Structure and Assembly of Bacteriophage T4 Tail Fibers

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Abstract

The structure and assembly of bacteriophage T4 tail fibers was examined as a model system for studying assembly of multiprotein structures. The function of six genes (34-38 and 57) is necessary for assembly of tail fibers. The role of each of these genes in assembly was examined by isolating and characterizing whole fibers and four precursors which accumulate in lysates of mutant phage-infected bacteria. Each isolated structure showed a single major electrophoretic component on polyacrylamide gels indicating near homogeneity. Electron microscopic examination of these isolated structures revealed that the whole fiber consists of two halves; one, requiring genes 57 and 34 for its assembly, is a rod 690 x 27 Å with a knob on one end and an antigen A; the other, requiring genes 35, 36, 37 and 57 for its assembly, is a rod 690 x 26 Å containing antigens B and C (designated BC'). The antigen B is introduced into this rod by the action of gene 36 which increases the length of a half fiber precursor,C, the product of genes 37,38 and 57, from 560 to 690 Å. The resulting BC precursor is converted to BC' under the control of gene 35, a step necessary to allow interaction with the A half fiber, but one making no morphological or serological change in the fiber.

The subunit composition of the isolated fibers and their precursors was examined by dissociation at 100°C and gel electrophoresis in the presence of the anionic detergent sodium dodecyl sulfate (SDS). A was found to contain a major polypeptide of molecular weight 150,000. C, BC and BC' were

each found to contain a major polypeptide of molecular weight 123,000. Minor components were also present but they were not reproducible. Because the major polypeptides were so large they could be resolved on SDS gels of crude lysates of mutant infected cells. These gels showed that amber mutations in gene 34 eliminated the 150,000 polypeptide and amber mutations in gene 37 eliminated the 123,000 polypeptide. This indicates that these polypeptides are the products of genes 34 and 37 (P34 and P37). The other tail fiber genes did not affect the synthesis of P34 and P37 except that amber mutations in gene 36 reduced the amount of P37 by 40%, suggesting that these genes are co-transcribed. Molecular weight estimates of the fiber precursors show that there are two copies of P34 in the A half fiber and two copies of P37 in each of the other half fibers.

Mutations in genes 57 and 38 affected the apparent solubility of P34 and P37 and allow these polypeptides to be dissociated in SDS at 37°C. This is consistent with P57 controlling the dimerization of P34 leading to the A half fiber and P38 and P57 controlling the dimerization of P37 leading to the C half fiber.

A new apparatus for destaining acrylamide gels electrophoretically and a new method of fractionation and scintillation counting of radioisotope-labeled acrylamide gels are also described.

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General Introduction

Our understanding of the way genes specify the synthesis of individual proteins is a significant achievement of molecular biology. Synthesis of individual proteins, however, is not generally sufficient for their function inside the cell. Few intracellular proteins function as single polypeptide chains; many enzymes and structural proteins associate into functional complexes of several or many polypeptides. Understanding the structure of these complexes and the mechanisms of their formation is a current challenging problem in molecular biology.

The structure and assembly of many complexes are being actively studied. Recent reviews cover work on viruses, flagella, bacterial pili, microtubules, ribosomes, muscle fibers, membranes, cell walls and fibrin clots (1) and also serum complement (2), enzyme complexes (3) and protein quaternary structure (4). The assembly of many of these protein complexes has been studied by isolating the complex, dissociating it with mild denaturing agents and studying the reassembly of the dissociated parts into the functional complex. The most complicated structure which has been dissociated and reassembled successfully is the *E. coli* 30S ribosomal subunit (5,6,7). The isolated 30S subunit can be dissociated into individual polypeptides and then reassembled into a functional unit by mixing these with the

ribosomal RNA. This implies that all the information required to specify the complete structure is contained in the RNA and polypeptide chains which comprise the assembled structure. This property indicates that the 30S ribosomal subunit is a self-assembling structure. Many enzyme complexes (3), some simple viruses (1) and perhaps bacterial flagella (1) may also be self-assembling.

On the other hand there are several systems which are not self-assembling. By this I mean that all the information for their assembly is not contained in their structural polypeptides. The insulin molecule is the simplest example of a non-self-assembling system. Insulin cannot be reassembled from its two polypeptides because it is normally formed by cleavage of a larger polypeptide , proinsulin (8). Some information for assembly is lost in the process of proinsulin cleavage. Other systems in which structural polypeptides are cleaved in the course of assembly include polio virus (9) and the head of bacteriophage T4 (10).

Another way in which information other than that in structural polypeptides can be used to assemble complexes is to modify a polypeptide after its synthesis. The formation of covalent cross links between the polypeptides of structural proteins (11) and the addition of carbohydrates to membrane proteins (12) are examples of such modification. Polypeptides to which carbohydrates have been added may or may not be self-assembling after the carbohydrate addition.

Further understanding of both self- and non-self-assembly is desirable because functional complexes are widespread and the control of their formation and function may be an important element in cellular control of metabolism and development.

An advantageous system for studying assembly of protein complexes is the bacteriophage T4. T4 is a large bacteriophage of molecular weight approximately 2.5×10^8 about half of which is protein and half DNA. The phage consists of a DNA filled head attached to a tailwhich has six long slender tail fibers attached to its base. At least 46 genes are involved in the morphogenesis of this structure (13). The complexity of the phage morphology and the large number of essential morphogenetic genes suggest that assembly of the phage may illustrate various ways in which information can be supplied to determine organization of protein structures. The major experimental advantage of T4 is that the availability of mutants in the morphogenetic genes allows experimental interruption of the assembly process. By studying the interrupted step in assembly one can infer the normal function of the defective gene. Direct studies of some of the interrupted steps are possible because they can be completed <u>in vitro</u> by mixing extracts of cells infected with mutants defective in complementary assembly genes(14). The different extracts supply the missing gene products to each other allowing assembly to proceed.

At least sixteen steps in assembly can be carried out in vitro (13). Isolation of assembly intermediates which accumulate when these steps are mutationally blocked has allowed the steps to be ordered into a pathway of assembly. For example genes 3 and 18 are involved in tail assembly (15). If tail precursors are isolated from a 3-defective extract by zone sedimentation they complement an 18-defective extract in vitro implying that the gene 18 product (P18) has acted on them. In contrast, if tail precursors from an 18-defective extract are isolated by zone sedimentation they do not complement a 3-defective extract, indicating that P3 has not acted on them. Therefore the sequence of action of these gene products is P18 then P3 (15).

The sequence does not reflect temporal control of gene expression because the gene products are synthesized simultaneously. Instead the sequence represents control of the order of interaction by the gene products themselves. Other steps have been sequenced in a similar manner aided by electron microscopic examination of the precursors (13-17) and these can be ordered into the pathway of phage assembly shown in Figure 1 (13). The figure indicates that the phage is assembled in three separate parts, head, tail, and tail fibers, which are joined together to form the complete virus. In order to learn how interactions lead to ordered assembly and to learn how individual gene products contribute to assembly, I chose to study the assembly of the

Fig. 1. Pathway of T4 morphogenesis.

The numbers represent the genes involved in each step of assembly. Solid arrows indicate steps which can be completed *in vitro*. Broken arrows indicate steps which have not yet been shown to work *in vitro*, but whose sequence is inferred by electron microscopic examination of defective lysates. The structures shown as intermediates are observed in electron micrographs of lysates of cells infected with phage carrying mutations in the genes controlling the step following the structure.

tail fiber in detail.

The tail fibers were chosen for several reasons: their assembly is the simplest branch of the assembly pathway; their antigens have been extensively studied and a simple quantitative assay for antigenicity is available; the assembled fiber can be easily assayed for biological activity *in vitro*; the genes controlling fiber assembly have been examined genetically in detail and many mutations in these genes are available.

The study of fiber assembly has focused on three specific questions:

(1) Does each gene product required for tail fiber assembly become incorporated into the completed fiber or do some of them act as catalysts of assembly?

(2) What kind of bonds hold the structural polypeptides of the tail fiber together?

(3) What imposes the obligate ordering of the steps in fiber assembly?

The general approach which I have taken to answer these questions is to isolate and structurally characterize the assembled tail fiber and four apparent intermediates in its assembly. Part I of this thesis is a manuscript, presently in press in the *J. Mol. Biol.*, describing the method of isolation of these structures and their characterization by gel electrophoresis, serology, electron microscopy and assembly *in vitro*. Part II is a manuscript, submitted to

J. Mol. Biol., which continues the characterization of the isolated fiber structures by determining their major polypeptide subunits their genetic control, and some of the properties of these subunits before and after assembly. The General Discussion relates the results of these studies to the specific questions posed above and suggests further experiments to answer the questions more fully. Part III described apparatus and methods which were developed in the course of these studies to facilitate the analysis of tail fiber subunit structure by acrylamide gel electrophoresis.

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Assembly of Bacteriophage T4 Tail Fibers: II.[‡] Isolation and Characterization of Tail Fiber Precursors

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Summary

Complete T4 tail fibers and four precursor structures (half fibers) were purified from lysates of mutant-infected E. coli cells, using serum blocking assays for tail fiber antigens to monitor the isolation procedures. Each of the final preparations showed a single major electrophoretic component on polyacrylamide gels. The purified structures, designated whole fibers and A, BC', BC, and C half fibers on the basis of their antigenic determinants were further characterized by electron microscopy and assays for ability to produce viable phage in vitro when combined with appropriate extracts of mutant-infected cells.

Purified whole fibers are similar in dimensions and appearance to the tail fibers seen on complete phage, and can be attached quantitatively to tail fiberless particles in vitro to produce active virus. The whole fiber is composed of one A and one BC' half fiber, each about 690 Å in length and 45 Å in width, joined end to end at an angle of about 160°. The isolated A half fiber carries a knob at one end, which is also visible at one end of the whole fiber. Since the A half is proximal to the tail in the completed phage, the knob presumably represents the point of tail fiber attachment to the baseplate. The A half fiber may not be a normal intermediate in whole fiber assembly, since it is not visible in most crude extracts containing A

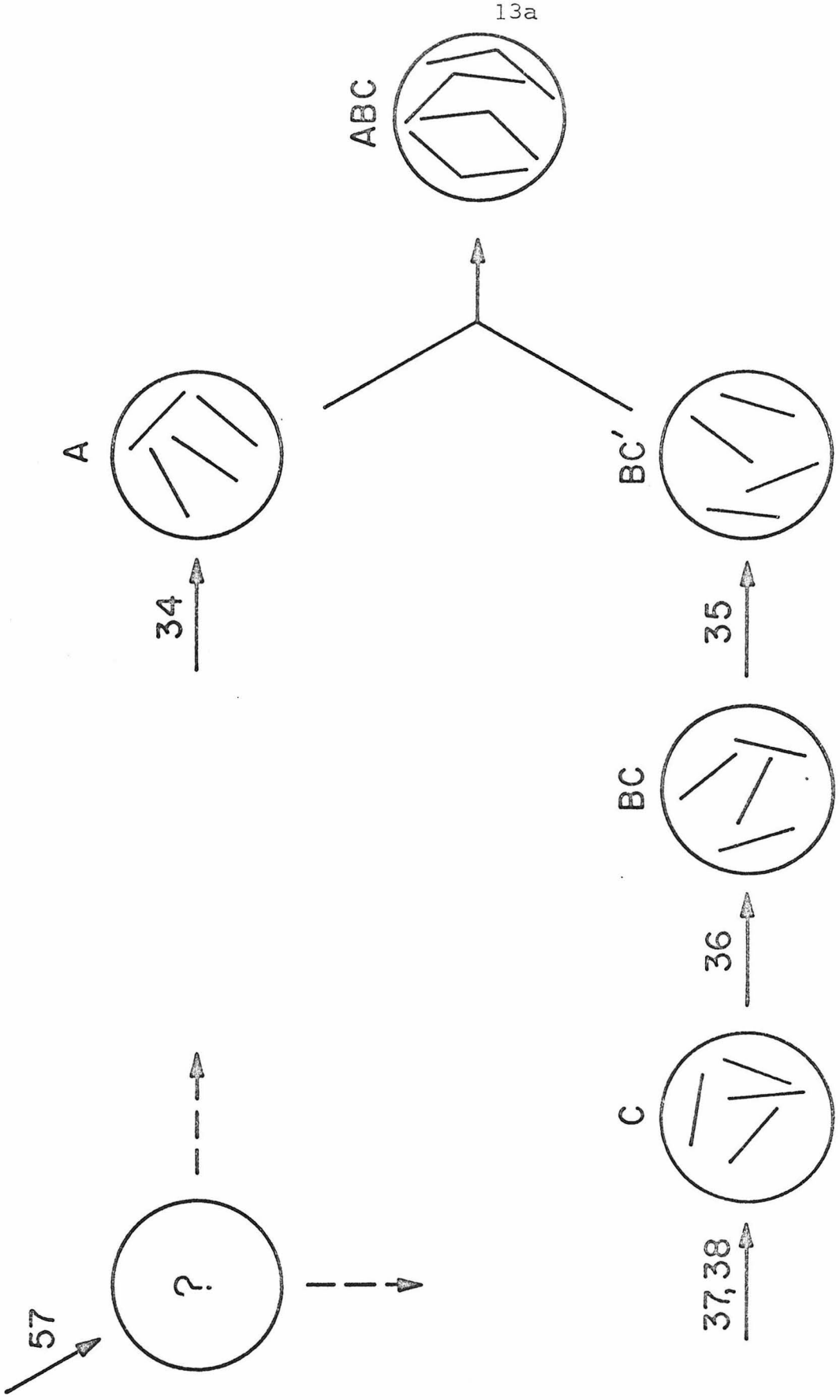
antigen, and retains little *in vitro* activity through the purification procedure. The BC' half fiber, by contrast, appears to be the normal precursor of the distal half of the whole fiber; it is visible in crude extracts and retains its *in vitro* activity in the purified state. BC, the probable precursor of BC', is inactive *in vitro* when purified but indistinguishable from BC' in dimensions and serological properties. C, the probable precursor of BC, is also inactive *in vitro* when purified. Besides lacking the B antigen it is shorter than BC, being only 560 Å in length.

1. Introduction

Bacteriophage T4 has six tail fibers which are necessary for its attachment to the host bacterium (Kellenberger *et al.*, 1965; Simon & Anderson, 1967 *a,b*). Their formation is under the control of at least six phage genes, (Epstein *et al.*, 1963; Edgar & Lielausis, 1965). Five of these (genes 34, 35, 36, 37 and 38) are located in a cluster on the genetic map and the sixth (gene 57) is unlinked (Edgar & Lielausis, 1965).

Edgar & Wood (1966) demonstrated that several steps in assembly of the tail fiber could be carried out *in vitro* by combining extracts made from bacteria infected with phage defective in different tail fiber genes (*in vitro* complementation). The tail fiber precursors present in such extracts were characterized by their appearance in electron micrographs, antigenic properties and complementing ability *in vitro* by King & Wood (1969), who proposed the following sequence of gene product interaction in tail fiber assembly based on their observations (Fig.1). Genes 57 and 34 control the formation of a structure carrying an antigen designated A which, as we shall show below, can appear as a half fiber in electron micrographs. Genes 57, 37 and 38 cooperate to produce a similar structure carrying a second antigen, designated C. Gene 36 controls the addition to C of a third antigen, B, to produce the BC half fiber. Gene 35 controls

FIG. 1. The pathway of T4 tail fiber assembly. (Adapted from King & Wood, 1969). Numbered arrows indicate the genes controlling the various steps; circled symbols represent the electron microscope morphology of the structures which accumulate when the pathway is blocked by mutations in the succeeding gene. The antigens associated with each structure are shown by the letter above the circles.


