adsorption with 37-defective lysate is S37 (=anti-C).

Phage mutants

All phage mutants are described either in Epstein et al. (1963) or Edgar and Lielausis (1965), except for ra41, a mutant of the rIIA cistron. The mutant ra41 was used as a reference phage. It is convenient to use an rII mutant for reference phage rather than wild type since a restrictive host is available. In serum blocking experiments this permits selective plating for the tester phage in the presence of high concentrations of viable phage from the lysate.

Sucrose gradient centrifugation

Gradients were prepared and centrifuged as described in Part I, but fractions were generally collected with a tube piercing device that yielded about 300 drops from a 5 ml. gradient. When hemoglobin was used as a centrifuge marker the sample to be layered on the gradient was made up to about 2% in hemoglobin. The optical density of the fractions containing the hemoglobin peak were read in a Bausch and Lomb Spectronic 20 colorimeter at 418 mu using tube adaptors to permit reading of 0.3 ml. samples. $S_{20,w}$ values were calculated using a value of 4.3 for hemoglobin. Due to the uncertainty in identifying the peaks of the hemoglobin and antigen distributions the $S_{20,w}$ values are only accurate to

within about ± 15%. In most experiments a marker was not employed and it was simply noted that the antigens were found in the expected fractions. $S_{20,w}$ values calculated for these experiments using the Martin and Ames (1961) procedure, assuming $\bar{v} = 0.725$, yielded similar values to those obtained from the hemoglobin determination.

Serum blocking assays of sucrose gradient fractions

The serum blocking assay is most sensitive in the region in which the blocking antigen is roughly stoichiometric with its antibody. The experiments described below were performed so that this condition existed in the samples containing fractions around the antigen peak. (For a lysate containing 2-4 x 10^{10} phage/ml., or equivalent, 0.1 ml. was layered onto a 5 ml. gradient, and after centrifugation 0.7 - 0.9 ml. fractions were collected into 1 ml. of serum, $k \cong 0.025$. The titer refers to serum after overnight incubation at 48°.)

The reaction between phage and antibody is slowed by the presence of sucrose, so that fractions from the bottom of the gradient give systematically higher estimates of the antigen present than fractions from the top of the gradient. However, the error in these experiments due to differential sucrose concentrations was of the order of a few per cent and did not affect the results.

Presentation of data

In order to convert the primary survival of tester phage data into per cent serum blocked, as was done in Part I, it is necessary

to determine a baseline for the titer of the serum, without added sample, under the conditions of the experiment. In a number of experiments such controls were not included, and consequently the data are presented as survival of tester phage without converting to per cent serum blocked. The tester phage survival, P/P_o, is plotted logarithmically; since $\ln(P/P_o) = -kt$, (k = serum titer, t = time of incubation), the ordinate is directly proportional to the per cent serum blocked in the incubation mixture, and therefore to the amount of blocking antigen in the sample. The only difference between this method of plotting the data, and the plot of per cent serum blocked, is that the serum blocking curves obtained with different antisera are not normalized to the same baseline.

Note that the numbers on the ordinate refer to the survival of tester phage; for most of the experiments 10% tester survival is roughly equivalent to blocking of half the serum by the sample.

Preparation of lysates and extracts

Lysates were prepared as described in Part I, except that superinfection was rarely employed. Extracts were prepared as described in Part I. Extracts are 2×10^{-2} in $MgSO_4$, whereas lysates in K medium are 10^{-3} M in $MgSO_4$. Since in a number of cases results from experiments with lysates were compared with results from experiments with extracts, a control experiment was performed to determine if the tail fiber components were sensitive to the $MgSO_4$ concentration in this range. Lysates (amE17, gene 9) were prepared in high (2×10^{-2} M) and

low (4×10^{-4} M) $MgSO_4$, in K medium, and then each of these centrifuged through sucrose gradients made up in high and low $MgSO_4$ BU. The resulting fractions were assayed for serum blocking power; no differences were detected among the antigen sedimentation patterns in the four cases.

Media and chemicals

Tris buffer was 0.1 M and was adjusted to pH 7.4 with HCl. Hemoglobin was obtained from Sigma Chemical Corp. and was from beef blood, 2 x recrystallized. Cesium chloride was obtained from Harshaw Chemical Corp.

RESULTS

(i) Serological characterization of the phage particles from tail fiber$^-$ lysates

The tail fiber antigens which accumulate in cells infected with the various tail fiber mutants might be free, or associated with the phage particles present in these cells. Lysates made with mutants defective in each of genes 34, 35, 36, 37, 38, and 57, were therefore centrifuged through sucrose gradients to separate the inactive phage particles from precursor material. The resulting gradient fractions were then assayed for antigens able to block the specific antisera. In each case the fractions were assayed for those antigens which were present in the defective lysate; e.g., fractions derived from centrifugation of a 34-defective lysate ($A^-B^+C^+$) were assayed for B and C

antigens. The background viable phage present in the defective lysates - due primarily to leakage and unadsorbed phage - were used as centrifuge markers; these phage represented less than 0.1% of the number of fiberless phage particles present in the lysates and were in far too low concentration to be detected serologically.

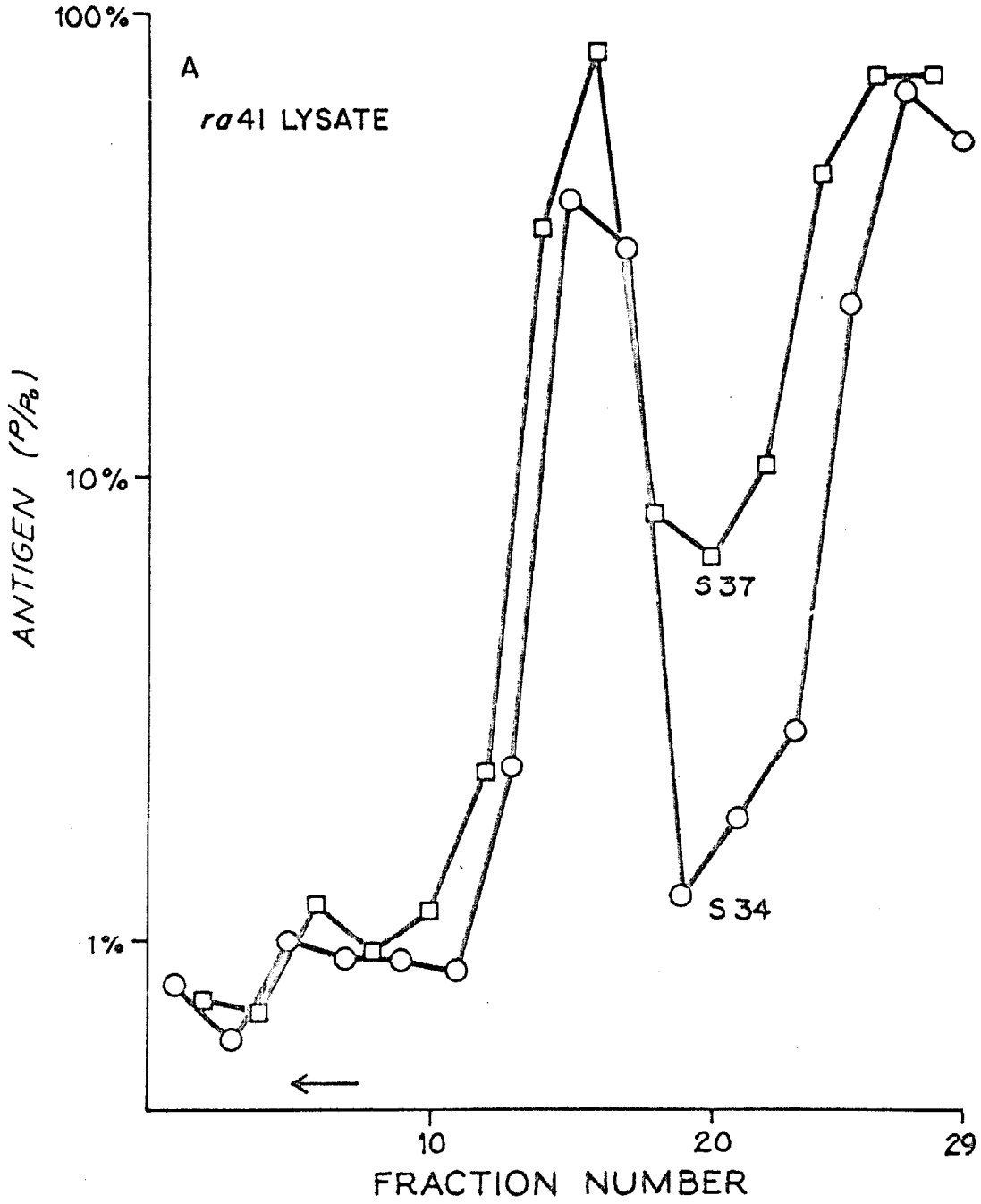
The results of such experiments with an ra41 reference lysate and with 34-, 36-, and 37-defective lysates are shown in Fig. 17. In the reference lysate a significant portion of the total antigen sediments with the phage (Fig. 17,A). However, in the defective lysates, no detectable antigen was found associated with the particles; all the antigen remained at the top of the centrifuge tube (Fig. 17, B-D). The same results were found with 35-, 38-, and 57-defective lysates, though with the 57-defective lysate low levels of antigen were found associated with the particles. This result is in keeping with the observations of Eiserling, Bolle, and Epstein (1967) that particles from 57-defective lysates have 1-2 tail fibers/particle.

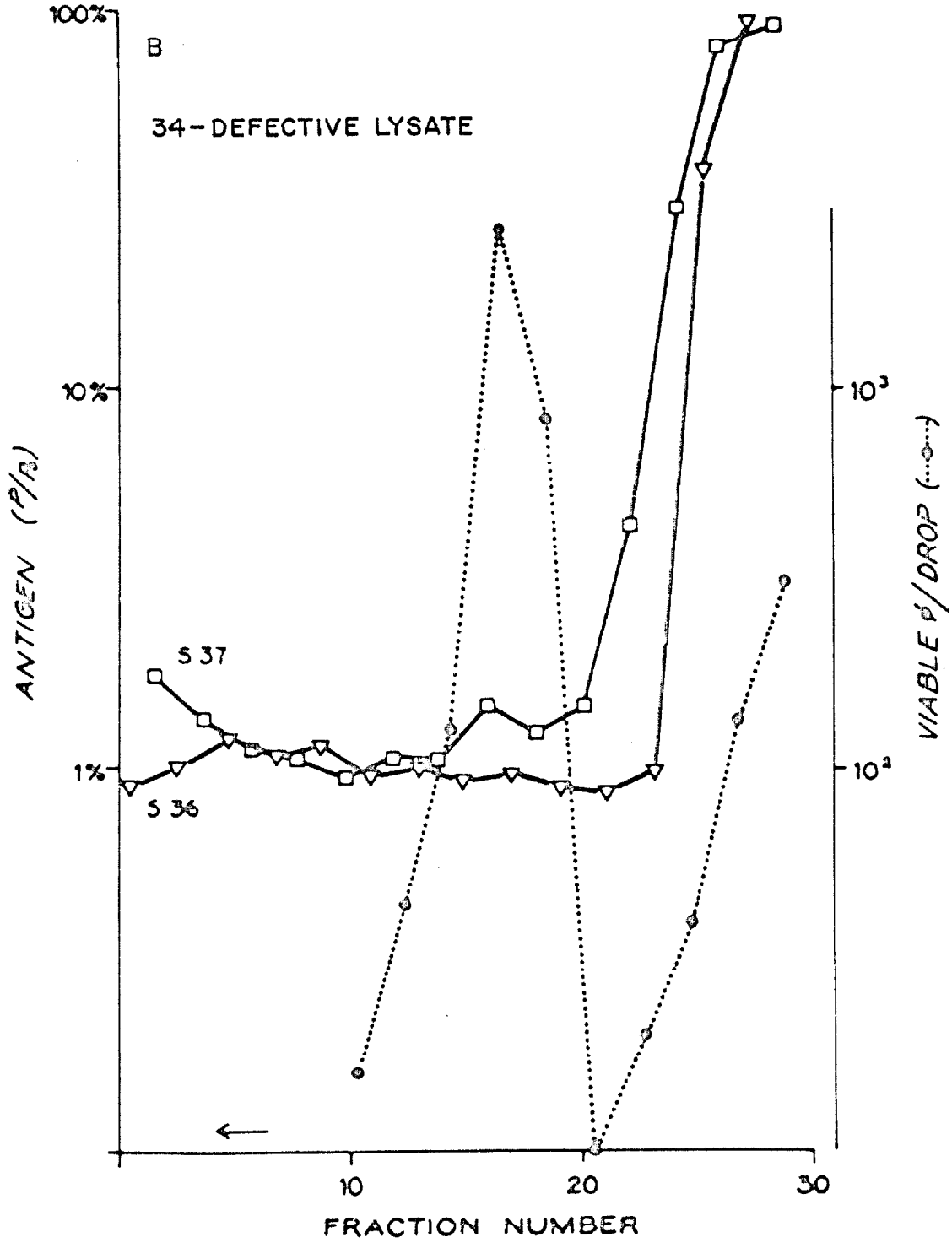
The results of these experiments are summarized in Table 7. They indicate that the products of all six tail fiber genes must interact prior to the attachment of any of the antigens to the particle, suggesting that only a completed tail fiber can attach to the phage particle.

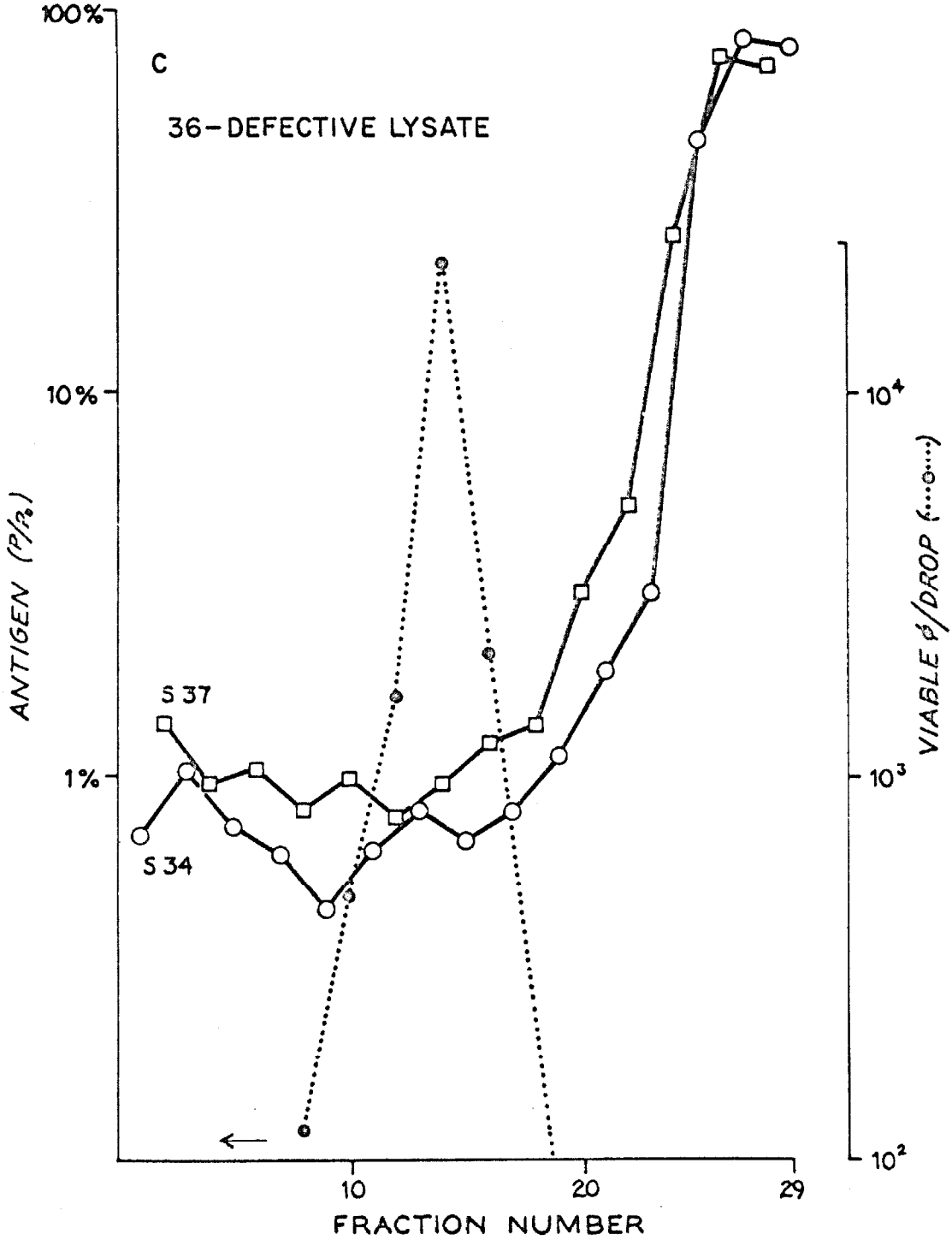
(ii) Sucrose gradient centrifugation of the tail fiber antigens

To characterize the sedimentation behavior of the free tail fiber antigens, experiments similar to those just described were performed, but the lysates were centrifuged for longer periods of time. These

Figure 17. Assay of phage particles from ra41 lysate and 34-, 36-, and 37-defective lysate for associated tail fiber antigens. Lysates were prepared without superinfection and allowed to lyse spontaneously. Samples of 0.2 ml. were layered on sucrose gradients made up in BU, 10^{-2} M in $MgSO_4$, and centrifuged for a few minutes at 37,000 rev./min., 20-24°C. Ten drop fractions were collected alternately into anti-A and anti-C (A,C), or anti-B and anti-C (B), or anti-A and anti-particle serum (D), and assayed for serum blocking activity. (The anti-particle serum was described in Part I of this thesis.) Between every two fractions in the region of the background phage peak, one drop was collected into 3% sucrose in H broth (to prevent osmotic shock) and assayed for viable phage. In (D) the shift of the particle antigen peak with respect to the peak of background viable phage is expected. The anti-particle serum measures the majority of the particles present; these lack tail fibers and thus sediment somewhat faster than the infrequent viable phage which have tail fibers (Kellenberger et al., 1965). (A) ra41 lysate. (B) 34-defective lysate. (C) 36-defective lysate. (D) 37-defective lysate. O = A antigen. ∇ = B antigen. □ = C antigen. X = particle antigen. ● = background viable phage.


100%
A
ra 41 LYSATE
10%
1%
ANTIGEN (P/P0)
S 37
S 34
10
20
29
FRACTION NUMBER


100%
B
34-DEFECTIVE LYSATE
10%
1%
S 37
S 36
ANTIGEN (P/S)
10^3
10^2
VIABLE P'/DROP (---)
10
20
30
FRACTION NUMBER


100%
C
36-DEFECTIVE LYSATE
10%
1%
ANTIGEN (P/P₀)
S 37
S 34
10⁴
10³
10²
VIABLE ϕ/DROP (.....)
10
20
29
FRACTION NUMBER

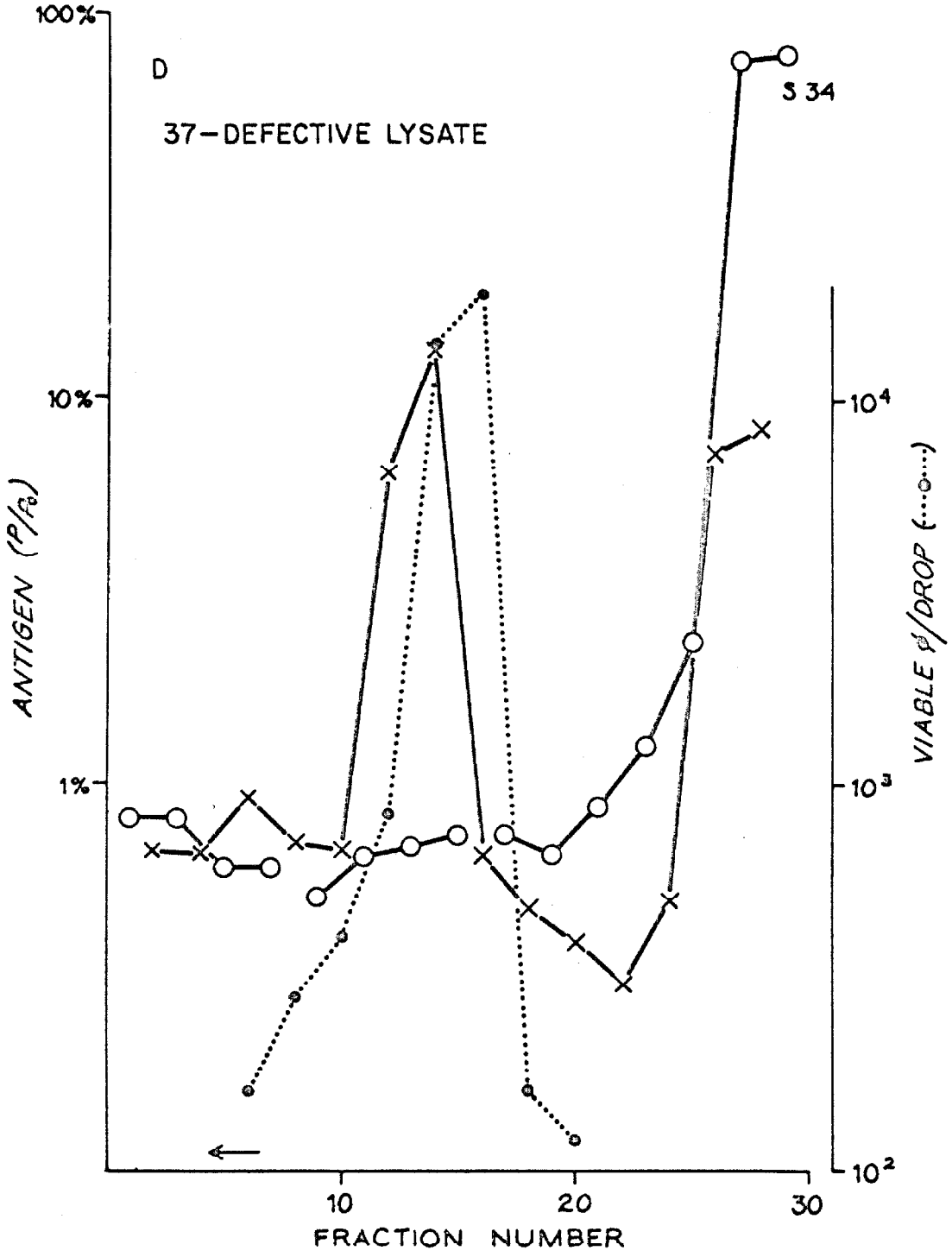

100%
10%
1%
D
37—DEFECTIVE LYSATE
S 34
ANTIGEN (P/P0)
VIABLE Ø/DROP (....)
10⁴
10³
10²
10
20
30
FRACTION NUMBER

TABLE 7

Serological characterization of particles from tail fiber⁻ lysates

Lysate Defective gene	Mutant	Particle-associated antigen		
		A	B	C
rII	ra41	+	+	+
34	amB265	(0)	0	0
35	amB252	0	0	0
36	amE1	0	(0)	0
37	amN52:amB280	0	(0)	(0)
38	amC290	0	(0)	0
57	amE198	low	low	low

This table summarizes the results of Figure 17 and similar experiments. 0 = antigen not present in gradient fractions containing particles. (0) = antigen not assayed for, since it was absent from the whole lysate. The amount of antigen associated with the particles from 57-defective lysate represented roughly 5-10% of the amount associated with the ra41 phage.

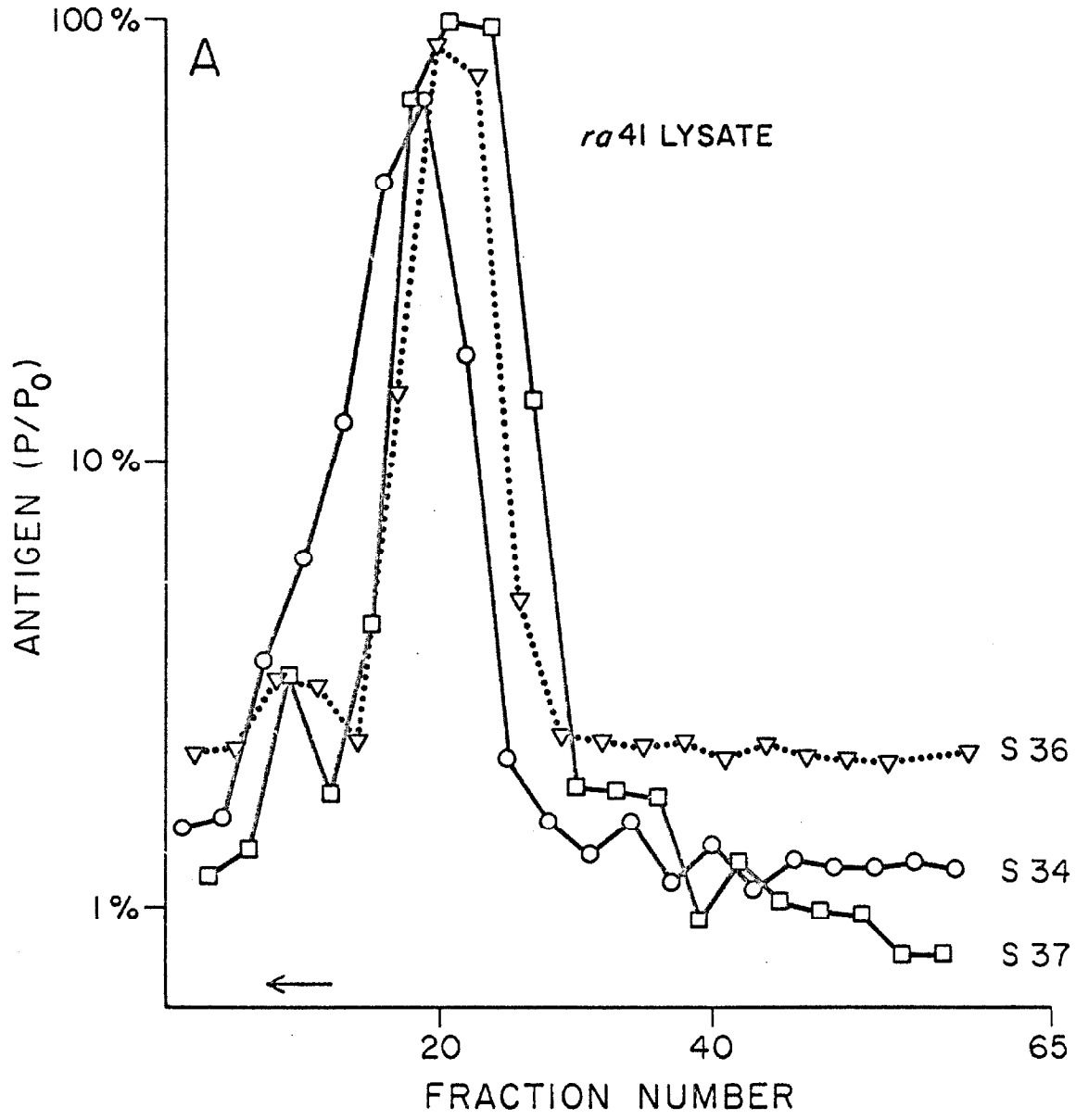
experiments were of a qualitative nature; centrifuge markers were not generally included, so that small differences in sedimentation behavior would have been unnoticed. Furthermore, since serum blocking activity is not a linear function of antigen concentration, the areas under the peaks are only rough measures of antigen concentration.