57
?
ABC
A
BC'
BC
C
34
35
36
37,38

the conversion of BC to BC'. This step does not affect the antigenic properties of the structure but is necessary to allow interaction with the A half fiber. A and BC' combine to produce the whole fiber with its characteristic central kink. Throughout this paper fiber structures will be referred to by their letter designations in Figure 1.

Elucidation of this sequence represented a first step toward understanding the molecular details of fiber assembly. As a continuation of this work, we report in this paper methods for purifying each of the structures shown in Figure 1. We have characterized these by serological analysis, electrophoresis on polyacrylamide gels, electron microscopy and by assembly into complete tail fibers in vitro. Evidence that the isolated structures represent normal intermediates in fiber assembly is discussed. A preliminary report of this work has been published (Ward & Wood, 1968).

2. Materials and Methods

(a) Media. See King & Wood (1969)

(b) Bacteria and mutant bacteriophage

In addition to the E. coli strains described by Wood & Henninger (1969), strain Bb (from S. Champe) was used as the restrictive host for preparing all lysates; strain G (λ) (restrictive for both amber and rII mutants) was used as the plating indicator in serum blocking assays, and strain CR63 (λ) (permissive for amber mutants; restrictive

for *rII* mutants) was used as the plating indicator for *in vitro* complementation assays.

Phage *amber* mutants derived from the standard type T4D were obtained from the collection of R.S. Edgar. Some of the multiple mutants used have been described by Wood & Henninger (1969); the remainder were constructed by appropriate crosses and are described in Table 1.

(c) *Specific antisera*

In the earlier stages of this work sera specific for the antigens A or B and C were prepared by adsorption of a hyperimmune anti-T4 rabbit serum (donated by J. Flatgaard) with appropriate antigen-defective lysates (Edgar & Lielausis, 1965; King & Wood, 1969). Adsorbed anti-A was the gift of J.King. Subsequently, specific hyperimmune rabbit antisera were prepared directly by injection of purified antigenic components (Fraction III A and Fraction IV BC', see Tables 2 and 3). Both of the sera obtained inactivate T4 with first order kinetics to a survival of less than 0.01. The first order inactivation rate constants (*k*) for the undiluted sera under conditions described below are 1.1×10^4 min^{-1} and 7.5×10^3 min^{-1} for anti-A and anti-BC' respectively. The inactivation of phage by these sera is blocked completely by whole phage and by the purified tail fiber antigens used to prepare each serum. More than 90% of the neutralizing activity of the anti-BC' serum is directed

Table 1. T4 multiple mutants

Multiple mutants were constructed by appropriate crosses of single amber mutants from the collection of Dr. R.S. Edgar. The genotype of each mutant was verified by measurement of genetic complementation and wild-type recombinant production in crosses of the multiple with appropriate single amber mutants.

TABLE 1

T4 multiple mutants

Mutant Designation	Amber mutations	Defective Genes	Experimental Use
SXO	E18, B17, N120	18, 23, 27	Source of ABC†
SX1	E18, B17, N120 A455, rdf41*	18, 23, 27, 34, rII	Source of BC'†
SX6	E18, B17, N120 N52, rdf 41*	18, 23, 27, 37, rII	Source of A†
SX7	E18, B17, N120 A455, B252, rfd41*	18, 23, 27, 34, 35, rII	Source of BC†
SX8	E18, B17, N120, A455 E1, rfd41*	18, 23, 27, 34, 36, rII	Source of C†
EX121	B252, E1, N52, C290	35, 36, 37, 38	Source of crude A‡
EX111	E1, N52, C290	36, 37, 38	Source of crude A and P35‡
X2J	N52, B280	37	Source of crude A, P35, P36‡

* Deletion of rII cistrons A and B.

† For purification.

‡ For *in vitro* complementation.

against antigen C, and this serum was used to measure C antigen. When this serum is adsorbed with excess Fraction II purified C the 8% neutralizing activity remaining is directed against antigen B. This adsorbed serum was used to measure B antigen and is similar to the anti-B serum described by King & Wood (1969).

(d) Serum blocking assays

The specific antisera were used to measure the antigen in various fractions during purification by two types of serum blocking assay. Both were carried out at 46 to 48°C in SB buffer (0.98 g Na_2HPO_4, 0.42 g $NaH_2PO_4 \cdot H_2O$, 0.6 g $MgSO_4$, 1 g bovine serum albumin per liter H_2O). The more sensitive and reproducible of the two is the end-point serum blocking assay (DeMars, 1955; Edgar & Lielausis, 1965). Serum was diluted to $\underline{k} = 0.1\ min^{-1}$. Several five-fold dilutions of the sample to be assayed were made in the serum and incubated 10 to 16 hr, which is sufficient for completion of the blocking reaction. The residual $\underline{k}$ in each tube was determined by adding 2000 tester phage, incubating for 46 min at 48°C, and then plating the entire contents of the tube on G (λ) indicator bacteria to measure the surviving fraction of tester phage (P/P_o). Standards were prepared in parallel using known amounts of rII phage in the blocking reaction, and the results plotted as P/P_o versus rII phage concentration on log-log coordinates. Using this standard curve,

values of P/P_o for each sample assayed were converted to phage equivalents of serum blocking power (SBP). The SBP values so obtained are linearly related to antigen concentration. One unit of SBP was defined for each antigen as 10^{12} phage equivalents. This assay can measure as little as 10^{-4} units of antigen, is reproducible within 15% and takes about 24 hr.

When more rapid assays were desired, SBP was determined by comparing rates rather than final levels of serum inactivation by blocking antigens (Israel, Anderson & Levine, 1967). The tester phage and blocking antigen were added simultaneously to serum at a $\underline{k}$ of 0.1 and plated after 46 min. Partially purified antigen preparations previously calibrated by end point SBP assays were used as standards, and SBP of samples was determined by comparison to a standard curve as above. This kinetic assay can measure about 5×10^{-3} units of antigen, is reproducible within 20%, and requires about 6 hr. It was used for preliminary assay of fractions during the purification, while the end-point assay was used to determine final recoveries.

(e) Electrophoresis on polyacrylamide gels

Gel systems at two pH values were used. The pH 4.3 system was essentially that of Reisfeld, Lewis & Williams (1962); the pH 9.5 system was that of Davis (1964). The acrylamide concentration in all gels was 4% with a 38:1 ratio

of acrylamide to methylene bis-acrylamide (Eastman). Samples were applied in 10% glycerol and no sample or stacking gels were employed. Gels were stained with Coomassie Brilliant Blue (Mann R250) and destained electrophoretically by the method of Ward (1970). To assay the distribution of antigens following electrophoresis, gels were fractionated by extrusion through a syringe as described by Ward, Wilson & Gilliam, (1970).

(f) Preparation of crude lysates

E. coli strain Bb was grown in K medium to a cell concentration of 4×10^8/ml. at 30°C. The cells were infected at a multiplicity of 4 phage/cell and superinfected twice at multiplicity 2 at 15 and 25 min after infection. Depending on the fiber structure desired, the appropriate multiply mutant phage was used for infection as described in Table 1. Two hours after infection chloroform was added to lyse the cells. Ten min later DNase (Sigma DN-c) and RNase (Sigma) were added to 1 µg/ml. and egg white lysozyme (Sigma) to 10 µg/ml. After an hour of incubation at 30°C lysates were stored overnight at 5°C. This extended lysis procedure helps to prevent the fiber structures from adhering to cell debris.

(g) Purification of tail fiber structures

A similar method was employed for purifying each of the

structures C, BC, BC', A and ABC. Use of the multiply mutant phage described in Table 1 simplifies the purification of fibers by eliminating phage heads, tails and baseplates from the lysates. All steps were carried out at 0 to 4° C unless otherwise listed.

(i) Low speed centrifugation

The crude lysates were warmed to 30°C for 10 min to ensure complete DNA digestion, and then centrifuged at 20,000 x g for 15 min to remove cell debris. The pellets were resuspended in distilled water and recentrifuged twice, and the supernatant solutions were combined (Fraction I).

(ii) Partitioning in a two-phase aqueous polymer system

A modification of the technique described by Albertsson (1960) was employed. Solid NaCl was added to Fraction I to a concentration of 0.47 M. Solid polyethylene glycol (PEG) (Carbowax 6000, Union Carbide) was then added to a final concentration of 7% (w/v) for the preparation of ABC, BC' and BC, 8% for C, and 9% for A. A 20% solution of sodium dextran sulphate 500 (NaDS) (Pharmacia) was then added to a final concentration of 0.2%, and the mixture was shaken gently. After standing for 12 hr the mixture separated into a small bottom phase containing the NaDS and a top phase containing PEG. The top phase was siphoned off and centrifuged 10 min at 20,000 x g to recover any bottom phase or

interface material not previously separated. The bottom phases and interface material, still containing some top phase, were then combined with as little mixing as possible and centrifuged at 20,000 x g for 10 min to compress the interface into a tight band. The top and bottom phases were withdrawn as completely as possible and the interface cake, containing most of the fiber structures, was resuspended in buffer I (0.01 M Tris (hydroxymethyl) aminomethane, pH 7.1; 0.005 M $MgSO_4$, 0.005 M β-Mercaptoethanol). Excess NaDS was removed by precipitation at 0°C with 0.15 vol. of 3 M KCl. After 2 hr, the mixture was centrifuged at 20,000 x g and the pellet discarded. The supernatant solution was then centrifuged at 200,000 x g for 1 hr to remove ribosomes and other remaining cell debris, and the resulting supernatant solution was recovered (Fraction II).

(iii) DEAE-cellulose chromatography

Fraction II BC', BC and C were diluted three fold into buffer I and applied to packed DEAE-cellulose (Cal Biochem Cellex D) column previously equilibrated with buffer I. (Dialysis of Fraction II preparations gave erratic recoveries). All three structures pass through the column unretarded.

Fraction II A and ABC were dialyzed 1 hr against buffer I in a rapid dialysis apparatus (Hoefer) and then applied to DEAE-cellulose columns as above. These structures were

eluted by applying either buffer I containing 0.15 M NaCl or a linear gradient to 0.25 M NaCl. Both elute at 0.1 M NaCl.

Column fractions containing antigen were pooled and concentrated in a pressure cell with a Diaglo XM 50 filter (Amicon Corp.) (Fraction III).

(iv) Hydroxylapatite chromatography

Fraction III C, BC and BC' preparations contained some PEG and a minor protein contaminant which were removed by chromatography on hydroxylapatite (Clarkson Chemical Co.). The preparations were dialyzed against buffer II (0.98 g Na_2HPO_4, 0.42 g $NaH_2PO_4 \cdot H_2O$, 0.6 g $MgSO_4$, 0.35 ml. β-mercaptoethanol per liter of H_2O) and applied to a column previously eqilibrated with buffer II. PEG is unretarded by the column. Antigen was eluted by a linear gradient to 0.25 M phosphate. Structures BC and BC' elute at 0.075 M phosphate and C elutes at 0.15 M. The antigen-containing fractions were pooled and concentrated by pressure dialysis (Fraction IV).

Structures A and ABC were not chromatographed on hydroxylapatite.

(h) Preparation of infected-cell extracts

For determination of the biological activity of the fiber structures, infected-cell extracts were prepared as described previously (Edgar & Wood, 1966) except that the

infected cells were superinfected at 15 and 25 min, and aeration was continued during the cooling to 4°C prior to centrifugation at 40 min after infection. For complementation with structures C and BC the extracts were used immediately after freezing and thawing once. For complementation with A, BC' or ABC the extracts were frozen and thawed twice, and then centrifuged (4°C) at 30,000 x g for 20 min to remove cell debris. The in vitro complementation reactions were carried out as described in the legend to Table 7.

(i) Electron microscopy

T4 phage particles of 10^{11} phage/ml. or tail fiber structures at 0.15 to 0.3 units of SBP/ml. were applied to carbon support films and stained with uranyl acetate as described previously (Wilson, Luftig, & Wood, 1970). Catalase crystals were added to the fiber preparations to provide an internal calibration standard for measurement of absolute dimensions (Luftig, 1968).

Grids were examined at 80 kV in a Siemens Elmiskop I equipped with a 200 μ condenser aperature, a 50 μ objective aperature and a pointed filament. All phage components were photographed at 45,000 X magnification on Kodalith LR 70 mm film. Dimensions were measured on the negatives using a Bausch & Lomb 7X reticular calibrated in 0.1 mm divisions. Determinations of absolute dimensions were based on measurements of catalase crystal striations in the same field as

the fibers, assuming 88 Å per striation (Luftig, 1968).

3. Results

(a) Purification of tail fiber structures

The purification of each of the tail fiber structures is summarized in Table 2. The purification procedure was first developed to prepare BC' and was applied with only minor modifications to the other four fiber structures. As indicated in the table, the recovery of antigen was highest for BC' and lower for the other structures. The recovery varied from preparation to preparation, due largely to variation in the partitioning step. The final specific activities of the structures were similar and reproducible. The overall purification was 25 to 100 fold depending on the specific activity of the crude lysate.

The purified structures can be stored at 4 to 6°C with no loss in antigenicity over a period of several months. They do, however, aggregate slowly under these conditions and lose *in vitro* activity. Storage in liquid nitrogen preserves the *in vitro* activity of Fraction III BC' for several months.

(b)Characterization by antigenicity

The SBP of each isolated structure was tested against three antisera: anti-A, anti-B and anti-BC' (essentially anti-C). The results are summarized in Table 3 and are

Table 2. Summary of the purification of tail fiber structures

The first column lists the steps of the purification as described in Materials and Method (g). Starting volumes of crude lysate were typically 1 liter, but the procedure has also been applied to 30-liter lysates, using a Sorvall centrifuge equipped with a KSB continuous flow system for the first two centrifugations. Specific activities are expressed as units (10^{12} phage equivalents) of SBP per mg of protein. C, BC, and BC' were assayed using anti-BC' sera. No correction is made for the lack of B antigen in C preparations, which makes the values of C antigen determined about 5% too low. A was assayed using anti-A serum and ABC was assayed using anti-phage serum. Protein was assayed by the method of Lowry et al., (1961) using bovine serum albumin (fraction V, Sigma) as a standard. A one-liter lysate yields 0.1 to 0.5 mg of purified protein. (-- means not measured).

TABLE 2

Summary of the purification of tail fiber structures

Fraction	Volume ml	C		BC		BC'		A		ABC	
		Specific Activity Units/mg	Recovery %	Specific Activity Units/mg	Recovery %	Specific Activity Units/mg	Recovery %	Specific Activity Units/mg	Recovery %	Specific Activity Units/mg	Recovery %
Crude Lysate	1000	0.4	100	1.2	100	0.7	100	0.8	100	3.6	100
I. 20Kxg Lysate	1100	0.6	70	--	56	0.7	81	0.7	55	4.8	--
II. Interface (200Kxg spnt)	10	10	11	5	15	25	47	4.0	16	21.0	30
III. Deae Eluate	12	47	5	45	15	50	39	43.0	8	~80.0	10
IV. Hydroxylapatite Eluate	5	--		--		60	34				

TABLE 3

Antigenicity of purified structures

SBP Antigen	Structure				
	C	BC	BC'	A	ABC
A	---	---	0.035	100	100
B	0.7	84	86	0.21	---
C	100	100	100	0.21	79

Table 3. Antigenicity of purified intermediates

Fraction III purified structures were assayed for SBP with anti-A, anti-B, and anti-BC' sera. Antigen levels ($\pm$ 15%) are expressed relative to the SBP of the major antigen in each preparation, which has been normalized to a value of 100. (--- means not measured).

consistent with the antigenic studies of Edgar & Lielausis (1965) and King & Wood (1969) in that the purified structures show essentially the same antigen ratios as the crude defective lysates from which they were isolated. This finding supports previous evidence (King & Wood, 1969) that antigens B and C are carried on the same structure.

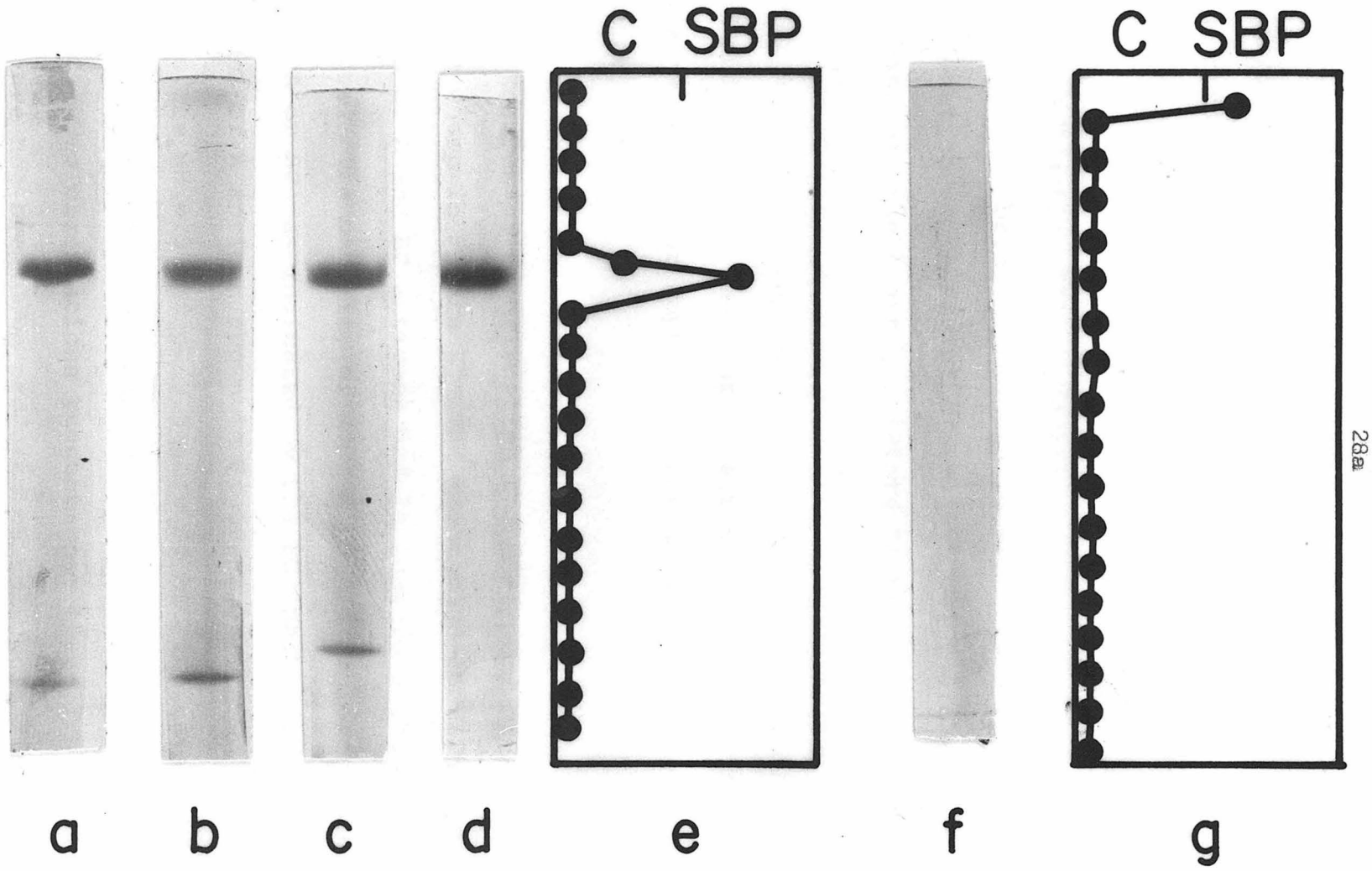
(c) Characterization by electrophoresis on polyacrylamide gels

(i) C, BC and BC'

The homogeneity of the Fraction III preparations was examined by electrophoresis on polyacrylamide gels at pH 4.3 and 9.5 (Plate I). At pH 4.3, C, BC, and BC' each show a major band with the same mobility and a minor band that migrates at the salt front (Plate I, a,b,c, respectively). Electrophoresis of Fraction IV BC; (Plate I d) shows that the minor band is removed by hydroxylapatite chromataography. Gels prepared in parallel to those shown were fractionated and assayed for SBP using anti-C serum. The results for Fraction III BC' are shown in Plate I e. Similar results for C and BC demonstrate that the major band on each of the pH 4.3 gels is the antigenic fiber structure.

A stained pH 9.5 gel of BC' and the SBP assay of a parallel fractionated gel are shown in Plate I f and g, respectively. No bands are visible, and the only antigen recovered is in the top gel fraction. This suggests that at basic Ph the antigen aggregates and only penetrates the

Plate I. Polyacrylamide gels of C, BC, and BC'. (a) Fraction III C, pH 4.3; (b) Fraction III BC, pH 4.3; (c) Fraction III BC', pH 4.3; (d) Fraction IV BC', pH 4.3; (e) C SBP assay of gel prepared in parallel to (c). (f) Fraction IV BC', pH 9.5; (g) C SBP assay of a gel prepared in parallel to (f).


C SBP
C SBP
a
b
c
d
e
f
g

top of the gel. In some fraction III preparations a non-antigenic minor band is visible at the salt front under these conditions.

(ii) A and ABC

When Fraction III preparations of A were electrophoresed at pH 4.3, the broad band shown in Plate II a was obtained. Fractionation and assay of a parallel gel indicated that this band was the A antigen (Plate II b). The broadness of the band suggests that the antigen may be aggregating. A gel of A at pH 9.5 is shown in Plate II c. A sharp major band and two minor bands are observed. Thus this fraction contains some minor impurities but does not seem to aggregate at pH 9.5.

A gel of Fraction III ABC at pH 4.3 is shown in Plate II d. Results of fractionation and SBP assay of a similar gel are shown in Figure 2. The major band contains equal amounts of A and BC antigens and is therefore the ABC structure. The mobility of ABC at pH 4.3 is lower than either A or BC' as indicated by the BC' marker and the shoulder of A antigen shown in Figure 2.

As the pH 4.3 and pH 9.5 gels of ABC indicate, there is a small amount of free A and BC' present in the Fraction III ABC. Two observations suggest that these free half fibers are not breakdown products of the whole fiber but rather were co-purified with it from the crude lysate. First, gels of an ABC preparation stored six months at 4 to 6°C

Plate II. Polyacrylamide gels of A and ABC. (a) Fraction III A, pH 4.3; (b) A SBP assay of gel prepared in parallel to (a); (c) Fraction III A, pH 9.5; (d) Fraction III ABC, pH 4.3.

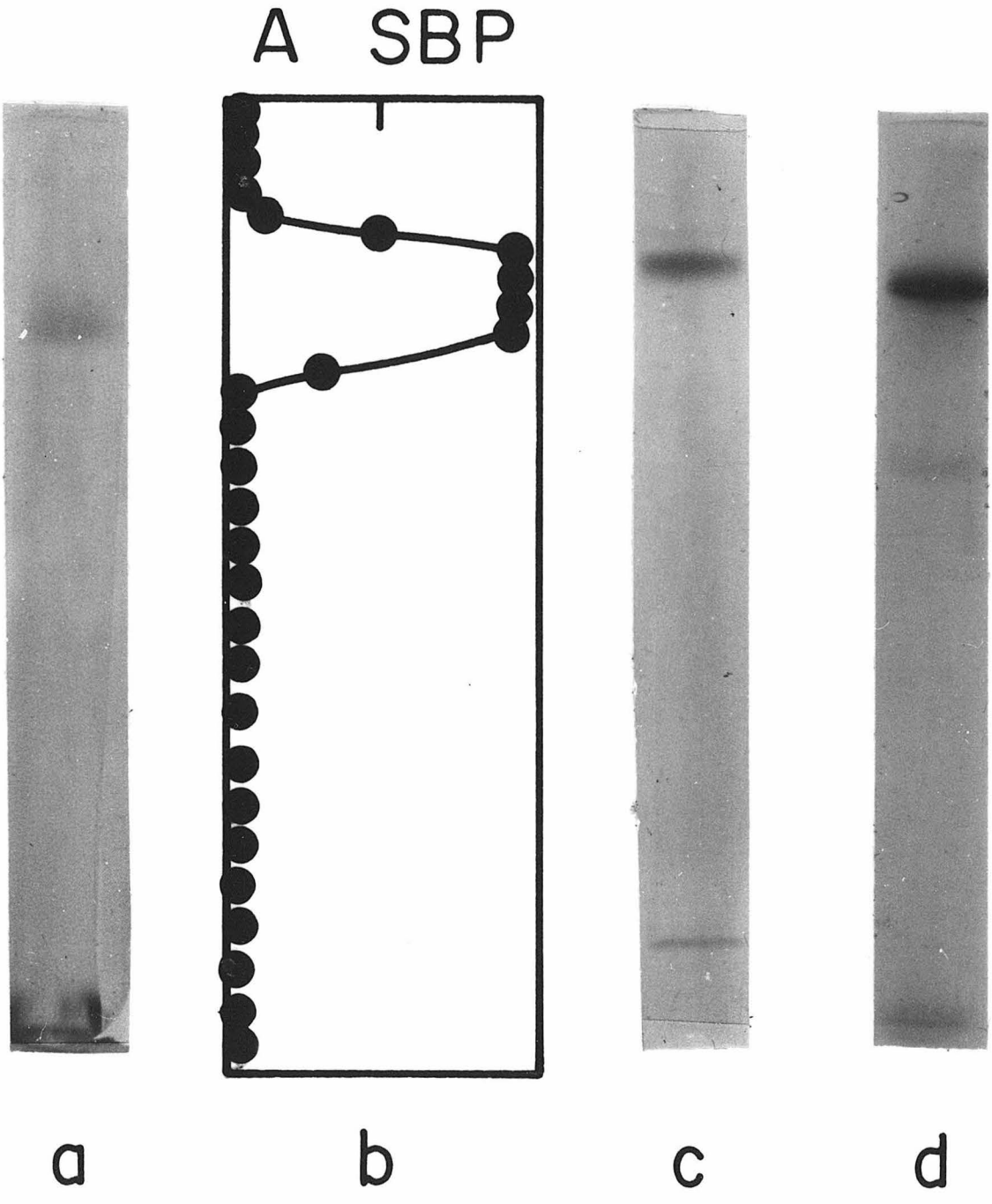
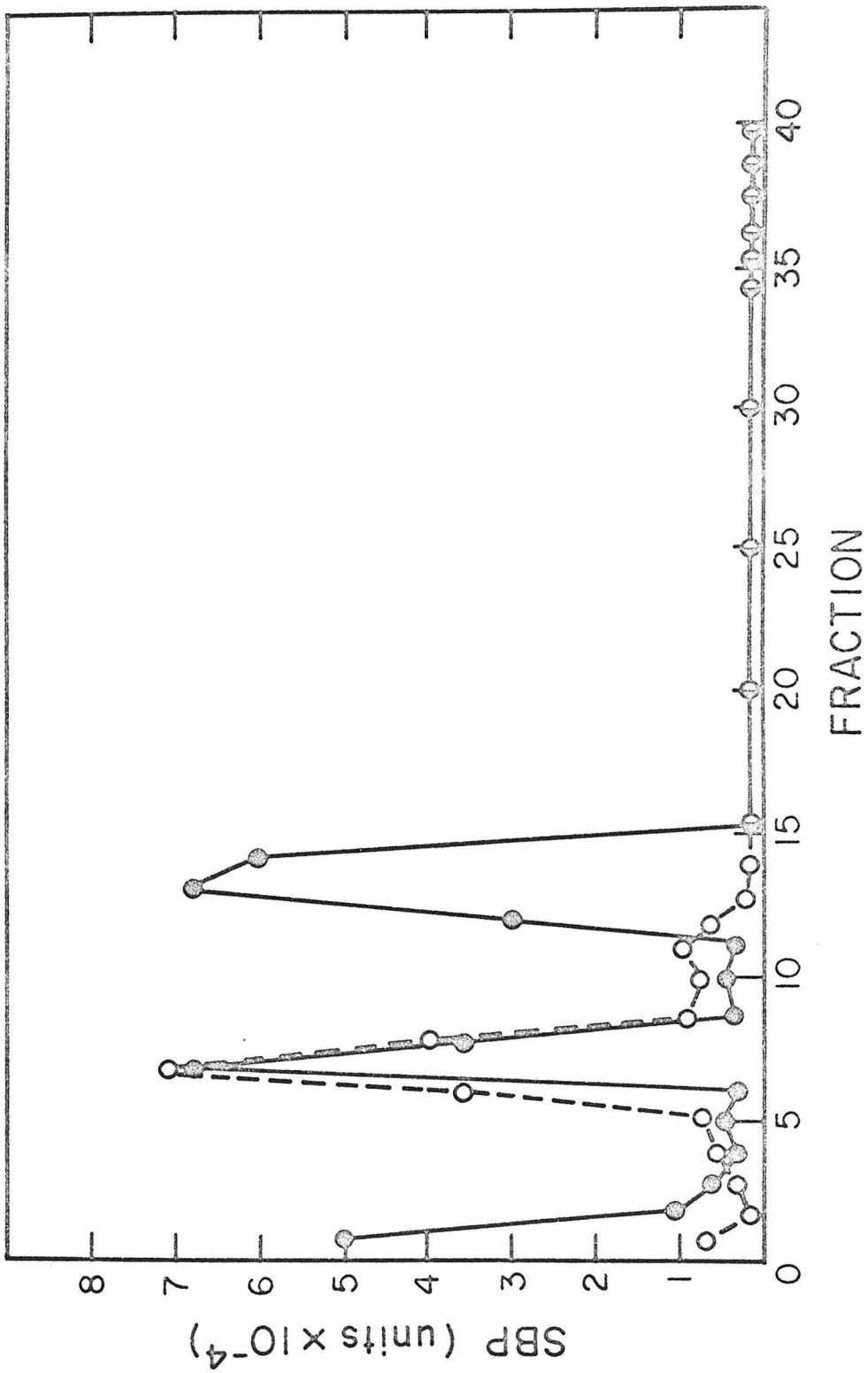

A
SBP
a
b
c
d

FIG. 2. Fractionation of a pH 4.3 gel of ABC. 0.014 units of ABC plus 0.008 units of BC' were applied to a gel at pH 4.3. After electrophoresis the gel was fractionated and assayed for both A and C SBP power. A SBP ---o---; SBP ——o——.