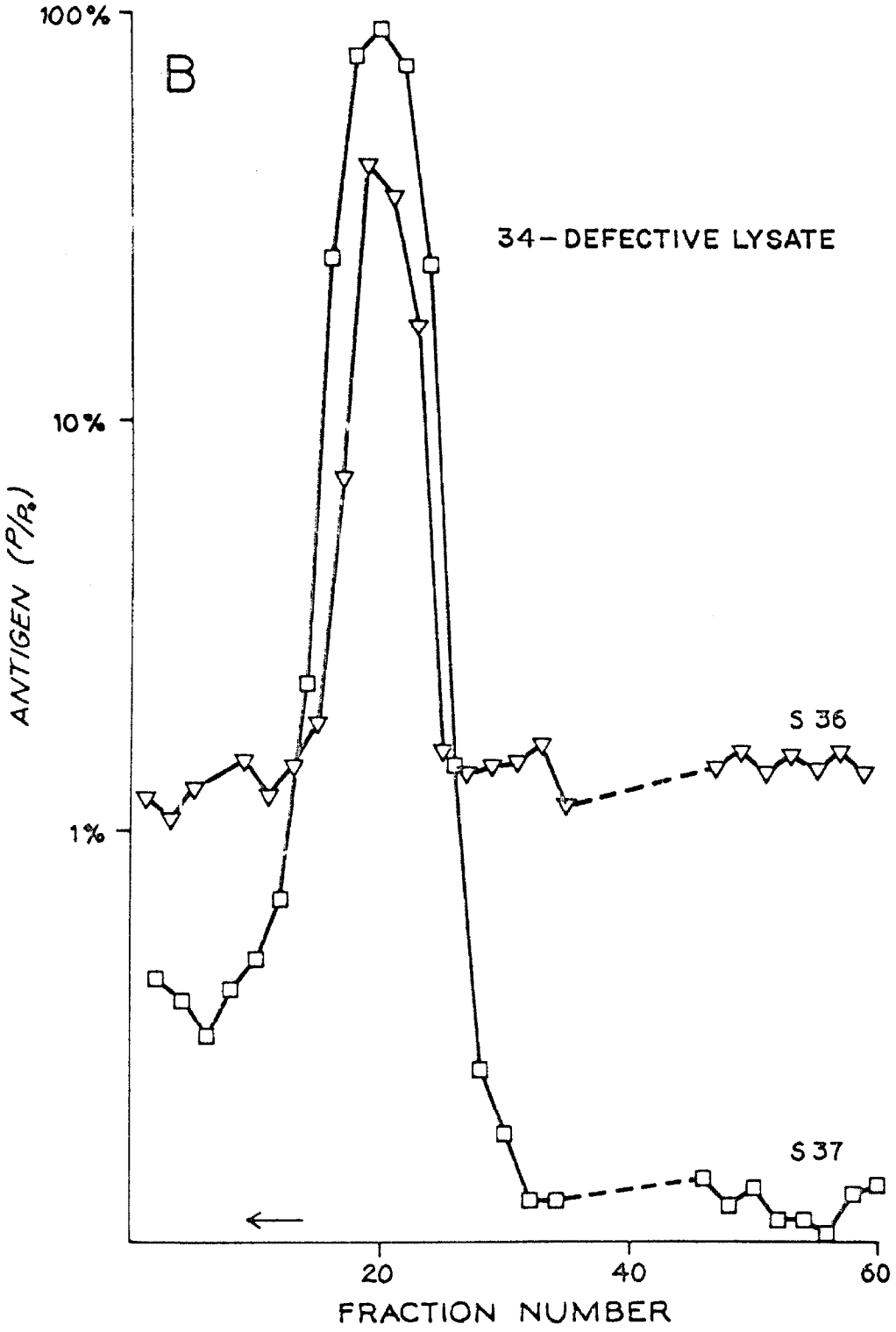
The results of these experiments, with respect to the presence or absence of the antigens in the gradient fractions from mutant lysates, were in complete agreement with those of Edgar and Lielausis (1965) summarized in Table 6. That is, those antigens present in the whole lysate were also found in the sucrose gradient fractions of the lysate. The antigen sedimentation patterns are shown in Figure 18.

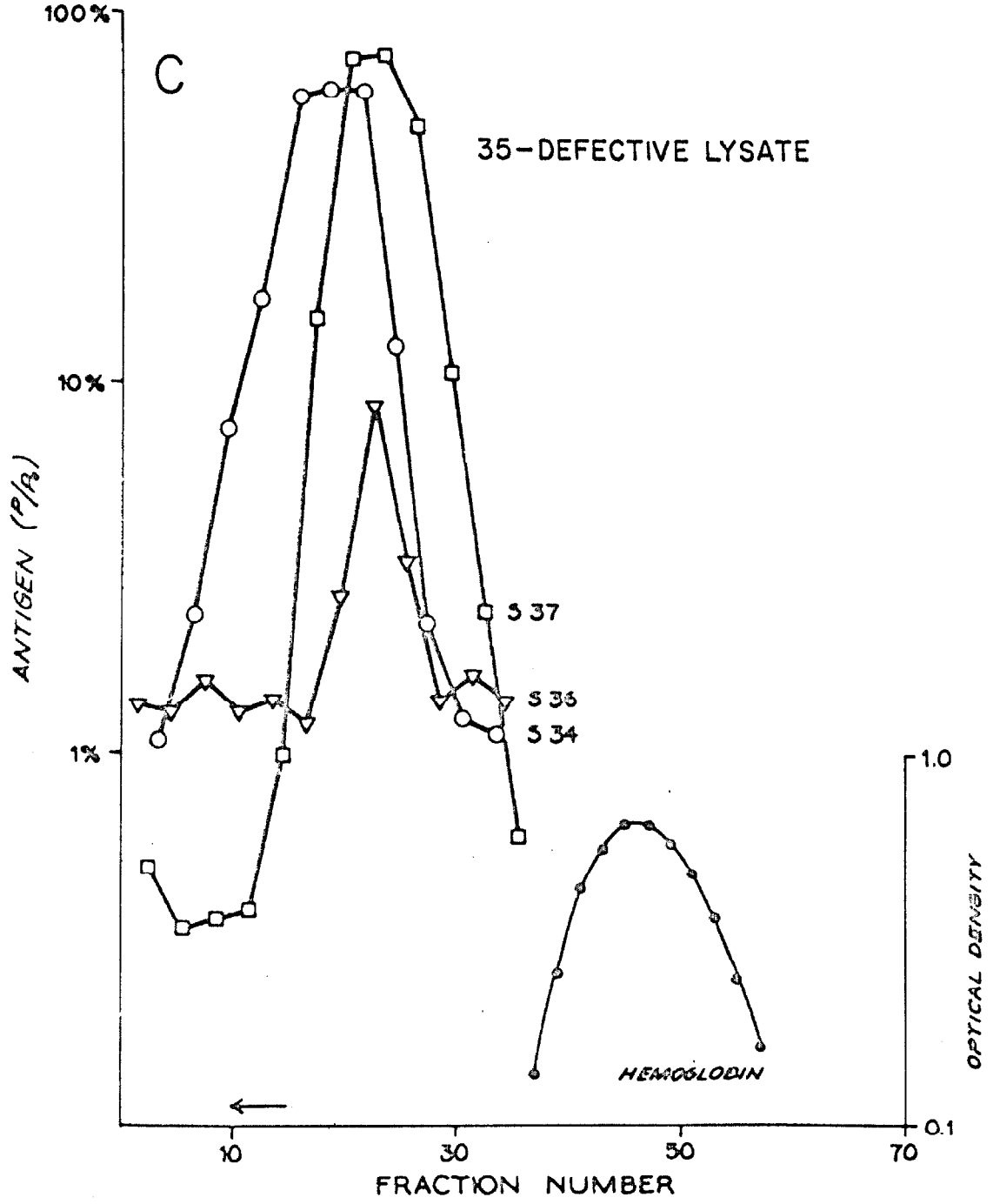
The sedimentation pattern obtained with an ra41 reference lysate is shown in Figure 18,A. All three antigenic activities sedimented very similarly. All the serum blocking antigen recovered from the gradient was between approximately 8 and 10S. Within the limits of error of the experiment, the B and C antigens appeared to sediment coincidently. However, the A antigen appears to migrate somewhat faster than the B and C antigens; in seven independent experiments the ratio of distances sedimented by A and BC was 1.07 (range, 1.06-1.08). The B and C serum blocking activity will subsequently be referred to as 8S, and the A activity as 9S. The small peak which may be present at about 10S and containing all three antigens will be discussed in a subsequent section.

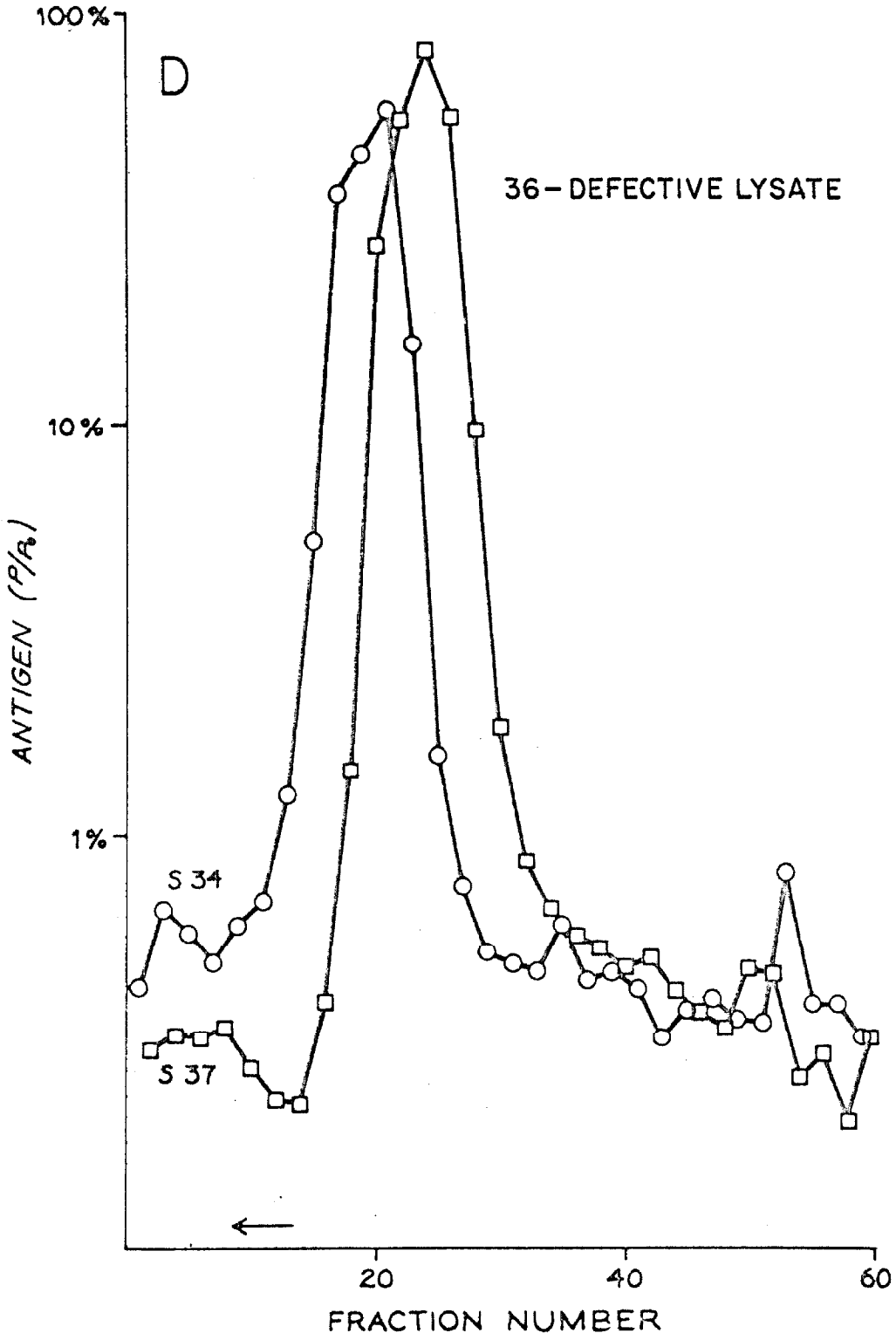
57-defective lysates were not included in these experiments. In the following discussion it should be kept in mind that the function of gene 57 is apparently required for the normal formation of all three tail fiber antigens.