SBP (units x 10^-4)
0
1
2
3
4
5
6
7
8
0
5
10
15
20
25
30
35
40
FRACTION

showed no increase in the half fiber bands. Second, by repeated DEAE chromatography of an ABC preparation, all of the contaminating BC' could be removed. After brief storage at 4 to 6°C and rechromatography, free BC' did not reappear. These observations suggest that the association of BC' and A to form ABC is not readily reversible under our conditions.

(d) Characterization by electron microscopy

(i) C, BC and BC'

Plate III shows electron micrographs of negatively stained Fraction III C, BC and BC'. All appear as straight rods with little reproducible fine structure. No differences were seen between Fraction III and Fraction IV BC' fibers (not shown).

Histograms of the length distributions of the three structures are shown in Figures 3 a,b, and c, respectively. As summarized in Table 4, the average lengths are, for C, 562 $\pm$ 30 A°, for BC, 685 $\pm$ 43 A°, and for BC', 697 $\pm$ 44 A°. Thus C is clearly shorter than the other two. As an additional check on this result the length distribution of an equal mixture of C and BC' was determined (Fig. 3 d). It is seen to be clearly bimodal, with the means corresponding to the lengths of C and BC'.

All three fibers vary somewhat in width along their length. However, all were found to have a mean width of 47 $\pm$ 10 Å.

Plate III. Electron micrographs of C, BC, BC'. A, B and C show Fraction III purified C, BC and BC' respectively.

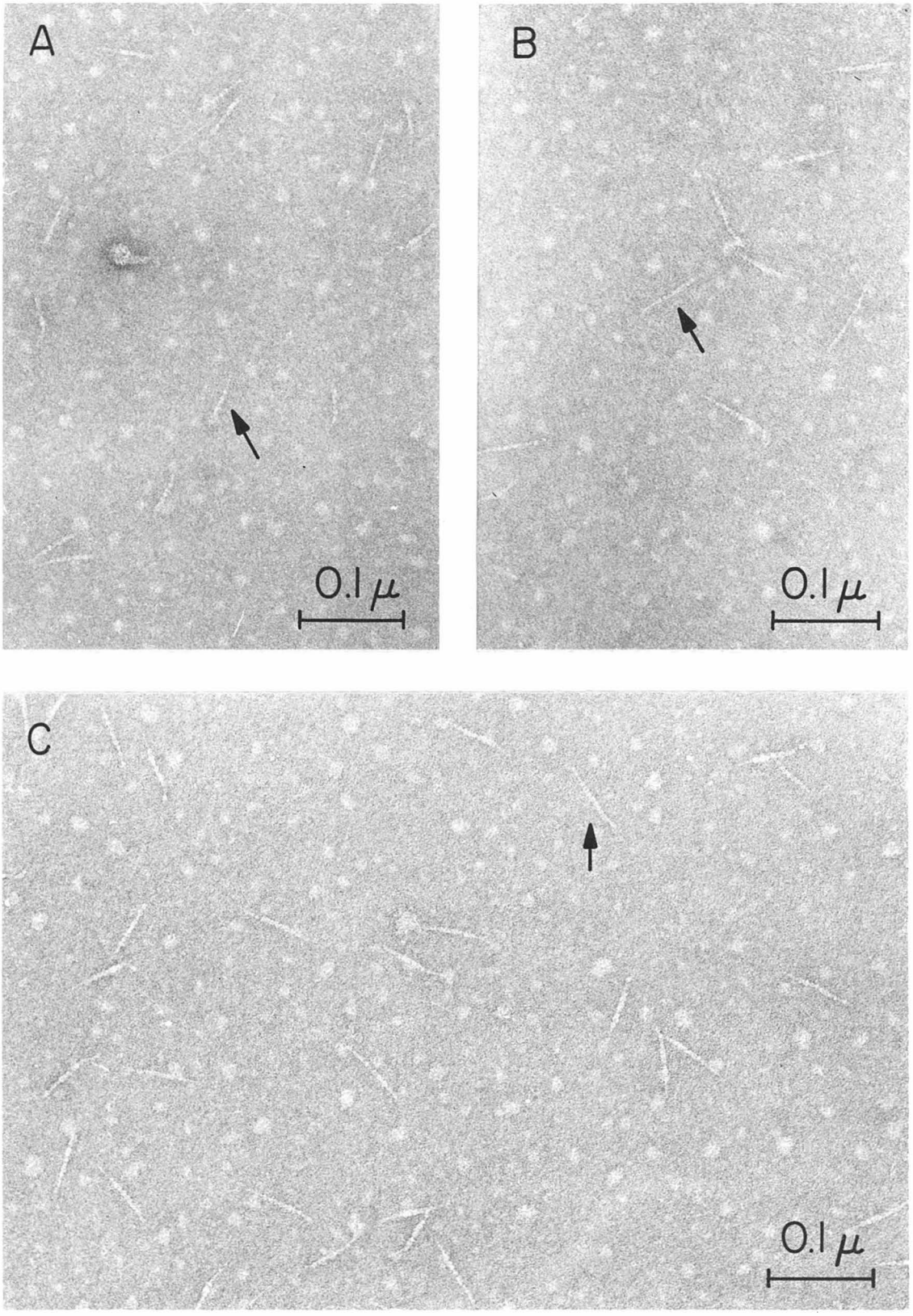

A
0.1 μ
B
0.1 μ
C
0.1 μ

FIG. 3. Length distribution of structures C, BC and BC'. The distribution of lengths of C, BC and BC' are shown by histograms a,b and c respectively. Histogram d shows the distribution of a 1:1 mixture of C and BC'. The mean and standard deviation of each distribution are given in Table 4.

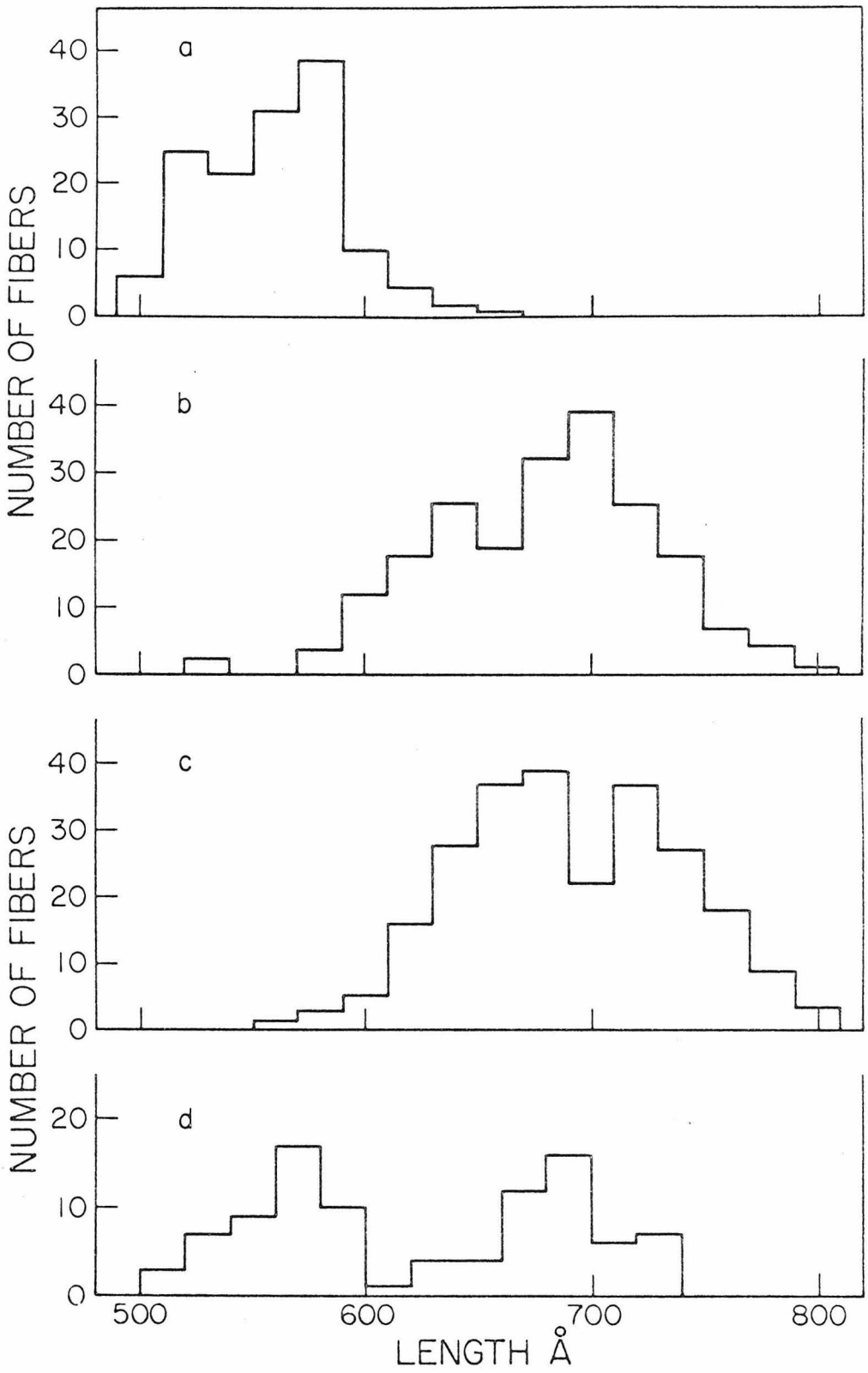

a
b
c
d
NUMBER OF FIBERS
NUMBER OF FIBERS
0
10
20
30
40
500
600
700
800
LENGTH Å

TABLE 4

Lengths of purified structures and attached tail fibers

Structure	Number Measured	Length (Å)
C	144	562 ± 30
BC	202	685 ± 43
BC'	246	697 ± 44
A	99	681 ± 31
Knob	45	122 ± 15
Knobbed Half of ABC	50	677 ± 40
Unknobbed Half of ABC	50	677 ± 40
Proximal Half of Attached Fiber	69	680 ± 40
Distal Half of Attached Fiber	69	676 ± 40

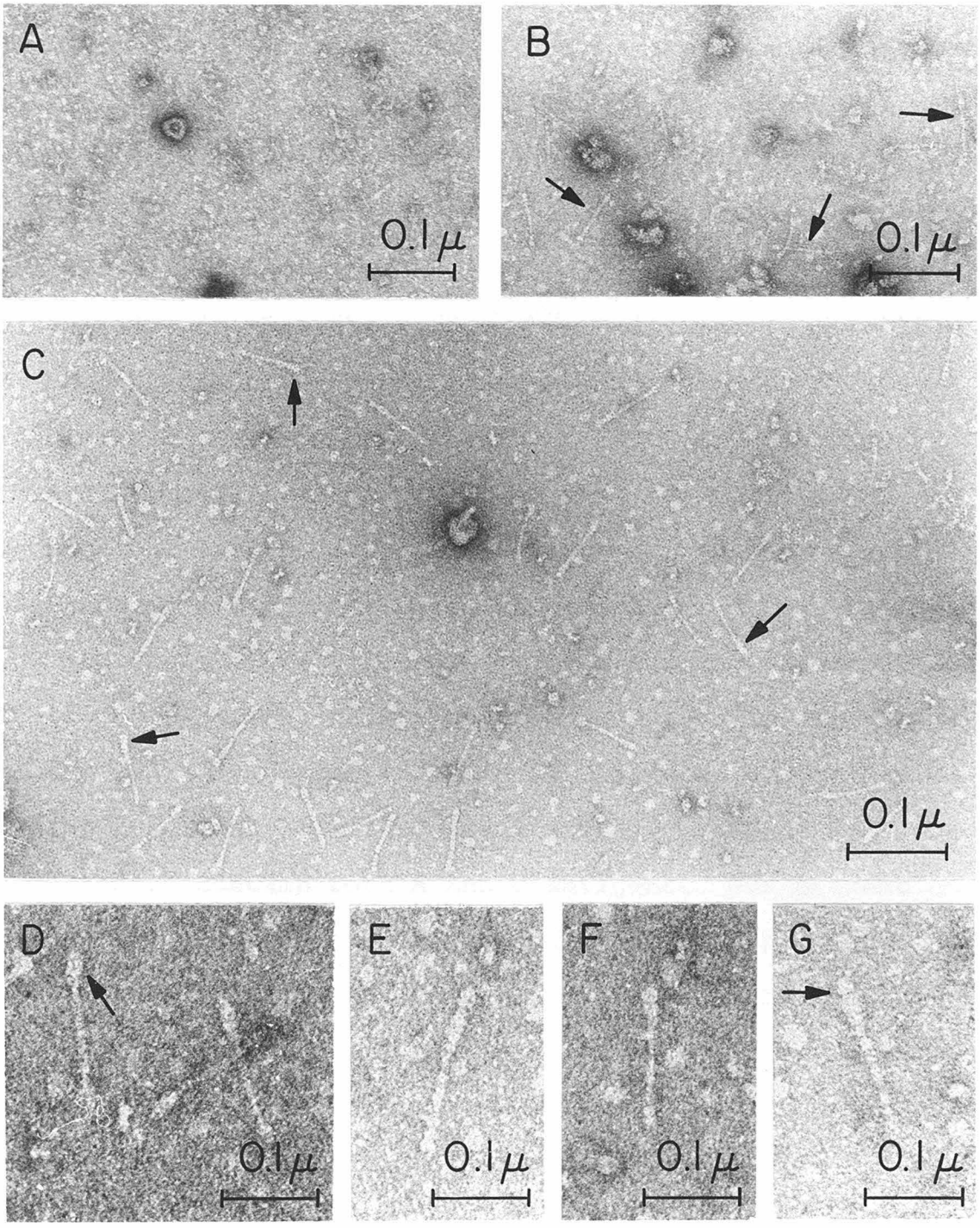
Table 4. Lengths of purified structures and attached tail fibers

Dimensions were determined as described in Materials and Methods (g) from electron micrographs similar to those shown in Plates III, IV and V. The proximal half of attached fibers was measured from the central kink to the edge of the baseplate.

(ii) A

Eiserling, Bolle & Epstein (1967) have reported that no fiber-like structures were visible in electron micrographs of lysates prepared with mutant phage defective in gene 37, although these lysates contain normal amounts of A antigen. However, micrographs of Fraction III A antigen (Plate IV C) showed large numbers of fiber structures with the appearance of half tail fibers. To explore this apparent discrepancy, we prepared micrographs of crude lysates, crude extracts and a Fraction II preparation of A from cells infected with phage SX6. All preparations were adjusted to the same A antigen concentration. Plate IV A shows that appearance of the crude lysate. Fibers similar to those in Plate IV C are not visible. Similar results were observed with the crude extract and the Fraction II antigen (not shown). When fiber-like structures were counted, all three preparations were found to contain less than 10% the number of fibers found in the Fraction III preparations. A one to one mixture of Fraction III A antigen and the crude lysate (Plate IV B) showed clearly visible fibers in roughly the numbers expected for Fraction III material alone. This indicates that if fibers were present in the crude fractions they would have been visible. We conclude that only a small fraction of the A antigen in crude preparations is present as fiber-like structures, although this fraction has been found to vary somewhat from one preparation to another

Plate IV. Electron micrographs of A. A is a crude lysate. B is the same crude lysate mixed 1:1 with Fraction III purified A. C is Fraction III purified A. The arrows indicate the knob discussed in Results d (ii) and counted in Table 6. D, E, F, G show enlargements of the individual fibers with arrows indicating the knobs.


A
0.1μ
B
0.1μ
C
0.1μ
D
0.1μ
E
0.1μ
F
0.1μ
G
0.1μ

(see King & Wood, 1969, note in proof). The presumed conversion of the antigenic material into the form of fibers must occur during the chromatography or the subsequent dialysis and concentration of the A antigen.

It is evident that the Fraction III A fibers are different from the C, BC or BC' fibers (compare Plate IV C to Plate III), in that the former show a distinctive swelling or knob at one end. This structure is indicated by arrows in Plate IV C and is more clearly visible in enlargement (Plate IV D,E,F, and G). The frequency of these knobbed ends on the various tail fiber structures is shown in Table 5. The knob is found almost exclusively on the A half fiber and the ABC whole fiber (see below), at a freqency of about 60%. It is not clear why this figure is less than 100%. King & Wood (1969) also found knobs on some fibers in zone sedimentation fractions of a 36-defective extract, which would be expected to contain both A and C antigens. Our present results suggest that the knobbed structures represented A half fibers rather than artifacts as proposed by these authors. It would therefore appear that their zone sedimentation procedure as well as our method of purification allows the A antigen to form fiber structures.

The length of the A half fiber (Table 4) is 681 $\pm$ 31 Å, the same as that of BC'. The length of the knobbed portion is 122 $\pm$ 15 Å. The width of the fiber is 47 $\pm$ 15 Å and the width of the knob is 68 $\pm$ 12 Å.

(iii) ABC and complete phage

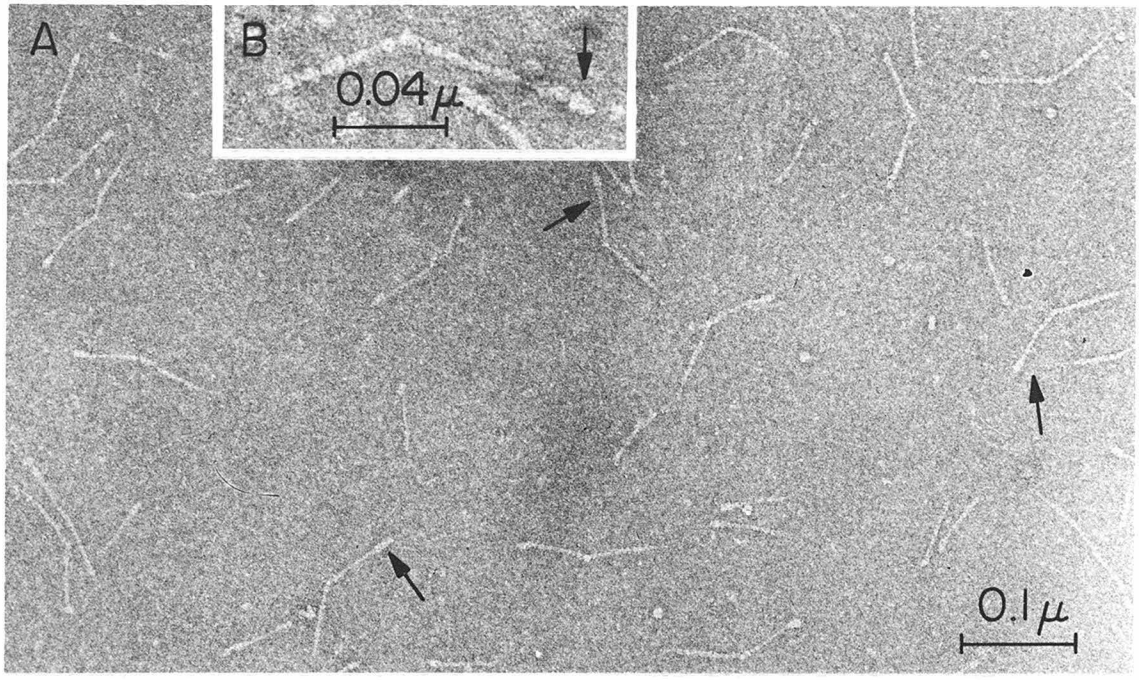
Plate V A is a micrograph of Fraction III ABC showing the characteristic sharp central kink of the complete tail fiber (Brenner et al., 1959). Free A half fibers are also visible. A knob similar to that found on the A half fiber is visible at one end of about 60% of the whole fibers (Table 5).

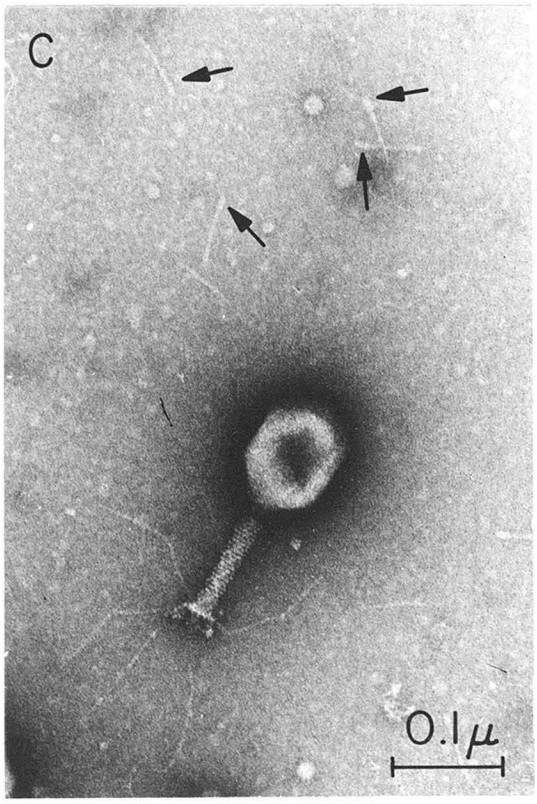
The lengths of the knobbed and unknobbed halves of the fiber, measured from the central kink, are identical within error and equal to the lengths of free A and BC' (Table 4). The length of the fibers on complete phage, measured from plates similar to Plate V B, were found to be identical to the length of free ABC as shown in Table 4.

The angle between the two halves of the whole fiber was measured for both isolated ABC and complete phage. Both gave mean values of about 160° (Table 6).

In order to determine which end of the whole fiber attaches to the phage baseplate, whole phage were examined for knobs visible on the distal tips of their tail fibers. Plate V C shows mixture of phage and purified A. The knobs are visible on the purified A but not on the distal tips of the phage tail fibers. This suggests that the knobbed A end of the tail fiber attaches to the baseplate. A fortuitous picture of a baseplate from degraded phage with half fibers attached, showing knobs at the point of attachment,

Plate V. Electron micrographs of ABC and phage. (A) shows Fraction III ABC. The arrows note the knobs on one end of thes fibers. (B) An enlargement of a single fiber. (C) Phage mixed with Fraction III A. The knobs are visible on the free A as noted by the arrows and not visible of the distal tips of the phage tail fibers. (D) A fortuitous picture of a phage baseplate with fibers attached derived from sonicated phage. (Kindly supplied by Fred Eiserling). The arrows show knob-like structures attached to the baseplate.


A
B
0.04μ
0.1μ


C
0.1μ

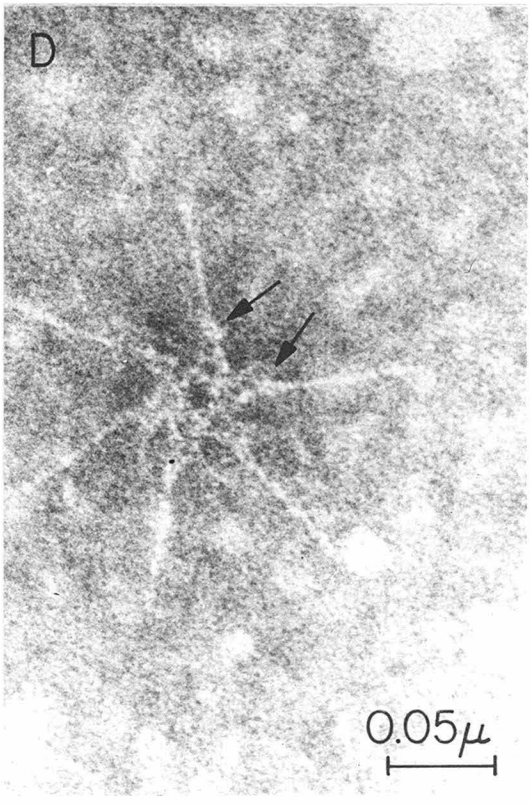

D
0.05μ

TABLE 5

Frequency of knobs on fiber structures

Structure	Number Observed	% With Knobs
C	183	6
BC	175	3
BC'	484	3
A	237	56
ABC	234	59
Fibers on Phage	173	3.5
A in Presence of Phage	165	58

Table 5. Frequency of knobs on fiber structures

Features designated as knobs are indicated by arrows in Plates IV and V. For the determination of knobs on fibers attached to phage, purified A half fibers were mixed with purified phage and photographed together (Plate V C). The last entry in the table indicates the percent of free A half fibers on which knobs were seen.

TABLE 6

Angle between halves of the whole fiber

	Number Measured	% With Bend In Center	Angle (°)
ABC	213	86	157 ± 11
Fibers on Phage	190	76	156 ± 13

Table 6. Angle between halves of the whole fiber

The angle between the two halves of the whole fiber was measured on enlargements of negatives using a protractor. The precision of measurement was about ± 5°. The distribution of angles about the mean shown was roughly symmetrical. Fibers not included in the table (approximately 20%) appeared either to have two kinks, or to have the kink displaced toward one end of the fiber.

supports this notion (Plate IV D). More convincing evidence that the A half fiber is proximal to the baseplate and the BC' half fiber is distal has been presented by Wilson, Luftig & Wood (1970) and Yanagida & Ahmad-Zadeh (1970).

(e) Biological activity

The ability of the purified structures to activate fiberless particles to plaque-forming phage in the presence of the necessary gene products was determined in two ways. First, the initial rate of activation of fiberless particles was measured under assay conditions in which the rate was dependent on the concentration of antigen being tested and other gene products were in excess (rate assay). Second, the number of fiberless particles activated to plaque-forming phage by limiting antigen was determined after the attachment reaction had gone to completion (end-point assay). Interpretation of the latter assay depends upon the way fibers distribute among the attachment sites on the particle baseplate and upon the plating efficiency of particles with less than six fibers. The method of analyzing these factors described in the Appendix enabled us to calculate the fractions of purified antigen which can be converted to active fibers *in vitro*. These two assays were used to compare the activity of the purified structures to the activity of the structures in crude extracts before purification (Table 7). The rate and end-point assays gave comparable results.

Table 7. Biological activity of purified structures

Crude defective extracts for *in vitro* complementation assays were prepared from non-permissive cells infected with the multiply mutant phage shown in the second column. All assays were carried out at 30°C in a volume of 0.1 ml. The reaction mixtures contained 0.02 M $MgSO_4$, 0.01 M N-Tris (Hydroxymethyl) Methyl-2-Amino ethane sulfonic acid, pH 7.1, 0.002 M dithiothreitol, and 0.001 M K_4EDTA. For both rate and end point assays the purified antigen was present at 0.01 to 0.1 units/ml., and the complementing antigen at 1.0 unit/ml. P63 in the extracts was in excess (Wood & Henninger, 1969). For the rate assays, fiberless particles were at 2×10^9/ml. or less, and for the end point assays, at 5×10^{11}/ml. or more. Aliquots of the mixture were assayed for infectious phage at 10 to 30 min after initiation of the reaction for rate determinations, and at 6 hr (at which time the increase in titer had ceased) for end point determinations.

Since the concentration dependence of the rate of phage activation is complex, relative activities were determined by comparing the crude and purified antigen concentrations in reaction mixtures which gave identical rates. The end-point assay for both crude and purified antigen was analyzed as described in the Appendix.

TABLE 7

Biological activity of purified structures

Structure	Complementing Extract		Relative Activity Purified / Crude	
	Mutant	Source of	Rate	End Point
ABC	XF1	P63	1.0	0.9
A	SX1	BC', P63	0.05	0.12
BC'	EX121	A, P63	0.85	0.8
BC	EX111	A, P35, P63	<.05	<.03
C	X2J	A, P35, P36, P63	<.05	<.07

Most of the activity of the ABC and BC' structures are retained throughout the purification. The activity of A is greatly diminished by purification and the structures C and BC retain no activity after isolation. This loss of activity of C and BC was not surprising, however, since the activity of these structures in crude extracts is unstable. After several days storage, even at -160°C, crude preparations of C and BC lose their *in vitro* activity although their antigenicity is unaffected. The loss of A activity during purification is more difficult to explain, since the activity of A in crude extracts is fairly stable. This discrepancy is further discussed below.

The *in vitro* activity of Fraction III ABC, BC' and A is lost after several weeks storage at 4 to 6°C. BC' activity can be retained by storage at -160°C.

4. Discussion

The interpretation of our characterization of the purified structures depends upon the assumption that these structures are normal intermediates in phage assembly. Alternatively, they might be aberrant by-products which accumulate when the normal assembly sequence is blocked by mutation. Examples of such aberrant structures in head and tail assembly are discussed by Edgar & Lielausis (1968). The evidence presented in this paper, as well as the lipopolysaccharide binding studies of Wilson *et al.*, (1970) on

purified fiber structures, provide some basis for deciding between these alternatives.

The ABC structure has the A, C and presumably B fiber antigens which are present on the complete phage. Electron micrographs of isolated whole fibers and fibers attached to phage show identical dimensions and angles at the central kink. The purified structure is active *in vitro* and can be quantitatively attached to phage. Since it is not readily broken down to A and BC' it probably attaches in its assembled form as surmised by King & Wood (1969). The isolated structure also interacts with *E. coli* lipopolysaccharide (Wilson *et al.*, 1970). These results all support the assumption that ABC is a normal intermediate in tail fiber assembly.

The BC' structure carries two of the antigens present on complete phage. It retains most of its *in vitro* activity throughout purification. Its dimensions are the same as those of the distal half of the phage tail fiber, and it also interacts with *E. coli* lipopolysaccharide (Wilson *et al.*, 1970). Thus BC' appears to be a normal intermediate in fiber assembly.

The A structure carries an antigen common to the phage, and has the same length and distinctive knob as the end of the whole fiber which attaches to the baseplate. However, the purified structure has little *in vitro* activity in comparison to that of the A antigen crude extracts. Moreover, active crude extracts show few visible fiber structures in

electron micrographs. The A half fiber seen in purified preparations therefore may not be a normal intermediate in assembly. A possible explanation for the results could be that the A antigen in fresh extracts is present in a form unrecognizable in electron micrographs. This antigen may be normally converted to the A half fiber only in the course of association with the BC' half fiber. In the absence of BC', this conversion can be triggered abnormally to produce a free A half fiber which can no longer be made into a functional whole fiber *in vitro*.

The purified BC structure represents a normal intermediate in fiber assembly by the criteria of antigenicity and electron microscopy. It is not active *in vitro*, however, so that its role in normal assembly remains uncertain. Since its activity in crude extracts is unstable, in contrast to A, it could be a normal intermediate which is inactivated by purification.

The purified C structure carries an antigen in common with the assembled fiber and interacts with lipopolysaccharide identically to BC' (Wilson *et al.*, 1970), but it appears shorter in electron micrographs than the completed half fiber. As in the BC case, the *in vitro* activity of C in crude extracts is very labile, so that the inactivity of the purified structure is difficult to interpret.