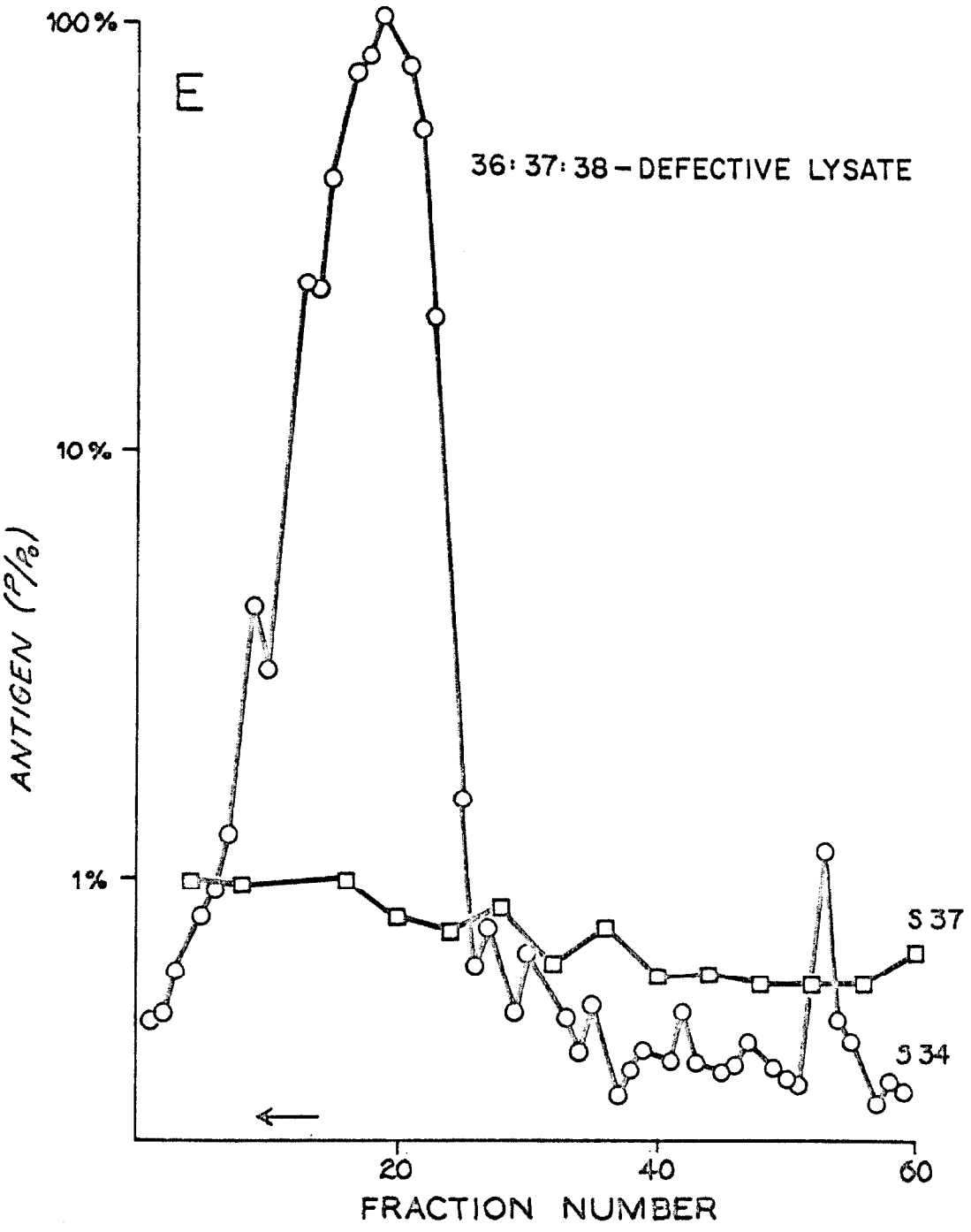
Figure 18. Sedimentation patterns of tail fiber antigens. Lysates were prepared in B/5 in K medium, except for the 34-defective lysate, which was prepared in H broth. Lysates were prepared and centrifuged independently. Samples of 0.1 ml. of the lysates were layered on sucrose gradients made up in BU, 10^{-3} M in $MgSO_4$, and centrifuged at 37,000 rev./min., at 4-6°C, for from 14 to 17 hours. Fractions were collected into the appropriate antisera and assayed for serum blocking activity. O = A antigen. ▽ = B antigen. □ = C antigen. ● = hemoglobin.

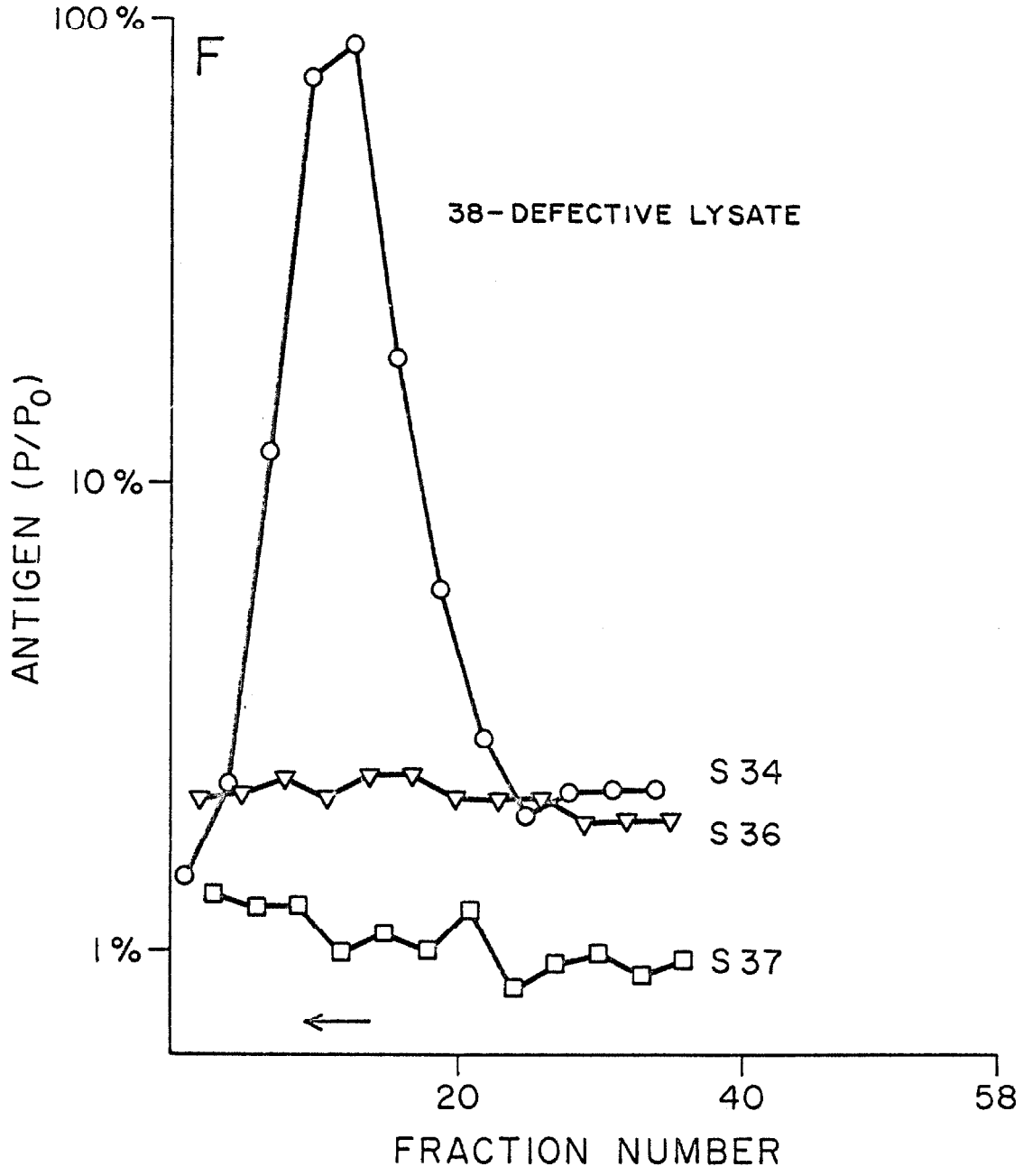

100 %
A
ra 41 LYSATE
ANTIGEN (P/P0)
10 %
1 %
S 36
S 34
S 37
20
40
65
FRACTION NUMBER


100%
B
34 – DEFECTIVE LYSATE
10%
ANTIGEN (P/P₀)
S 36
1%
S 37
20
40
60
FRACTION NUMBER


100%
C
35-DEFECTIVE LYSATE
10%
1%
ANTIGEN (P/B)
S 37
S 36
S 34
HEMOGLOBIN
1.0
0.1
OPTICAL DENSITY
10
30
50
70
FRACTION NUMBER


100%
10%
1%
D
36-DEFECTIVE LYSATE
S 34
S 37
ANTIGEN (P/P)
20
40
60
FRACTION NUMBER


100%
10%
1%
E
36: 37: 38 – DEFECTIVE LYSATE
S 37
S 34
ANTIGEN (P/P0)
20
40
60
FRACTION NUMBER


100 %
F
38-DEFECTIVE LYSATE
ANTIGEN (P/P0)
10 %
1 %
S 34
S 36
S 37
20
40
58
FRACTION NUMBER

The sedimentation patterns of the antigens from 35-, 36-, and 38-defective lysates and 36:37:38-defective lysate are shown in Figure 18, C-F. The A component from all these lysates sedimented at about 9S, as it did in the ra41 lysate. As expected, this 9S A component was absent only from 34-defective lysate (data not shown) and thus appears to be the product of gene 34.

The C antigen sedimented at 8S whether derived from 34-, 35-, or 36-defective lysate, or from the ra41 lysate (Fig. 18, A-D). It was absent from 38-defective lysate and from a multiple mutant lysate defective in genes 36, 37 and 38 (Fig. 18, E,F). A single mutant 37-defective lysate was not included in these experiments but presumably also would have lacked the 8S C antigen (since no C antigen is found in the whole 37-defective lysate). Thus the 8S C antigen is apparently the product of the actions of genes 37 and 38.

Antigen B, whose formation required the function of gene 36, in addition to genes 37 and 38, cosedimented with antigen C at 8S. In ten independent experiments in which the sedimentation properties of both B and C antigens were determined (using 34- and 35-defective, and ra41 reference lysates), the B and C antigens sedimented together. These observations together with the absence of antigen B in 37- and 38-defective lysates (C^-) suggest that B and C antigenic sites are on a single sedimenting structure.

The results of these experiments are summarized in Table 8; there appear to be at least two basic components of the tail fiber, an 8S BC component, and a 9S A component. BC, and C, its presumed precursor, have the same sedimentation coefficients.

TABLE 8

Sedimentation behavior of free tail fiber antigens

Lysate		Sedimentation of antigens, $S_{20,w}$		
Defective gene(s)	*am* Mutants*	A	B	C
*r*II	*ra*41	9	8	8
34	B25:A455	-	8	8
35	B252	9	8	8
36	E1	9	-	8
37	B262	9	-	-
34:35:36	B25:A455:B252:E1	-	(-)	8
35:37:38	B252:N52:B262	9	(-)	-
36:37:38	E1:N52:B262	9	(-)	-

*Except *ra*41

This table summarizes the results of Figure 18 and similar experiments. - indicates no detectable antigen. (-) indicates that the antigen was not tested for, since it was absent from the whole lysate. As noted in Materials and Methods, sedimentation coefficients were only determined with an accuracy of about ± 15%. The integral 8 and 9S values are used for convenience. Most experiments yielded somewhat lower values; 7-8S for the B and C antigens, and 8-9S for the A antigen.

(iii) Electron microscopy of gradient fractions

Eiserling, Bolle, and Epstein (1967) found that thin rods, with the dimensions of a half tail fiber, were present in T4 wild type lysates and in 34-, 35-, and 36-defective lysates, but were absent from 37- and 38-defective lysates. These observations, and the antigenic properties of the mutant lysates, suggest that the "half-fibers" correspond to the C and BC tail fiber components. To test this notion, concentrated extracts of mutant infected cells (prepared as described in Part I of this thesis) were centrifuged through sucrose or D_2O gradients and the 8S fractions examined with the electron microscope. Plate X demonstrates that the 8S fractions - from a mutant extract containing all the tail fiber components (9-defective extract) - had large numbers of rods with the dimensions of half tail fibers. Similar rods were also found in the 8S fractions from a 36-defective extract (Plate XI). These findings suggest that the C and BC components are indeed the thin rods identified by Eiserling, Bolle, and Epstein (1967). The resolution of the photographs is not good enough to determine if two morphological classes are present, though it is worth noting that in some cases small knobs with a diameter of about 20Å can be seen at one end of the rods. However, at present, C and BC are indistinguishable by either morphological or sedimentation criteria.

The A antigen has not yet been identified morphologically. Though the sucrose gradient fractions represent some purification with respect to the whole extract, many phage and bacterial proteins are still present;

Plate X. Structures resembling half tail fibers from gradient fractions of 9-defective extract. A 9-defective extract was centrifuged through a sucrose gradient and grids prepared from the fractions expected to contain the tail fiber antigens. The appearance of the rods was different on different grids prepared from the same sample. In some cases the rods appeared rather thick, as if perhaps globular proteins were sticking to them. Some of the rods in the photograph shown have this appearance; the width varies non-uniformly along the length. Tail fibers are too thin for their width to be accurately measured (~20Å x 1400Å). For this reason, it was not possible to decide whether the rods had the same width as tail fibers (x about 150,000).

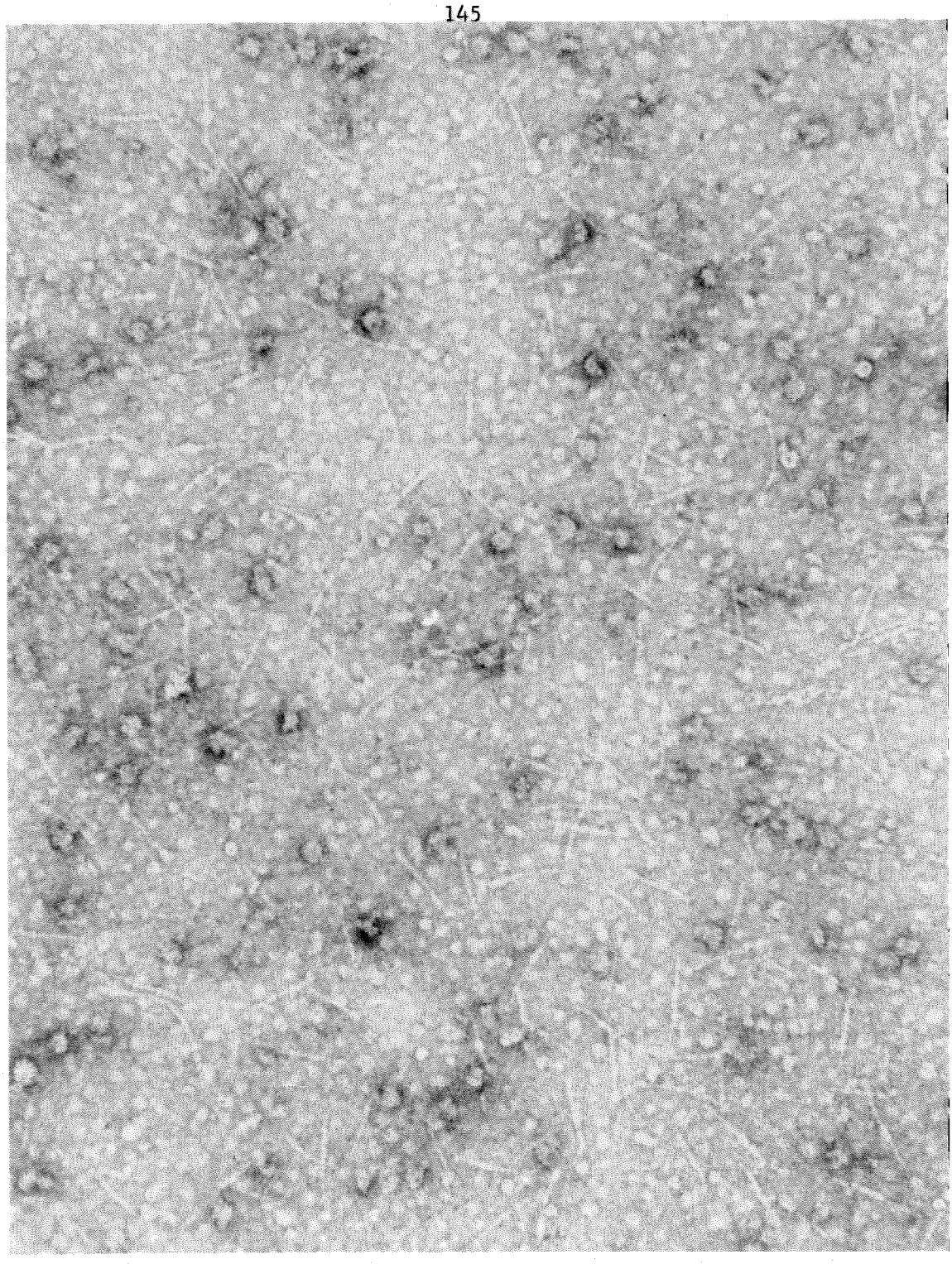
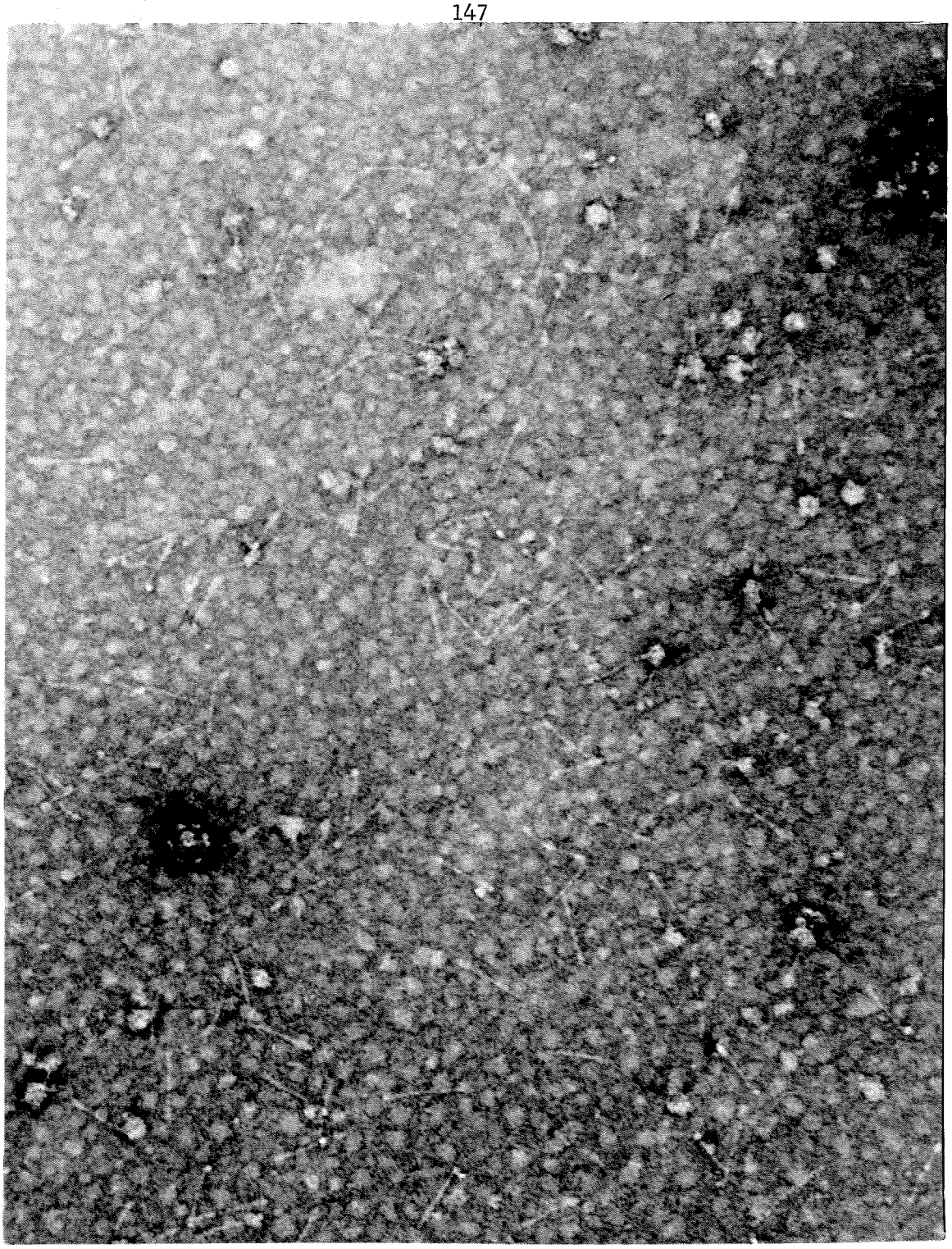


Plate XI. Structures resembling half fibers from gradient fractions of 36-defective extract. In an attempt to improve the resolution of the electron micrographs, a 36-defective extract was centrifuged through a D_2O gradient (20-90%) made up in 10^{-2} M ammonium acetate. Thus both sucrose and salt crystals (ammonium acetate is volatile) would be absent from grids prepared from such gradient fractions. Small knobs can be seen at one end of many of the rods. A 9-defective extract was also included in this experiment; many of the rods from this extract also had knobs at one end. However, these knobs may be an artifact due to the rods sticking to the grids with one end, and then bending against the grid on drying down. The stain might be more efficiently excluded from the interface between grid and rod end than between other contact surfaces. Such effects with phage cores can be seen in Plate III of Part I.

Large phage structures such as baseplates were infrequent on these grids. The baseplate in this photograph was included as an aid in focusing. (x about 150,000).



thus only molecules with a unique morphology, such as the C and BC rods, would be recognizable in the electron microscope.

(iv) Buoyant Density Centrifugation

In searching for improved separation of the A and BC components an rII lysate was subject to buoyant density centrifugation in cesium chloride, and the resulting fractions assayed for serum blocking antigen. Figure 19 shows that all three antigens band at a density of 1.3, the density of typical proteins. A similar result was found for the A and C antigens from a 36-defective lysate (results not shown).

(v) The ABC component

The observation that the phage particles which accumulate in cells infected with tail fiber mutants lack associated tail fiber antigen suggests that the fiber is formed prior to its attachment to the particle. The complete fiber might be expected to contain all three antigens, and to have sedimentation properties similar to the 8S rods, since the sedimentation of rod shaped structures is relatively insensitive to their length. As noted above, serological analysis of the sucrose gradient fractions from an rII lysate revealed a small peak which sedimented at about 10S and exhibited all three antigens. This material was a good candidate for the complete fiber, but was present in unexpectedly small quantity. However, if complete tail fibers were limiting in phage formation, they might not accumulate. In Part I of this thesis it was shown that only particles which have been acted upon by the gene 9

Figure 19. Buoyant density gradient analysis of tail fiber antigens. A lysate of ra41 was diluted by two with H broth and made up to a density of about 1.3 with cesium chloride. Three ml. of this solution was centrifuged for 28 hours at 35,000 rev/min. at room temperature. Fractions were collected into 2.5 ml. of dilution buffer and subsequently diluted 5-fold into antisera to determine serum blocking activity. Fractions from the top and bottom of the gradient were used for refractive index determinations, from which the density gradient was calculated. The density at the bottom of the liquid column was 1.42; phage particles were therefore pelleted. O = A antigen. ▽ = B antigen. □ = C antigen.

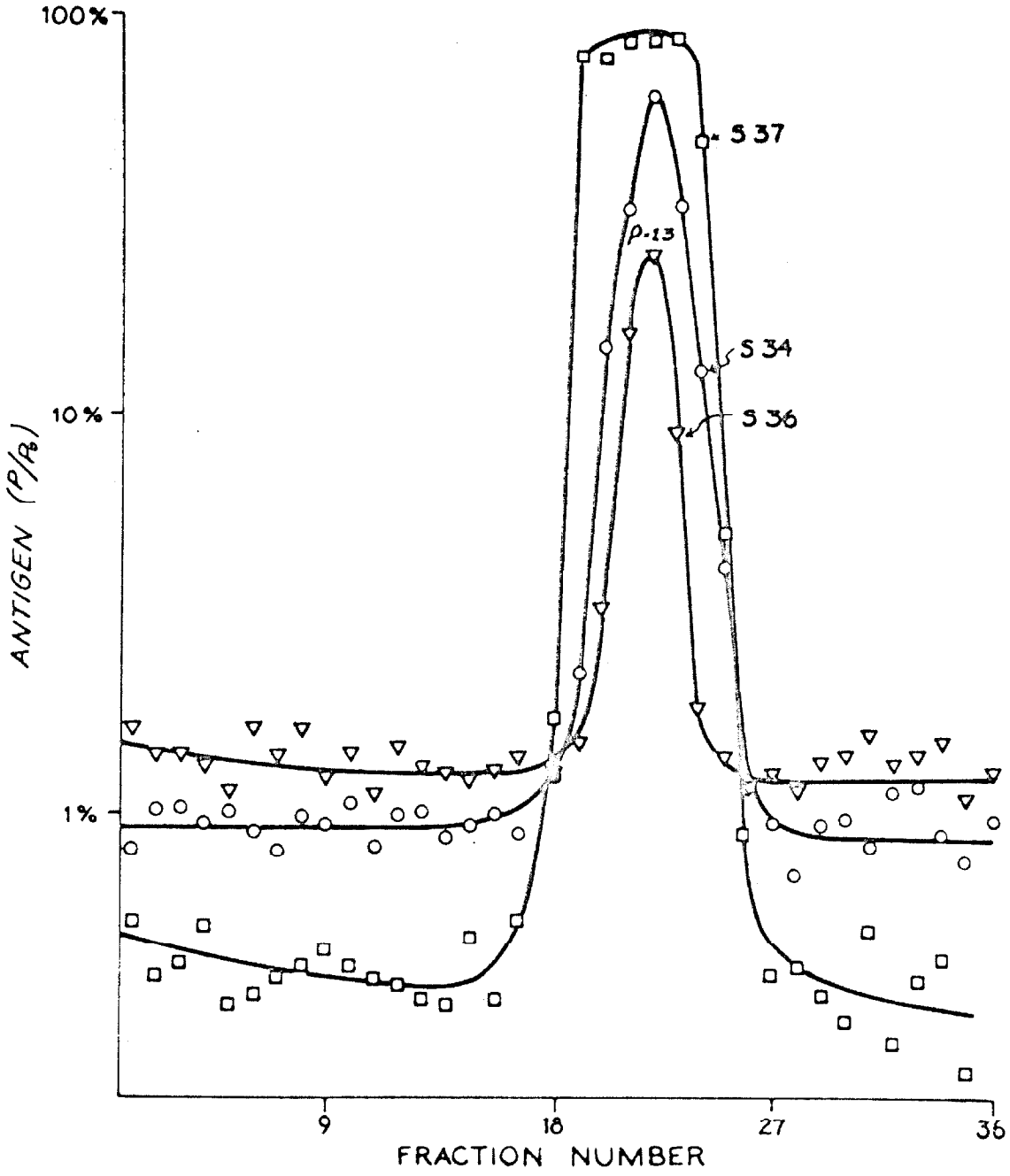

100%
10%
1%
ANTIGEN (P/B)
S 37
P-13
S 34
S 36
9
18
27
36
FRACTION NUMBER

Figure 20. Presence of a 10S ABC component in lysates lacking the substrate particle for fiber attachment. A 0.1 ml. sample of 9-defective lysate (amE17) was layered on a sucrose gradient made up in BU, 10^{-2} M in $MgSO_4$, and centrifuged for 17 hours at 37,000 rev./min., at 4-6°C. Four drop fractions were collected alternately into anti-A and anti-C sera (1 ml.) and assayed for serum blocking activity. O = A antigen. □ = C antigen.