The studies of purified structures presented here establish the role of the ABC fiber in normal assembly and

show that this whole fiber consists of one BC'half, assembled under the control of genes 57, 38, 37, 36, and 35, and one A half assembled under the control of genes 57 and 34. In normal assembly the A half fiber may be formed only in the presence of BC'. If it is assumed that the C and BC fibers represent normal intermediates, then gene 36 action, besides adding the B antigen, also increases the length of the C fiber by 130 Å. From the studies of Yanagida and Ahmed-Zadeh (1970) showing localization of the B antigen near the central kink of the whole fiber, it seems likely that the added material is at the end of the structure which subsequently attaches to the A half fiber. Gene 35 function may modify this site in preparation for A attachment, but if so the resulting structural alteration is too minor to be detected by the techniques we have employed.

Still unclear are the functions of genes 57 and 34 and genes 57, 38 and 37 in the early stages of forming the A and BC' half fibers, respectively. Information on the roles of these genes, derived in part from degradation of the structures purified here to their subunit polypeptides, will be presented in a subsequent paper (Ward & Dickson, in preparation).

APPENDIX

Analysis of End Point Fiber Attachment Reactions

All of the tail fiber structures described in this paper were purified on the basis of serum-blocking activity. In determining what fraction of these purified structures retain biological activity, that is, the ability to attach to and thereby activate tail-fiberless particles, the following theory was used.

Consider the attachment of the whole fiber, ABC, to the fiberless particle. Assume the following:

(1) There are six fiber attachment sites on the base-plate, and the fibers are attached to them randomly and one at a time.

(2) Particles with less than six fibers can form plaques with a lower-than-normal efficiency of plating.

(3) The antigenicity of the fibers is the same whether they are free or attached to particles. Therefore one phage-equivalent of free SBP represents six fibers.

Wood & Henninger (1969) have provided evidence supporting the first two assumptions and discussed their validity. The third assumption was tested by measuring the SBP of Fraction III ABC before and after attachment to fiberless particles. The SBP changed by $<20\%$.

In a reaction that has gone to completion in which

there are more fiberless particles than fibers, the probability that any attachment site on a fiberless particle is occupied by a fiber is given by the ratio of fibers to attachment sites:

$$p = \text{fibers}^* \,/\, \text{fiberless particles} \qquad \text{(i)}$$

where fibers^* represents the concentration of active fibers expressed in phage equivalents. This is the quantity we wish to determine. We can measure experimentally the fraction of fiberless particles which can form plaques, F. These two quantities are related as follows.

If the fibers are distributed randomly among the sites on the baseplates the fraction of particles in the mixture with n fibers, f_n, where n is an integer from 0 to 6, is given by a binomial distribution function:

$$f_n = \frac{6!}{n!(6-n)!}\, p^n (1-p)^{6-n} \qquad \text{(ii)}$$

The fraction of particles which can form plaques, F, is equal to the sum of the fractions f_n each multiplied by the probability that a particle with n fibers will form a plaque. Following Wood & Henninger (1969), we designate as c_n the fraction of phage with n fibers which will <u>not</u> form a plaque. Then F is given by the equation

$$F = \sum_{n=o}^{6} (1-c_n) f_n \qquad \text{(iii)}$$

Using the values of c_n determined to give the best fit

to the rate of activation data analyzed by Wood & Henninger (1969), namely $c_o = c_1 = 1$, $c_2 = 0.95$, $c_3 = 0.5$, $c_4 = c_5 = c_6 = 0$, we can write

$$F = 0.05\ f_2 + 0.5\ f_3 + f_4 + f_5 + f_6 \qquad \text{(iv)}$$

Substituting in the expression for f_n (ii) and combining terms gives the following polynomial relating F and p:

$$F = p^2(0.75 + 7p - 10.5p^2 + 3p^3 + 0.75p^4) \qquad \text{(v)}$$

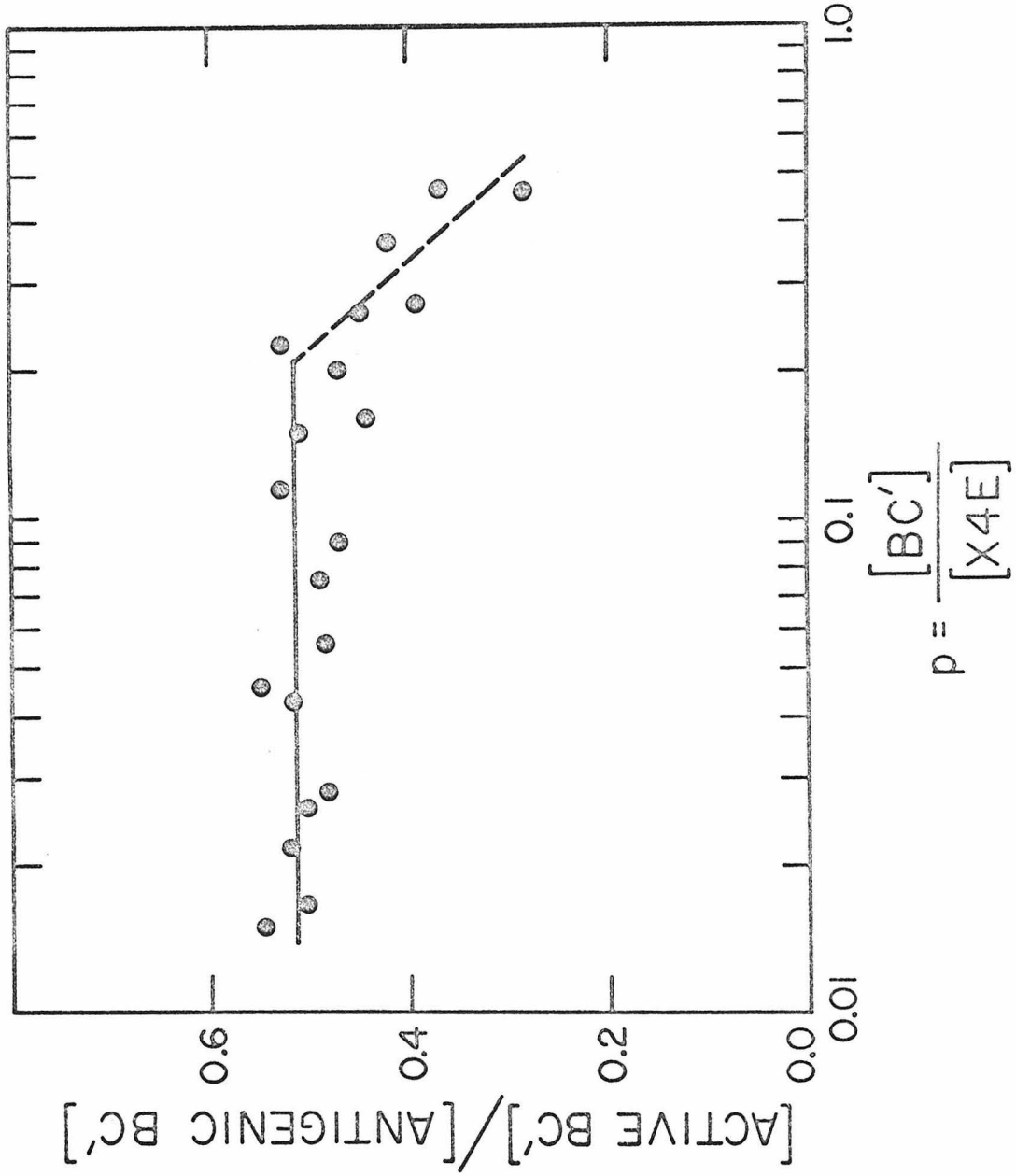
From (v) we can compute the value of p from an experimentally determined value of F. To do so an IBM 360/50 computer was programmed to compute F for assigned values of p using equation (v) and the results were plotted as F versus p on log-log coordinates. For each assay, values of p were determined for measured values of F from this plot. The concentration of active fibers in the assay was then determined from the value of p using equation (i).

This method can be used to measure concentrations of active fiber precursors as well as whole fibers. In vitro complementation is carried out with a large excess of crude extract containing the gene products required to convert the precursor to whole fibers, which then attach in the manner assumed above. This assumes that the fiber precursors C, BC, BC' and A are completely converted to ABC in the reaction mentioned. The method therefore gives a minimum estimate of the concentration of active fiber precursors.

The theory was tested by determining that the fraction

of antigenic fibers which were active *in vitro* in two independent SX1 (BC'-containing) extracts was independent of p. The value of p was varied in different assay tubes by varying both the fiberless particle concentration and the antigen concentration. The results shown in Figure 4 indicate that the active fraction predicted from the theory is nearly constant for values of p from 0.015 to 0.2. The decrease in predicted active fraction at values of p greater than 0.2 has not been further studied. The lower limit of the assay is determined by the background activity of the complementing extracts. The observation that a crude extract of BC' has only 50% active fibers may reflect inefficient conversion of BC' to ABC or may actually imply that some fibers are antigenic but defective in their ability to activate fiberless particles. In crude extracts containing ABC the fibers are approximately 100% active under these conditions.

FIG. 4. Test of end point attachment theory. The fraction of antigen which is active *in vitro* for crude extracts containing BC^1 was determined at various concentrations of fiberless particles and fiber antigen. The active fraction of antigen which should be independent of dilution is plotted along the ordinate. The abscissa shows the experimentally determined value of p based on equation (v).


[ACTIVE BC']/[ANTIGENIC BC']
0.0
0.2
0.4
0.6
0.01
0.1
1.0
p = [BC'] / [X4E]

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ASSEMBLY OF BACTERIOPHAGE T4 TAIL FIBERS:

III. GENETIC CONTROL OF THE MAJOR TAIL FIBER POLYPEPTIDES

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Summary

Purified preparations of complete T4 bacteriophage, tail fiberless particles, whole tail fibers, and four tail fiber precursors were dissociated by heating briefly at 100°C in 1% sodium dodecyl sulfate (SDS) containing 1% mercaptoethanol. Analysis of the dissociated structures by polyacrylamide gel electrophoresis in the presence of SDS and mercaptoethanol revealed two high molecular weight (150,000 and 123,000 daltons) polypeptides as major tail fiber components. Due to their high molecular weight, the two components could be easily identified by autoradiography of SDS gels of radioactively labeled infected cell extracts. The larger of the two was missing from extracts of cells infected with gene 34 amber mutants, and the smaller from extracts of cells infected with gene 37 amber mutants. It is concluded that the two components represent the products of genes 34 and 37 (P34 and P37), respectively. Molecular weight calculations indicate that two copies of each polypeptide are present in each complete tail fiber. By scintillation counting of the radioactive material in the P34 and P37 bands following gel fractionation, it was shown that gene 36 amber mutations reduce the synthesis of P37, implying that genes 36 and 37 are included in a single unit of transcription. Amber mutations in genes 38 and 57 were found to affect the apparent solubility of P34 and P37 and their resistance to dissociation in cold SDS, but not their synthesis. Based on these results, the previously reported pathway of tail fiber assembly (King & Wood, 1969) has been reformulated in more detail.

1. Introduction

Bacteriophage T4 provides an excellent system for studying genetic control of the assembly of a complicated biological structure. Several steps in assembly can be carried out *in vitro* and studies of these assembly steps have shown that most of them are strictly sequenced and can be ordered into a pathway of assembly (Wood, Edgar, King, Lielausis & Henninger, 1968). The phage head, tail and tail fibers are each assembled in independent branches of the pathway, and then joined together to complete the virus. In order to understand the molecular mechanisms of gene control of some assembly steps we have chosen to study the assembly of the tail fiber in detail.

The first paper in this series (King & Wood, 1969) described the sequence of gene product interaction in tail fiber assembly and identified several fiber precursors. In the second paper (Ward *et al.*, 1970) the assembled fiber and four precursors were isolated and characterized by electron microscopy, serology and assembly *in vitro*. The present study continues the characterization of the isolated fibers by determining their subunit structure by dissociation and polyacrylamide gel electrophoresis in the presence of the anionic detergent sodium dodecyl sulphate (SDS). The genes controlling synthesis of the two major polypeptides are identified and the assembly of these polypeptides into half fibers is examined by comparing their solubility and resistance to dissociation before and after assembly. The results of these studies are summarized by reformulation of the pathway of tail fiber assembly in greater detail.

2. Materials and Methods

(a) Bacteria and bacteriophage

Escherichia coli strains B and Bb were used as restrictive hosts for bacteriophage T4D *amber* mutants obtained from the collection of R. S. Edgar. Methods used for the preparation and purification of phage and tail fiberless particles are given in Dickson, Barnes & Eiserling, (1970).

(b) Purified tail fiber precursors and whole fibers

Four tail fiber precursors and whole fibers were prepared from lysates of mutant-infected cells as described by Ward *et al.* (1970a). These will be referred to by their antigenic determinants as C, BC, BC' and A (half fibers) and ABC (whole fibers).

(c) Radioactively labeled lysates of phage-infected cells

Procedure I. Strain Bb was grown to a cell concentration of 10^8/ml. as described by Ward *et al.* (1970a) except that no casamino acids were added to the medium. The cells were collected by centrifugation at 10,000 x g for 10 min and resuspended at 4 x 10^8 cells/ml. Two ml. aliquots of this suspension were warmed to 39°C, infected with phage at a multiplicity of 4, and aerated in a bubbler tube at this temperature. Twelve min after infection, 25 µC of a uniformly ^{14}C-labeled amino acid mixture (New England Nuclear) or 50 µC of ^{3}H-labeled amino acid mixture (New England Nuclear) were added. Fifty to 70% of the label was incorporated into acid-insoluble material within 6 min. Forty-five min after infection the cells were lysed as described by Ward *et al.*

(1970a). Lysates were dialyzed against 0.01 M phosphate buffer, pH 7.1 and stored frozen.

Procedure II. The method is described in detail by Dickson et al. (1970).

Both procedures gave identical results. Defective lysates will be designated by the number of the defective gene, e.g. 37-defective lysates.

(d) Electrophoresis on polyacrylamide gels containing SDS

Procedures for preparation and running of gels were similar to those of Maizel (1960) and are described in detail by Ward, Wilson & Gilliam (1970b) and Dickson et al. (1970). Prior to electrophoresis, extracts and samples containing phage or fiberless particles were frozen and thawed three times and treated with 0.01 M $MgSO_4$ and 1 µg/ml. DNase I for 1 hr at 37°C. All samples were then either dialyzed or diluted into 0.01 M phosphate buffer, pH 7.0, containing 1-2% SDS and 1-2% 2-mercaptoethanol. Phage structures were dissociated by heating the sample for 2 min in a boiling water bath. Longer heating times did not alter the results. Gels containing unlabeled proteins were stained by the method of Weber & Osborn (1969) with Coomassie brilliant blue R250 (Mann) and destained by the method of Ward (1970). Gels containing radioactive proteins were fractionated and counted as described by Ward et al. (1970b) or autoradiographed by the procedure of Fairbanks, Levinthal & Reeder (1965).

3. Results

(a) Large polypeptides in whole phage, tail fiberless particles and isolated tail fibers

Purified phage and purified fiberless particles were dissociated in SDS, electrophoresed on SDS gels and then stained and destained. As shown in Plate I, the fiberless particle pattern lacks two major bands, 19.0 and 21.0, which are found in the phage pattern (band numbering is that of Dickson et al., 1970). No other bands appear to be missing from the fiberless particle pattern, but the resolution in the lower part of the gel is not adequate to detect differences in minor components.