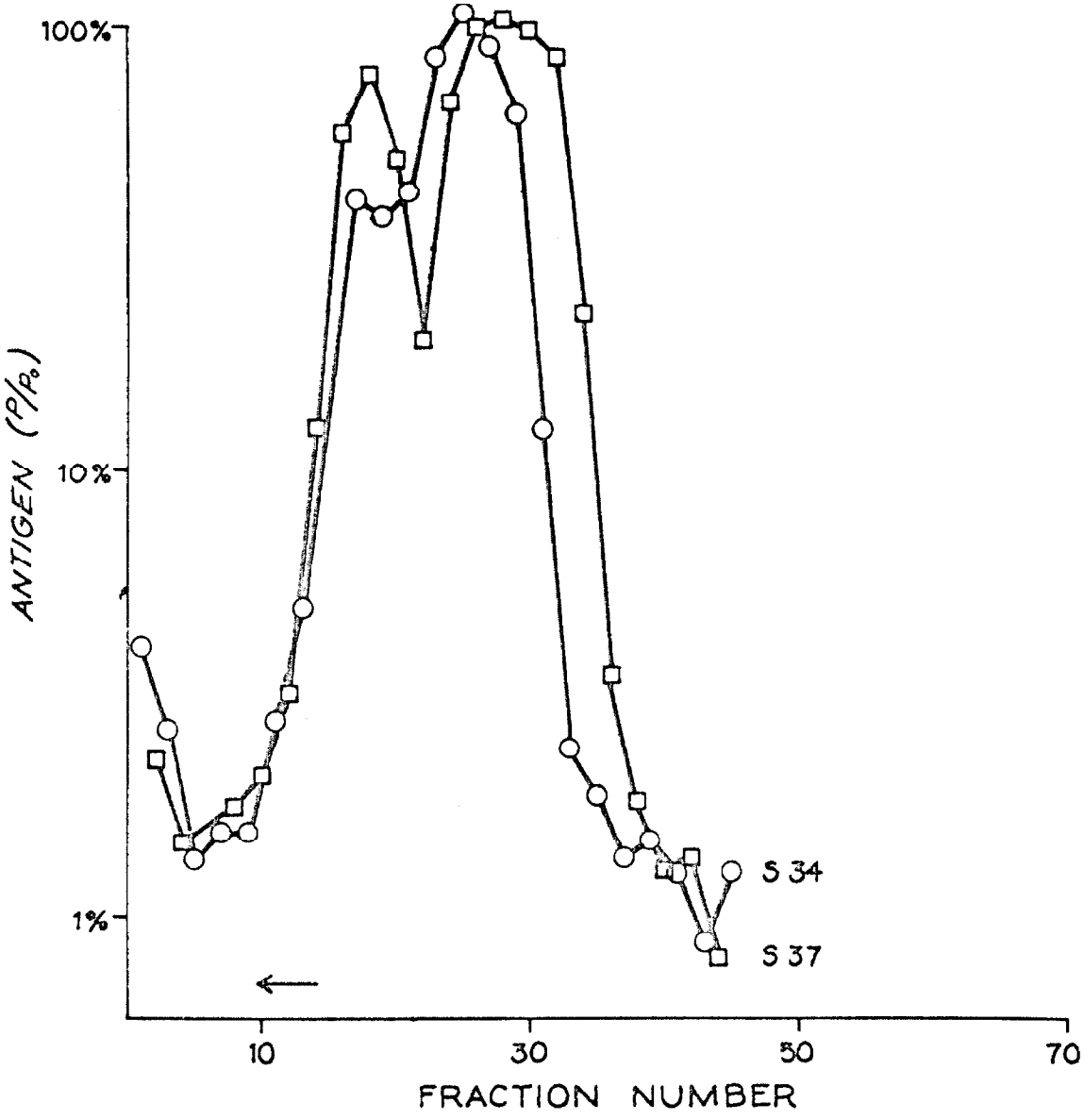

100%
10%
1%
ANTIGEN (%P₀)
S 34
S 37
10
30
50
70
FRACTION NUMBER

TABLE 9

Presence of a 10S ABC component in tail fiber$^+$ lysates

Lysate		Antigenic components			Ratio of distance sedimented	
Defective gene(s)	am Mutant(s)	9S A	8S BS	10S ABC	A/BC	ABC/BC
5:6:7:8	B256:B251:B16:N132	+	+	+	1.06	1.26
9	E17	+	+	+	1.07	1.25
16	N66	+	+	+	1.06	1.21
20	N50	+	+	+	1.08	1.25
23	B17	+	+	+	1.08	1.27

Experimental conditions as described in Figure 20.

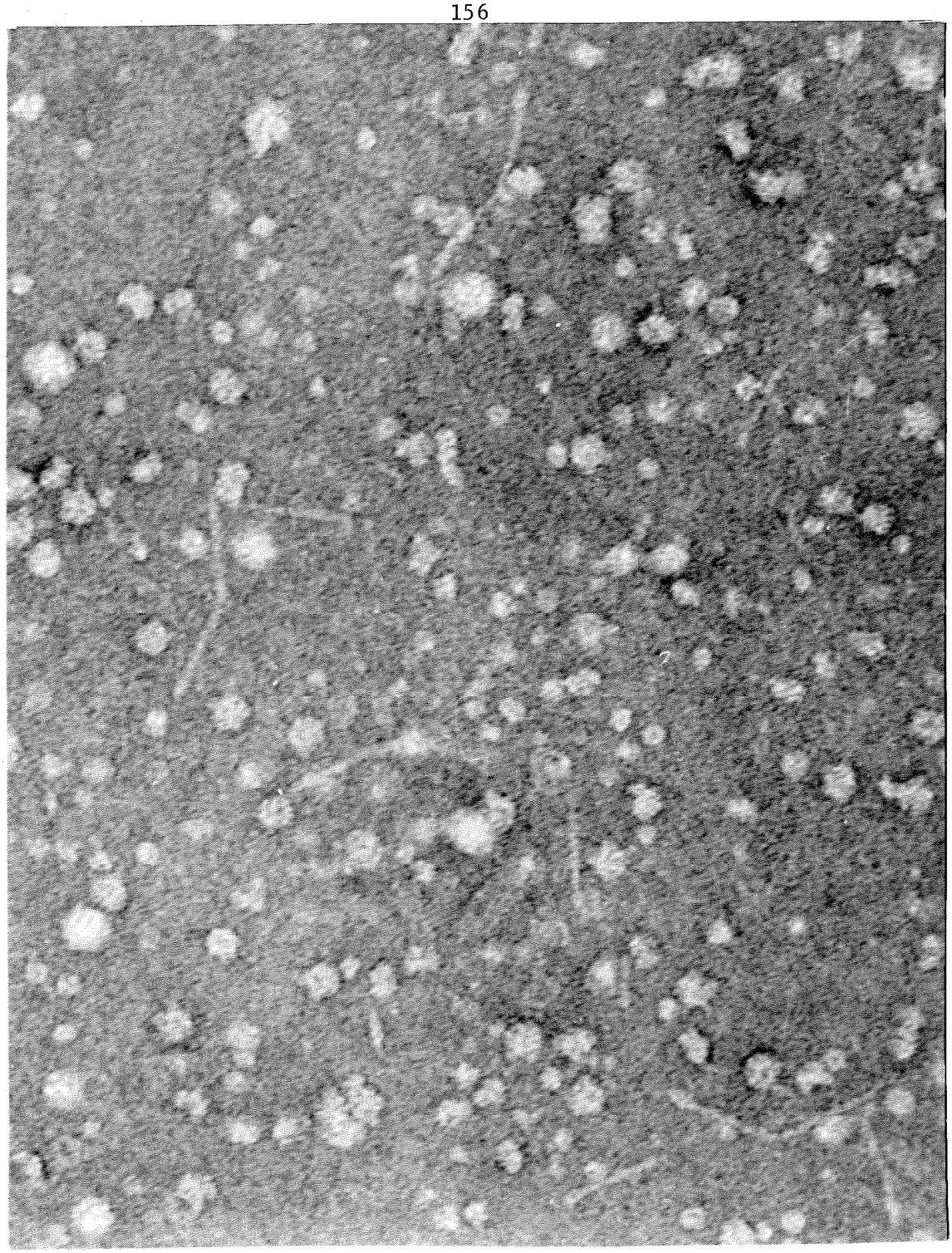
product are substrates for tail fiber attachment; free tails, or 9⁻ particles, are not substrates for fiber attachment. Therefore, one might expect tail fibers to accumulate in lysates made with mutants blocked in particle formation. Lysates prepared from a variety of such mutants were centrifuged through sucrose gradients and the fractions assayed for serum blocking activity. The antigen sedimentation pattern shown in Figure 20 was typical of the results of these experiments (summarized in Table 9). A 10S ABC antigenic peak was present and appeared to represent a considerably larger fraction of the total antigen than it did in the ra41 lysate. However, the 8S BC and 9S A components were still present in excess. (In these experiments the BC component was identified using anti-C only).

To test the idea that the 10S ABC component represents a complete tail fiber, the 10S fractions from a sucrose gradient of a 9-defective extract were examined with the electron microscope. Plate XII shows that complete fibers exhibiting the distinctive kink in the middle can be seen on grids prepared from such fractions, though, again, the half fibers are present in excess.

When the 10S fractions containing the ABC component (from 9-defective extract) were layered on a second sucrose gradient and recentrifuged, a peak of ABC activity again appeared at 10S, and was somewhat enriched relative to the A and BC peaks (data not shown). However, the A and BC peaks were still present in excess.

Returning to Figure 18,C, we note that 35-defective lysate yielded both A and BC components, but no 10S ABC component was present. Since

Plate XII. Structures resembling whole tail fibers from gradient fractions of 9-defective extract. Experimental conditions as in Plate X but grids were prepared from fractions at the front edge of the expected antigen distribution. (x about 260,000).



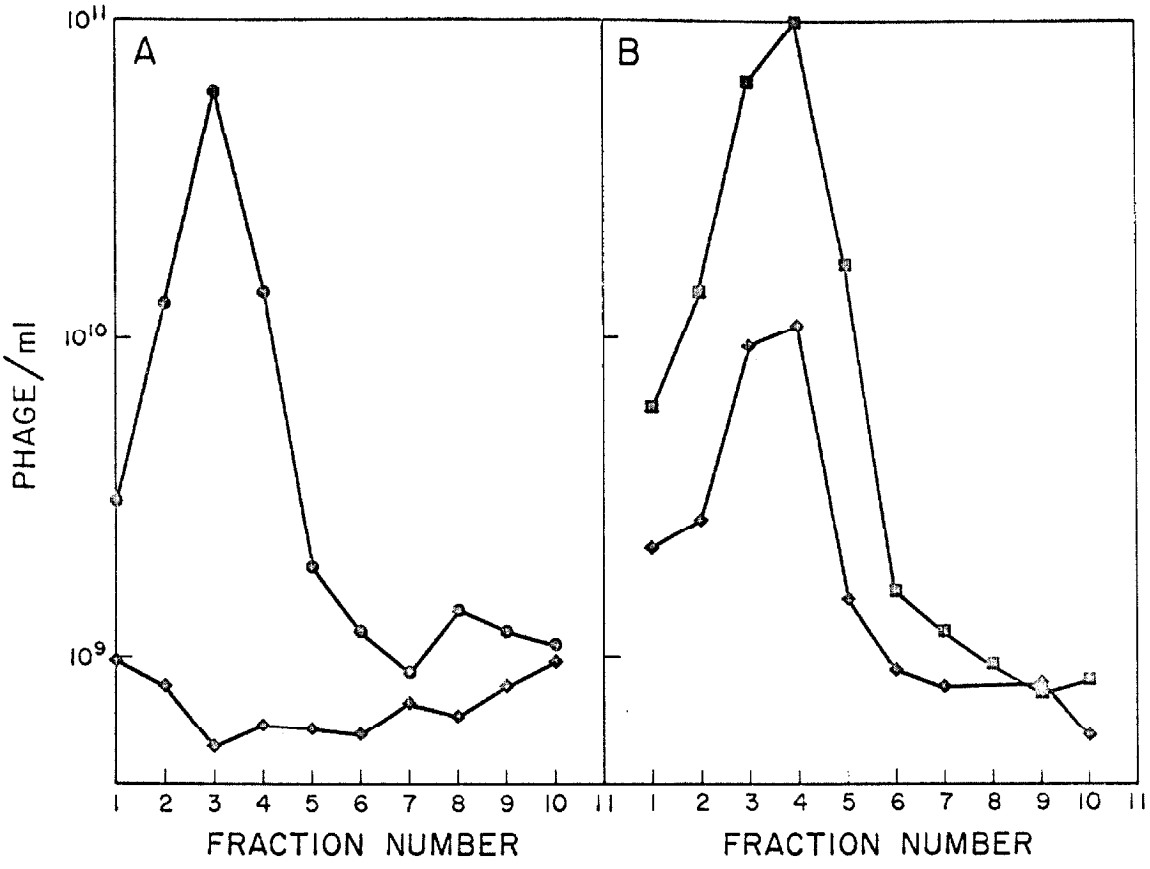
no antigen was associated with the particles from 35-defective lysate, the absence of the 10S component cannot be due to its attachment to the particle. Therefore the absence of the 10S ABC component from 35-defective lysates indicates that the function of gene 35 is required for its formation.