The above observation suggests that bands 19.0 and 21.0 represent structural components of the tail fiber. Isolated whole fibers were examined for these components after dissociation in SDS-mercaptoethanol. Their SDS gel pattern is shown in Plate IIa. Plate IIb shows the pattern of whole phage run in parallel and IIc shows the pattern from a mixture of whole fibers and phage. From these results it is clear that the two major polypeptides from isolated fibers coelectrophorese with bands 19.0 and 21.0 from complete phage.

To determine the molecular weight of bands 19.0 and 21.0 their mobilities on SDS gels were compared to those of protein standards of known molecular weight (Shapiro, Vinuela & Maizel, 1967; Weber & Osborn, 1969). Using this technique, and assuming that mobility is determined solely by molecular weight, the molecular weight of band 19.0 was found to be 123,000 $\pm$ 5% daltons and band 21.0 to be 150,000 $\pm$ 10% daltons. The standard curve used to obtain these values is given in Figure 2

Plate I. SDS gels of phage and fiberless particles.

Dissociated samples were electrophoresed on 7.5% acrylamide gels 10 cm long at 8 ma/sample then stained and destained. (T4) 2 x 10^{11} phage. (X4E) 2 x 10^{11} fiberless particles prepared from multiple fiber mutant X4E.

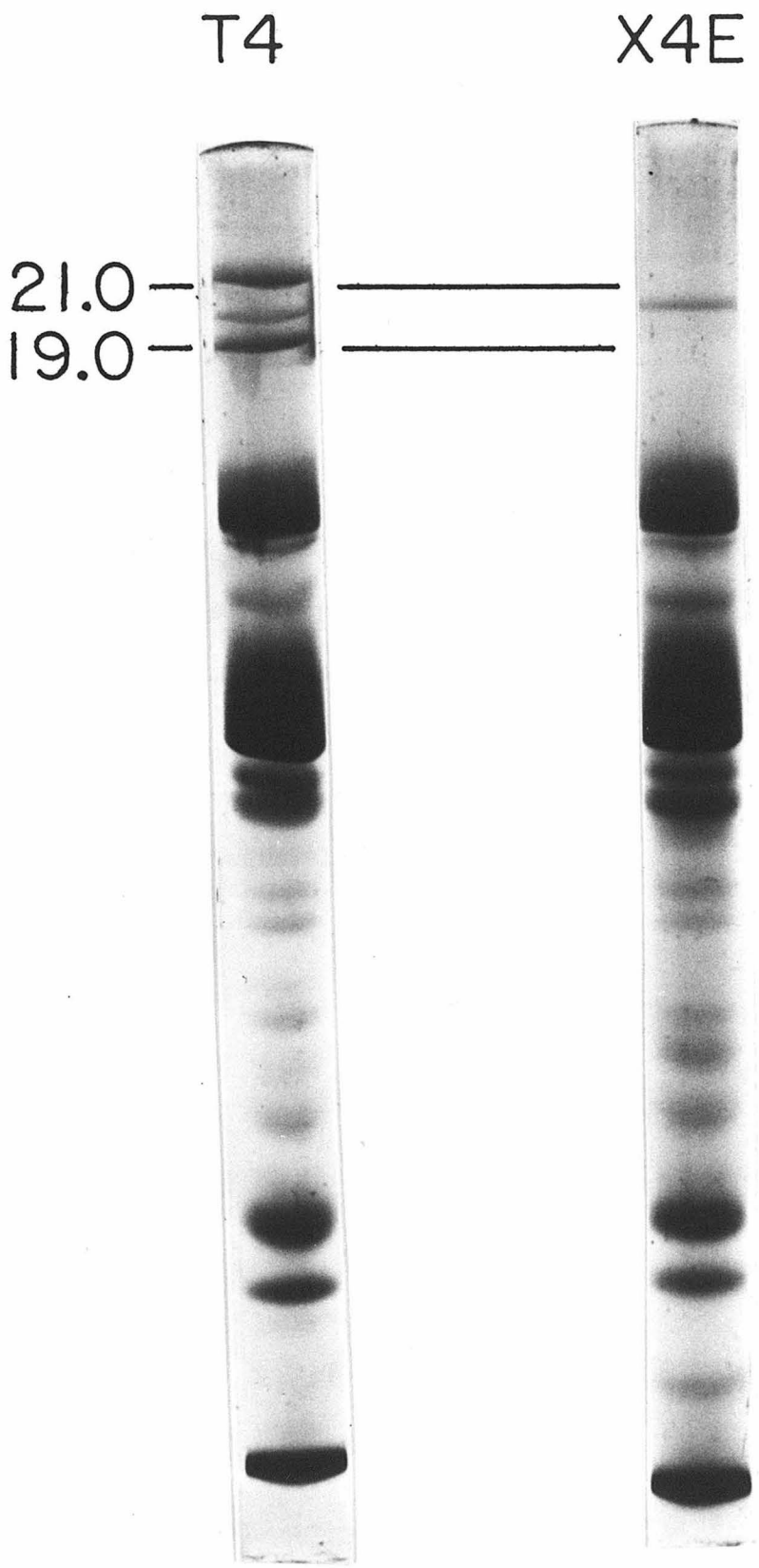

T4
X4E
21.0
19.0

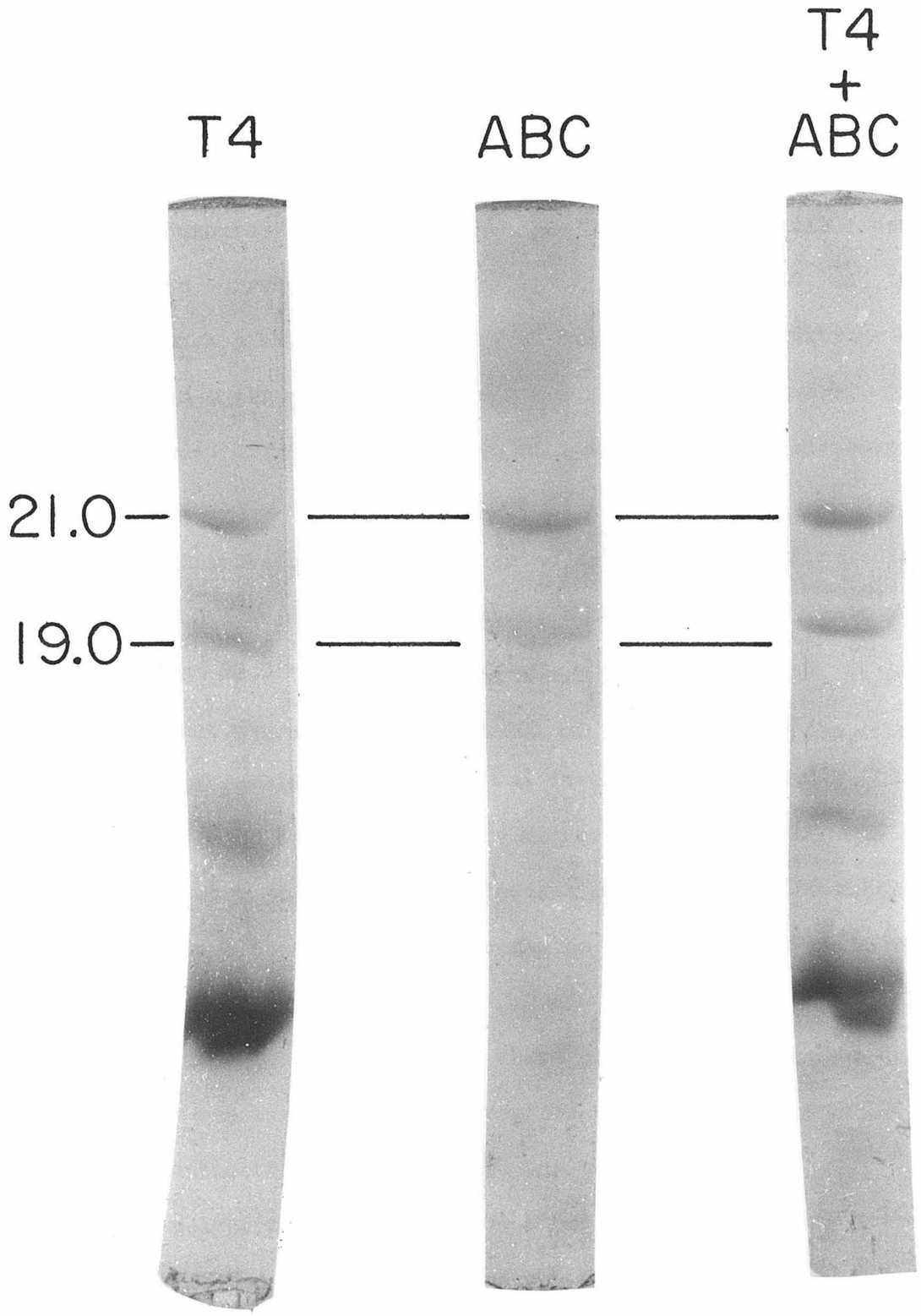
Plate II. SDS gels of phage and isolated ABC.

Samples were electrophoresed at 8 ma/sample for 280 min on 5% acrylamide gels 6.5 cm long containing 1% SDS then stained and destained.

(T4) 10^{11} phage

(ABC) 10^{11} phage equivalents of Fraction III ABC.

(ABC + T4) 10^{11} phage equivalents of Fraction III ABC plus 10^{11} phage.


T4
ABC
T4
+
ABC
21.0
19.0

of Dickson et al. (1970).

(b) Large polypeptides in tail fiber precursors

Plate III shows SDS gel patterns obtained with the purified tail fiber precursors C, BC, BC' and A. The C, BC and BC' half fibers each show a band corresponding in mobility to phage band 19.0, and the A half fiber shows a band corresponding to band 21.0. In addition the whole fibers and their precursors give a few minor bands; however, these have so far proven difficult to resolve and reproduce and will therefore not be discussed in this paper. When SDS gels of precursors labeled with a mixture of radioactive amino acids were fractionated and counted in a liquid scintillation counter, about 80% of the counts were in bands 19.0 and 21.0 for BC and C and about 90% for C and A. Thus the minor bands do not represent more than 20% of the protein in the fibers.

In one preparation of C which was investigated carefully, the mobility of the major band was slightly higher than that of band 19.0 from BC or BC', corresponding to a molecular weight of 114,000 rather than 123,000. As discussed below this may be an artifact of purification of the C fiber.

The high molecular weights of these components raise the question of whether they represent single polypeptide chains. Heating to 100°C in SDS and mercaptoethanol should break any non-covalent or disulfide bonds between polypeptides, but several other strong denaturing solvents were tested as well. After lyophylization, samples of purified BC' were resuspended in 100% formic acid, 80% phenol, 5% SDS in 8 M urea, 7 M guanidine hydrochloride, or 1 M hydroxylamine at pH 10 and incubated at

Plate III. SDS gels of isolated tail fiber precursors.

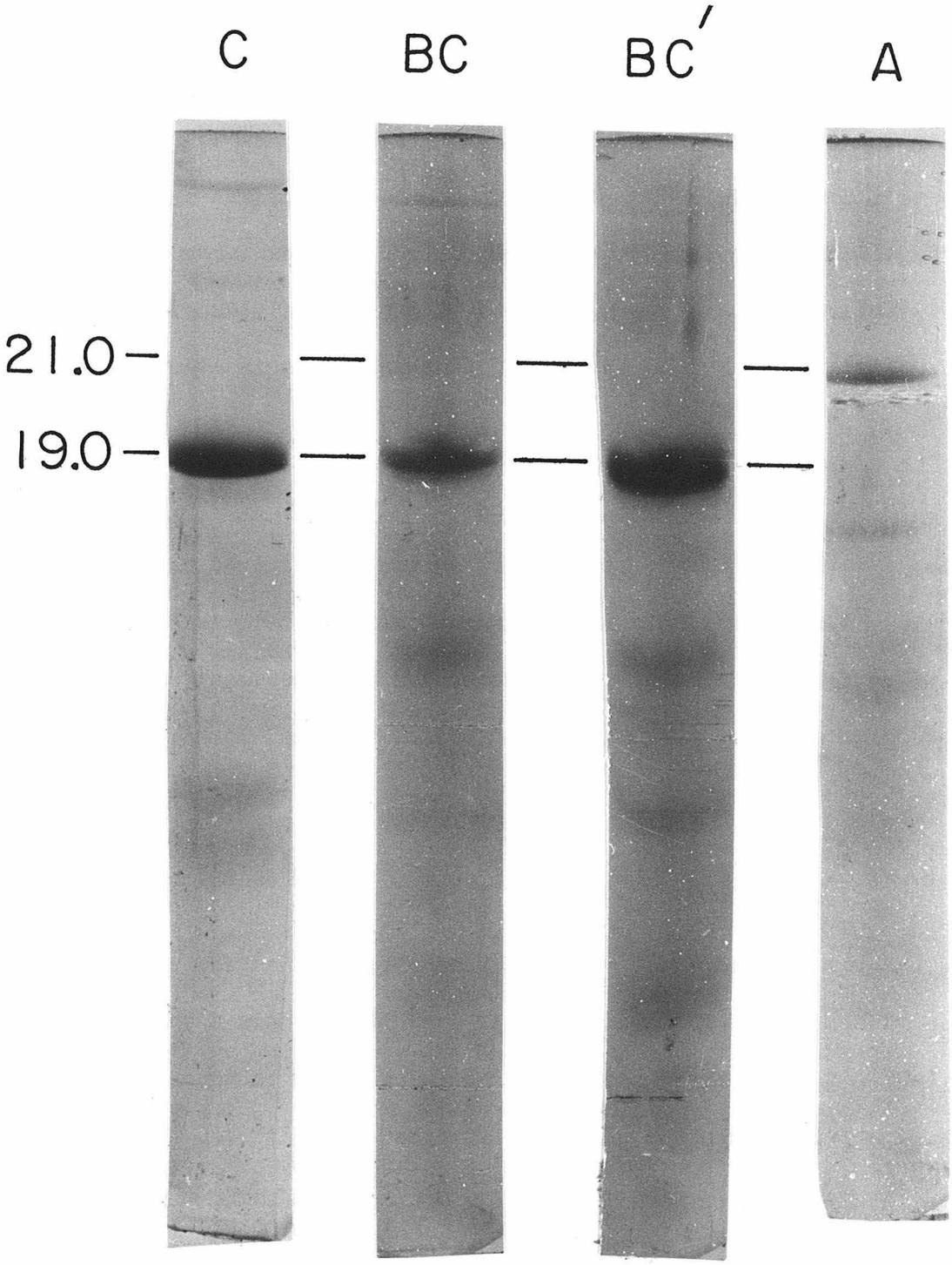
Samples were electrophoresed for 140 min at 8 ma/sample on 5% acrylamide gels 6.5 cm long containing 1% SDS, then stained and destained.

(C) 4×10^{11} phage equivalents of Fraction III C.

(BC) 7×10^{11} phage equivalents of Fraction IV BC.

(BC') 3×10^{11} phage equivalents of Fraction IV BC'.

(A) 10^{11} phage equivalents of Fraction III A.


C
BC
BC′
A
21.0
19.0

37°C for several hours. The formic acid and phenol samples were diluted, lyophylized, and then resuspended in 0.01 M phosphate buffer containing 1% SDS. The other samples were dialyzed against this buffer. SDS gels of each of these samples showed a band with the approximate mobility of band 19.0. We conclude that the 123,000 Mw component corresponding to band 19.0 is probably a single polypeptide chain. Similar studies have not been made with component 21.0 from the A half fiber.