(vi) In vitro complementation

Do the antigenic components isolated by centrifugation represent true intermediates in phage assembly, or aberrant byproducts of the assembly pathway? To resolve this question the in vitro complementation assay of Edgar and Wood (1966) was employed. By analogy with the serological experiments, extracts of mutant infected cells were centrifuged through sucrose gradients and the fractions assayed for their ability to complement appropriate mutant extracts in vitro, as in Part I. Figure 21,A shows an experiment in which a 37-defective extract was centrifuged through a sucrose gradient and the fractions incubated with a 34-defective extract and subsequently titered for viable phage (filled circles). The resulting peak of complementation activity (8-9S) presumably represents the A component. In Figure 21,B, a 34-defective extract was centrifuged through a gradient, and a peak of activity (8-9S) appeared which complemented 37-defective extract. This presumably represents the C and BC components.

Because the ABC component is not effectively separated from the A and BC components by centrifugation, it was not possible in these experiments to show that the ABC component was functional in vitro.

Figure 21. Assay of sucrose gradient fractions for ability to complement tail fiber$^-$ extracts in vitro. Donor extracts were diluted/1.5 with BUM and 0.3 ml. samples layered onto sucrose gradients made up in BUM. These were centrifuged for 15 hours at 37,000 rev./min., 4-6°C. After fraction collection, one drop of each fraction was incubated with one drop of tester extract for 4 hours at 30°C, at which time the mixtures were diluted and assayed for viable phage. The phage titers obtained are plotted on the ordinate directly. Control mixtures containing unfractionated donor extract + tester extract yielded titers of about 2×10^{11} phage/ml. The tester extracts alone contained 5×10^8 - 10^9 phage/ml. (A) 37-defective extract on gradient (B) 34-defective extract on gradient. ● = activity of fractions with 34-defective tester extract. ◇ = activity of fractions with 35-defective tester extract. ■ = activity of fractions with 37-defective tester extract.


A
B
PHAGE/ml
10^{11}
10^{10}
10^{9}
1 2 3 4 5 6 7 8 9 10 11
FRACTION NUMBER
1 2 3 4 5 6 7 8 9 10 11
FRACTION NUMBER

The 10S fractions containing the ABC component (from a gradient of 9-defective extract) complemented an extract derived from a multiple mutant defective in genes 34,35,37, and 38 (data not shown). However, since the 8S BC and 9S A components were also present in these fractions, they might have been responsible for the positive result.

The serological experiments indicated that the gene 35 product must interact with the A or BC components prior to fiber completion. Wood (personal communication), in attempting to isolate the 35 gene product, found it to be unstable in the absence of the BC component (that is, in 37-defective extract). Figure 21, B (filled diamonds) indicates that this is indeed the case; the ability to complement 35-defective extract cosediments with the ability to complement 37-defective extract. In Figure 21, A in which 37-defective extract has been centrifuged through the gradient, no 35^+ activity was recovered from any of the fractions; in the absence of BC, all the 35^+ activity was apparently lost during the 18 hours of centrifugation. These observations suggest that the 35 gene product interacts with the BC component, converting it to a structure competent to interact with the A component to form the 10S ABC structure. The stoichiometry of this association will be discussed below.

(vii) Experiments with temperature sensitive mutants

Edgar and Lielausis (1965) found that certain temperature sensitive mutants of genes 34, 36, and 37 exhibited serological phenotypes

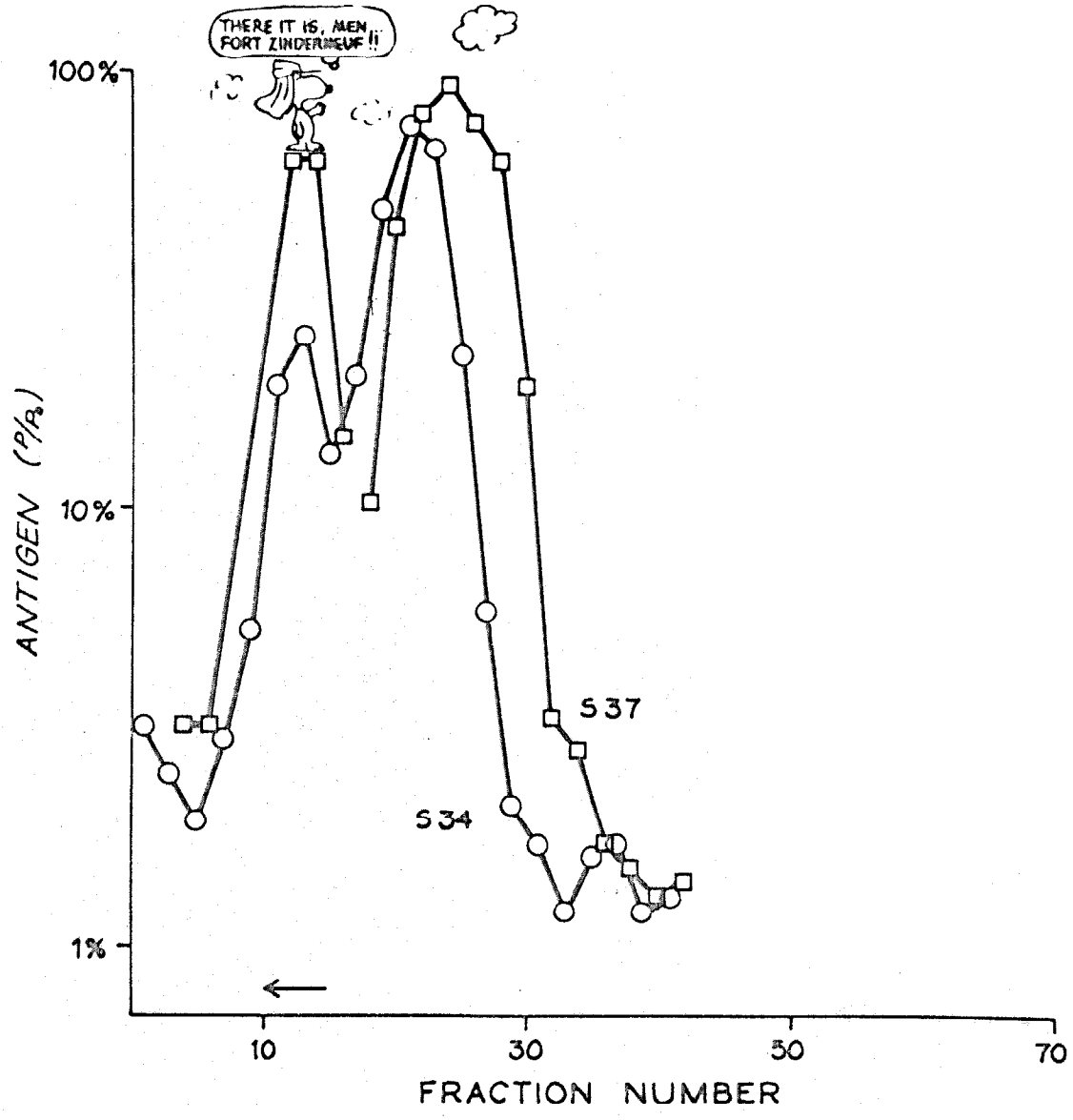
somewhat different from those of the amber mutants defective in these genes; though no viable phage were produced in cells infected under restrictive conditions with these mutants, the antigen controlled by the mutant gene was made. In the hope of finding mutants affecting in novel ways the assembly of the fiber or its attachment to the phage, I determined the antigen sedimentation patterns of lysates made with a number of these ts mutants. One mutant, tsN1, which maps at the extreme right end of gene 34, was of particular interest. Lysates made from this mutant exhibited the 8S BC and 9S A components, and also the 10S ABC component (Fig. 22). However, an experiment comparable to that described in Fig. 17 showed that the particles from such a lysate had no associated tail fiber antigen (data not shown). Apparently the tsN1 mutational lesion does not prevent the A component from joining with BC, but does prevent the attachment of the resulting ABC structure to the phage particle.

DISCUSSION

Assembly of the tail fibers

On the basis of their lethal phenotypes, six genes - 34, 35, 36, 37, 38 and 57 - and more recently a seventh - gene 63 - have been implicated in tail fiber formation and attachment. Based on studies of mutants defective in these genes by Edgar and Lielausis (1965), Edgar and Wood (1966), and Eiserling, Bolle and Epstein (1967) - and on those reported here - a pathway for the assembly of the tail fibers of bacteriophage

Figure 22. 10S ABC component from a lysate of tsN1. Experimental conditions as in Fig. 20. O = A antigen. □ = C antigen.


THERE IT IS, MEN, FORT ZINDERNEUF !!
100%
10%
1%
ANTIGEN (%)
S37
S34
10
30
50
70
FRACTION NUMBER

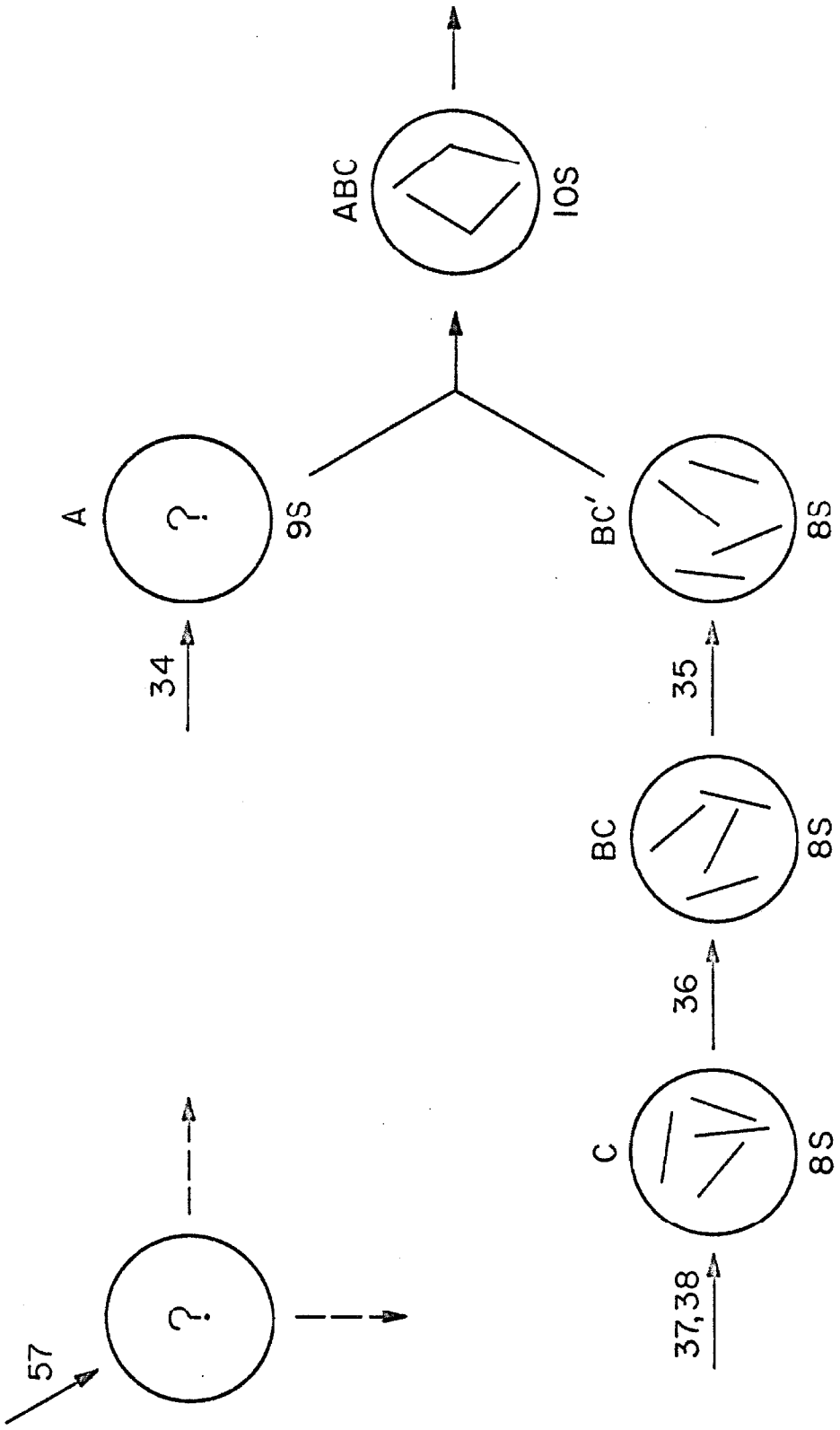
T4 is proposed (Fig. 23). The pathway describes a sequence of interaction of gene products, not an ordering in time of gene transcription or translation. (This point is considered further in the General Discussion).

The experimental observations - and the conclusions drawn from them - which have led to this picture of tail fiber assembly are summarized below.

It was shown in Part I of this thesis that the substrate for fiber attachment is the fiberless particle; none of the precursor structures, such as the tail, may serve as a substrate. A mutant lysate which lacks the substrate for fiber attachment would be expected to accumulate the last stable intermediate in the fiber assembly pathway prior to attachment to the substrate particle. Such lysates (Table 9) contain a component which sediments at 10S and exhibits all three antigens, A, B, and C. Examination of fractions containing this component revealed structures with the dimensions and kink characteristic of whole tail fibers. These observations indicate that 1) the 10S ABC fraction contains whole tail fibers, and 2) tail fibers are formed in cells infected with mutants blocked in head formation and in tail formation. The formation of the tail fiber can thus proceed in the absence of the formation of the substrate particle.

Tail fibers are not present in lysates made with mutants defective in genes 34, 35, 36, 37, 38, or 57. The products of these six genes are thus required for the formation of the whole fiber, and presumably determine its formation.

Figure 23. Pathway for T4 tail fiber assembly. Numbers over the arrows refer to genes controlling that step. Letters above boxes give antigenicity of strucure. Boxes show electron microscope morphology. Sedimentation coefficients are given below boxes.


57
?
37,38
C
8S
36
BC
8S
35
BC'
8S
34
A
?
9S
ABC
10S

None of the antigens present in mutant lysates defective for these genes are associated with the particles present in the lysates. Fiber precursor components apparently cannot bypass the 10S ABC assembly stage and attach independently to the particle. Thus the tail fiber is not assembled on the particle; apparently the complete fiber is first formed and subsequently attached to the phage particle.

Recently Wood (in preparation) has found that the attachment of the fiber to the particle is under the control of gene 63. The product of this gene exhibits properties expected of an enzyme which joins tail fibers to phage particles.

The sequence of steps leading to the formation of the fiber, as shown in Figure 23, was derived from the following considerations. Lysates made with mutants defective in any of genes 35, 36, 37, or 38 contain a 9S component with antigenic specificity. This component is absent in 34-defective lysates. Therefore this 9S A component is the product of gene 34, and is formed independently of the steps controlled by genes 35, 36, 37, and 38.

However, 34-defective lysates contain 8S material with antigenic specificities B and C. This material is a product of the activity of gene 35 as judged by the fact that the material can complement 35-defective extracts <u>in vitro</u>. That this material, termed the BC' component, is in fact the product of a sequence of steps controlled by genes 38, 37, 36 and 35 derives from the following observations.

Though 35^+ activity can be recovered from 34-defective extract, it is not recoverable from 37-defective extract under the same conditions.

The formation of stable 35^+ activity therefore requires 37 gene function and presumably the 37 and 38 gene product, the C component. This point, and the fact that the 35^+ activity cosediments with the B and C antigens at 8S indicates that they are on the same physical structure.

An 8S BC component is found in 35-defective lysate. Therefore the 35 step is not required for the formation of BC. Rather BC appears to be the precursor of BC'. 36-defective lysates contain an 8S component, but it lacks the antigenic specificity B. This material, the C component, appears to be the precursor of BC, since B is not formed in the absence of C (that is, in 37- and 38-defective lysates).

A prediction of the pathway as shown would be that the C and BC components would fail to complement 35-defective extract. This prediction has not been tested. Note that though the 35 step cannot be required prior to the 36 step, since 35-defective lysates contain B antigen, none of the arguments presented rule out the possibility that the 36 and 35 steps proceed independently of each other.

The role of gene 57 in fiber formation remains obscure. Since the function of gene 57 is required for the formation of all three antigens, gene 57 has been represented as controlling a step at the beginning of the pathway.

The nature of the step resulting in joining of A and BC has not been determined. This step may be spontaneous, or the gene controlling it may have escaped identification thus far. It should be noted that intermediates in the pathway may exist which have not been explicitly identified. For example, A and BC' may interact to yield a structure

which is antigenically A:BC, but is still perhaps morphologically a half fiber. This is discussed at further length in the next part of this discussion.

Structure of the tail fiber

Temperature sensitive mutants of both genes 34 and 37 exhibit intragenic complementation, indicating that the 34 and 37 proteins are multimers (Bernstein, Edgar, and Denhardt, 1965).

Host range mutants which result in the loss of the ability of T4 to adsorb to E. coli B map in gene 37 (R. J. Huskey, unpublished results; J. King, unpublished results). The host range difference between T2 and T4 maps in or near gene 38 (Russell, 1967). The 37 and 38 gene products may both become structural proteins of the C component or one may modify the other catalytically. Note that the 37 and 38 gene products have not been identified independently of each other, but only as a complex.

Genes 34 and 35 were originally thought to be unusually long because of the high recombination values found in crosses between mutants within either gene. However, subsequent studies indicate that the high recombination values were not due to the length of the genes but to unusually high recombination in this region of the T4 genome. Mosig (1966) measured the physical length of T4 genes and found that genes 34 and 35 were no longer than most T4 genes. Furthermore, Russell (1967) found that genes 34 and 35 of T2, which are functionally analogous to genes 34 and 35 of T4, did not exhibit unusually high recombination values in intragenic crosses.

Amber mutations in gene 34 are polar with respect to gene 35, indicating that these two genes are cotranscribed, and that the direction of reading is 34 $\longrightarrow$ 35 (Nakata and Stahl, 1967).

The gene 36 and gene 35 products have not been identified; only the products of their interactions with the C and BC components, respectively have been identified. Thus these gene products may act catalytically on the precursor components, or may combine with them without detectably altering their sedimentation behavior. The end product of the action of genes 35, 36, 37 and 38 is BC'. From morphological considerations it would appear that the tail fiber contains two such components.

It is not clear how many A components are in the completed fiber. However, regardless of the number, one might predict the existence of an A:BC half fiber intermediate. Such a structure might be expected to cosediment with the complete tail fibers at 10S. Since the arms of the whole fiber are not morphologically distinguishable from BC', any intermediate stage would probably also be indistinguishable. Thus none of the experiments I have performed would have detected such an additional intermediate structure. It may be, however, that such a structure is formed, but is converted to a complete fiber too rapidly to permit significant accumulation.

The formation of the tail fiber now appears to be a mutable subject for the study of the interactions among structural proteins during morphogenesis. A well developed genetic system exists including a variety of classes of mutants; amber, temperature sensitive, host range,

and cofactor requiring. Two highly specific assays exist for a number of the components in the pathway; the serological assay and the *in vitro* complementation assay. The former is likely to be insensitive to small disturbances in protein structure, while the latter is likely to be highly sensitive to such disturbances.

The complex assembly line leading to the formation of the tail fiber and the fiber's function as a specific adsorption and trigger organ, indicate that it represents a molecular machine deserving of further investigation.

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"Hallo!" said Piglet, "what are you doing?"

"Hunting," said Pooh.

"Hunting what?"

"Tracking something," said Winnie-the-Pooh very mysteriously.

"Tracking what?" said Piglet, coming closer.

"That's just what I ask myself. I ask myself, What?"

"What do you think you'll answer?"

"I shall have to wait until I catch up with it," said Winnie-the-Pooh.

GENERAL DISCUSSION

The investigations of T4 assembly by R.S. Edgar, W.B. Wood and others in this laboratory have now progressed sufficiently to reveal the general outlines of the phage assembly process. The combined complexity and accuracy of this process is impressive. Whether other biological structures, such as flagella, centrioles, and chloroplasts are assembled in an analogous manner will have to await further investigation. Nonetheless, I would like to discuss some general aspects of biological assembly processes, in light of the phage studies.

The Nature of Assembly Processes

Mechanisms for ensuring the correct self-assembly of complex structures can be divided into at least two general classes. In class (I) the parts of a structure are so designed that they fit together in only one arrangement; the structure is completely determined by its component parts. The single possible arrangement of the parts may be assembled sequentially, or non-sequentially. If sequenced, the absence of a part results in correct assembly of the structure up to the step requiring the missing part. Parts normally added subsequent to the missing one remain free, or, if the assembly line is branched, assemble correctly up to the point where they would interact with the missing product. If the assembly is not sequenced, all other parts assemble accurately in the absence of any missing part; addition of the missing part completes the structure. Both of these descriptions represent ideal cases.

In the second class (II) of mechanisms, correct assembly is determined by the sequential appearance in time of the parts. Contrary to class I, the parts can be assembled into more than one arrangement — though only one arrangement is correct. Correct assembly is achieved by having the parts become sequentially available in the right order. This mechanism differs from the first in that aberrant products of the assembly process are expected. Consider a structure 1-2-3. If any 1 or 2 remains unreacted after the first step, $1 + 2 \rightarrow 1 - 2$, the appearance of 3 will result not only in the formation of the correct 1-2-3 structure, but in aberrant 1-3 and 2-3 structures.

Note that these mechanisms cannot in general be easily distinguished from each other; the assembly process must be perturbed; for example, by removing a necessary part.

Class I assembly requires that the interactions between the parts of a structure be highly specific. However, the accuracy of the assembly is essentially independent of the concentrations of the parts.

In class II assembly, little specificity is required in the interactions between the parts. However, accurate assembly requires tight control over the concentrations of the parts and the change in these concentrations with time.

Class I accuracy depends on the energetic relationships between parts. Class II accuracy depends on the kinetic relationships between parts.

These mechanisms are of course not exclusive of each other; both may contribute to the accuracy of a single step, or to ensuring the

correctness of different steps in the same pathway.

The evidence now available suggests that phage assembly is essentially a class I process. Tail and tail fiber assembly represent examples of sequential class I assembly; the parts can only be assembled in a specific order. Removal of a required part (by mutation) does not result in the formation of aberrant structures, with the exception of polysheath. Examples of non-sequential class I assembly are the steps controlled by genes 11 and 12. Though the 11 and 12 gene products ordinarily interact with the baseplate early in tail assembly, particle assembly proceeds in their absence. Addition of 11 and 12 gene products to the incomplete particles yields viable phage.

Processes which appear to resemble class II assembly are evident in sheath and head formation. If necessary parts are missing, aberrant structures form; polyhead and polysheath. If the gene 66 product is missing the head subunits form into an aberrant small head. Thus both sheath and head assembly are sensitive to the concentration and time of appearance of certain other phage components.

However, closer examination reveals that these are not in fact examples of class II assembly. What is required in the formation of sheath is that the substrate be present at the same time as the other parts. No ordering of steps in time is required. This also appears to be true for the formation of the head. What is required is that the gene 20 product, for example, be present at the same time as the gene products necessary for head formation, in order for heads rather than polyheads to be formed. These are simply cases where the com-

ponents are not completely unreactive with respect to an aberrant assembly. In fact, polysheath and polyhead are formed only late in the phage growth cycle indicating that the sheath and head subunits do not efficiently assembly into the aberrant form.

These cases of aberrant assembly involve repeating subunit structures. Identical subunits differ from most parts in that they are their own substrates. They cannot be designed in terms of class I mechanisms since by definition repeating subunits can always form more than one possible structure.

One possible mechanism by which the oorrect assembly of repeating subunits could be assured is as follows. Consider a subunit "s" which is in equilibrium with an active state s', where s' tends to polymerize. Let A be the normal substrate for this polymerization. Since s' is its own substrate aberrant polymerization will go as the square, or higher power, of the s' concentration. If spontaneous polymerization is not to occur in the absence of A, the equilibrium between s and s' will have to be far over toward s. However, this is inefficient for two reasons; 1) a large energy barrier will be required between the two states, and 2) very low concentration of s' may also slow the formation of the correct structure. Clearly the solution to this problem is to maintain A in high concentration.

Other than the special case of repeating subunit structures, all available evidence indicates that aberrant structures are not found when steps in phage assembly are blocked: This is a strong argument for phage assembly being class I. Furthermore, phage are produced

throughout the latent period. Since all necessary proteins must be present in the cell by the time the first phage are produced, it is exceedingly difficult to imagine how phage assembly could be controlled by a class II process; T4 assembly does appear to be so designed that the parts fit together in only one way.

Regulation of Phage Protein Synthesis

The problem of optimally designing an assembly line with perhaps 100 steps is a formidable problem. Whatever the solution, its implementation will require a mechanism for allocating the total protein synthetic capacity among the various species of proteins required.

In certain cases regulating the stoichiometry of a pair of components is particularly important; for example, when a large number of proteins interact with a single substrate. Consider the formation of the sheathed tail; if the concentration of sheath subunits drops to half of the concentration required to complete all the naked tails present, the yield of sheathed tails will drop not by a factor of two, but by some much higher order. Therefore a large negative perturbation in the ratio of sheath subunits to naked tails would sharply decrease viable phage yield. A mechanism is required to coordinate the production of sheath subunits and naked tails. Other examples could be adduced; the ratio of core subunits to baseplates, the ratio of tail fibers to heads.

As noted in the Discussion to Part II, a number of pairs of T4 genes appear to be cotranscribed (Stahl, Murray, Nakata, and Crasemann,

1966). It may be that such cotranscription provides a mechanism coordinating the synthesis of a pair of polypeptide chains. Recently a model of the control of protein synthesis has been presented in which the transcription of a cistron is determined by the nature of the chain initiating ribosome attachment site of the messenger RNA (Yanofsky and Ito, 1966; Martin, Silbert, Smith, and Whitfield, 1966). In this model ribosomes initiate translation at the operator end of the messenger RNA. After translating the first cistron the ribosome has some definite probability of continuing on to translate the second cistron which is determined by the initiation region of the second cistron. Therefore the rates of synthesis of different proteins can be varied, But any pair of cotranscribed genes will be translated in some fixed ratio, regardless of the absolute level of protein synthesis, according to the nature of the two initiation sites.

A pair of cotranscribed genes may indeed control the ratio of sheath protein synthesis to core production. It was shown in Part I of this thesis that gene 18 controls a sheath structural subunit and gene 19 controls in part the formation of the core. I have performed preliminary experiments with amber mutants in these two genes which indicate that gene 18 amber mutants are polar to gene 19. Thus the 18 and 19 cistrons may form an operon for the purpose of controlling sheathed tail formation. Similarly the cotranscription of genes 34 and 35 (Nakata and Stahl, 1967) may coordinate the production of the A and BC' components of the tail fiber. The function of the other polar gene pairs identified - 48 → 54, 9 → 10, 13 → 14, and 53 → 5

(Stahl, Murray, Nakata, and Craseman, 1966; Stahl, personal communication; Edgar and Lielausis, 1967) - are not sufficiently well understood to determine if in these cases this interpretation of polarity is tenable.

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