(c) Estimation of the molecular weight of the undissociated tail fiber precursors

In order to estimate how many copies of the large polypeptides were in each fiber precursor we determined the approximate molecular weight of each precursor from its dimensions Assuming each fiber is a cylindrical rod of length = 2a (cm) and radius = b (cm) the molecular weight in daltons is given by

$$Mw = \frac{1}{\bar{v}} (2a)\pi b^2 N \qquad (1)$$

where $\bar{v}$ = fiber partial specific volume; $1/\bar{v}$ = fiber density (g/cm^3) and N = Avogadro's number. We used the fiber lengths determined by electron microscopy of negatively stained fibers (Ward et al., 1970a). The fiber radii determined by negative staining are unreliable, however, because of drying artifacts and uneven binding of stain. For example, the diameter of whole fibers is about 47 A^o when determined by staining with uranyl acetate (Ward et al., 1970a); whereas it is about 20 A^o when determined by staining with phosphotungstate (Brenner et al., 1959). Because of this variation we determined the radii of the fiber

precursors from the measured sedimentation coefficient ($s_{20,w}$) by deriving an equation relating $s_{20,w}$ to the fiber dimensions.

Bloomfield, Dalton & van Holde (1967) derive the frictional coefficient of a cylindrical rod of length 2a by approximating a rod as a linear array of spheres of radius b such that a = nb. Combining their equations 32 and 37 yields the following expression for the frictional coefficient of such a rod:

$$f = \frac{6\pi\eta(2a)}{2\ln\frac{2a}{b} - 0.2316 + \frac{b}{a}} \qquad (2)$$

η = viscosity of solvent

The A half fiber is not a cylindrical rod in electron micrographs but has a knob on one end (Ward et al., 1970a). Assuming this knob is present on the fiber in solution, it would tend to align the fiber during centrifugation with the direction of sedimentation reducing its frictional coefficient slightly. If the knob aligned the fiber perfectly the frictional coefficient would be reduced by about 30% (compare Bloomfield et al. equations 32 and 31). The knob is unlikely to have this large an effect so in the absence of precise calculations we estimate that the knob would reduce the frictional coefficient by 5% and include this correction in the determination of the molecular weight of A. Other uncertainties in the molecular weight determination are greater than this uncertainty in frictional coefficient.

Equation 2 was substituted into the equation defining the sedimentation coefficient, (Tanford, 1961, p. 365)

$$s = \frac{Mw(1-\bar{v}\rho)}{Nf} \qquad (3)$$

ρ = density of solvent

yielding,

$$s = \frac{Mw(1-\bar{v}\rho)(2 \ln \frac{2a}{b} - 0.2316 + \frac{b}{a}}{N\ 6\pi\eta\ (2a)} \qquad (4)$$

Substituting equation (1) for the molecular weight into equation (4) gives an equation relating the sedimentation coefficient to the fiber dimensions and partial specific volume:

$$s = \frac{\frac{1}{\bar{v}} b^2\ (1-\bar{v}\rho)(2 \ln \frac{2a}{b} - 0.2316 + \frac{b}{a})}{6\eta} \qquad (5)$$

Equation (5) was used to estimate the fiber radii from measured values of $s_{20,w}$, the measured length, and $\bar{v}$ determined from amino acid analysis, by programming an IBM 360/50 computer to calculate the radii which gave $s_{20,w}$ closest to the measured value. Then the molecular weights of each fiber precursor were calculated by equation (1). The results of these calculations are summarized in Table 1.

As noted in the previous section more than 80% of the molecular weight of the precursors is contributed by the large polypeptides. From the molecular weights of these polypeptides plus 10 or 20% we calculated that the molecular weight of each precursor would approximate the molecular weight in Table 1 only if there were two copies of the large polypeptides in each precursor. The approximate molecular weights would be C = 270,000; BC = BC' = 300,000; A = 330,000 if there were two copies of the large polypeptides in each precursor. These values are in agreement with those in Table 1.

TABLE 1

Precursor	Length A°	$s_{20,w}$ (sec x 10^{-13})	Diameter A°	Molecular Weight (daltons)
C	560 ± 30	7.9 ± .3	26 ± 1	260,000 ± 30,000
BC	690 ± 40	8.0 ± .3	26 ± 1	310,000 ± 35,000
BC'	690 ± 40	8.2 ± .3	26 ± 1	320,000 ± 35,000
A	690 ± 40	9.0 ± .4	27 ± 1	320,000 ± 40,000

Table 1. Estimation of the molecular weight of undissociated fiber precursors.

The lengths of each precursor determined by Ward et al. (1970a) are shown rounded off to indicate the BC, BC' and A are indistinguishable in length. The sedimentation coefficients were determined by preparative zone sedimentation in an SW 50 rotor of a Beckman L-2 ultracentrifuge using 5-20% sucrose gradients centrifuged at 47,000 rev./min for 8 hr. Labeled precursors were used and the peaks identified by liquid scintillation counting of fractions after collection by piercing the bottoms of the centrifuge tubes. Hemoglobin ($s_{20,w}$ = 4.3) and catalase ($s_{20,w}$ = 11.3) were used as markers to determine the sedimentation coefficients. The diameter of the fibers was calculated from the sedimentation coefficient using equation 5. For this calculation values of $\bar{v}$ for A (0.726) and BC' (0.734) were calculated from amino acid analyses of purified fibers (unpublished results) as described by Schachman (1959). BC and C were assumed to have the same $\bar{v}$ as BC'. From the length, diameter and $\bar{v}$ the molecular weights were calculated using equation 1. The uncertainty in the molecular weights results from propagation of the error in the measured parameters used for calculation.

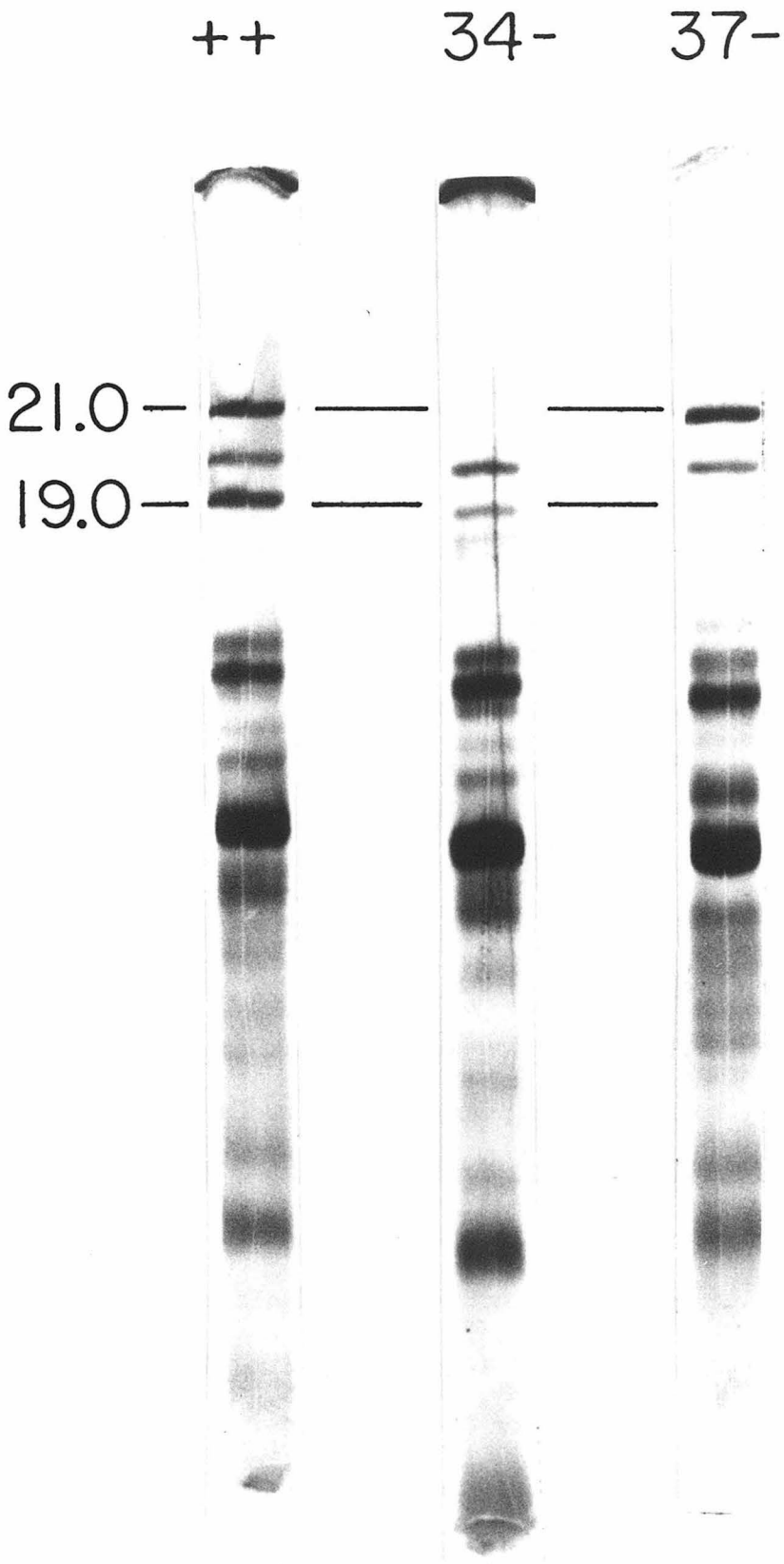
(d) Genetic control of the tail fiber polypeptides

To determine the gene control of the two large tail fiber polypeptides, crude lysates made from restrictive cells infected with phage carrying amber mutations in various tail fiber genes were examined for the presence of bands 19.0 and 21.0. Phage proteins were labeled with radioactive amino acids added to the infected cells as described in Materials and Methods. After lysis, dialysis and dissociation in SDS as above, the preparations were electrophoresed on SDS gels which were then sliced, dried and autoradiographed. Because amber mutations lead to termination of polypeptide synthesis at the amber codon (Sarabhai, Stretton, Brenner & Bolle, 1964), the polypeptide product of a gene carrying an amber mutation will be smaller than the normal gene product, and will therefore migrate faster on SDS gels. Thus amber mutations in the genes controlling the band 19.0 and 21.0 polypeptides will lead to elimination of these bands. New bands representing the amber peptides will appear, but may be obscured by the many other bands in the lower region of the gels. This method of inferring the gene control of polypeptides was first developed and applied to the study of protein synthesis in T4 by Hosoda & Levinthal (1969).

Plate IV shows autoradiographs of SDS gels made with wild type, 34-defective and 37-defective lysates. Band 21.0 is missing from the 34-defective lysate; thus the 150,000 Mw polypeptide comprising this band is the product of gene 34 (P34). Band 19.0 is missing from the

Plate IV. SDS gels of lysates.

Samples containing approximately 300,000 cts/min of ^{14}C were electrophoresed at 8 ma/sample on 7.5% acrylamide gels containing 0.1% SDS then the gels were sliced and autoradiographed for 100 hr. (++) wild type lysate; (34-) X2D (am A455, am B25) lysate; (37-) X2J (am N52, am B280) lysate.


++
34-
37-
21.0
19.0

37-defective lysate; thus the 123,000 Mw polypeptide comprising this band is the product of gene 37 (P37).

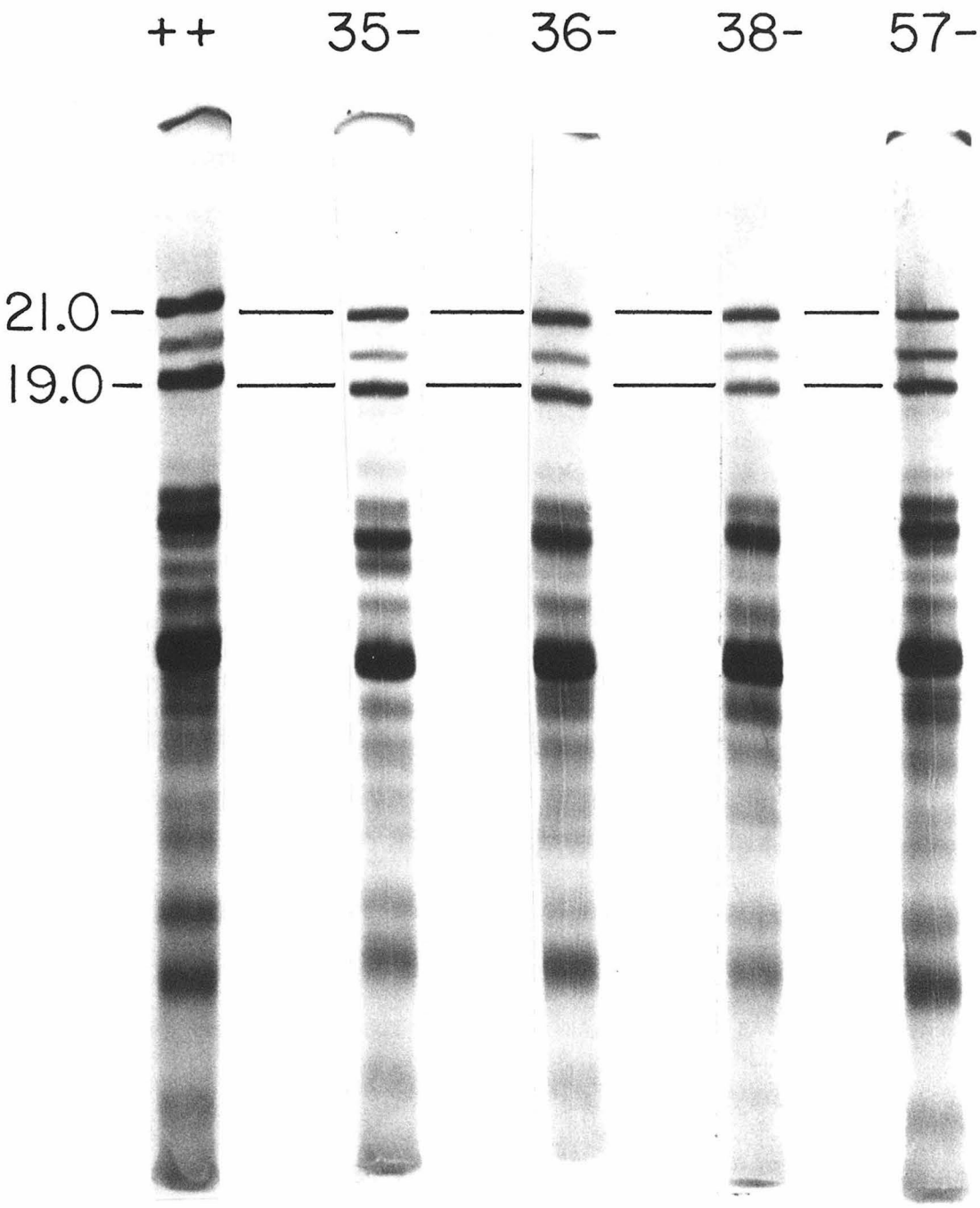
Plate V shows autoradiographs of 35-, 36-, 38-, and 57-defective lysates. Both bands 19.0 and 21.0 are present in these lysates, indicating that these genes do not grossly affect the synthesis of the P34 and P37. Fourteen different 36-defective lysates have been prepared with slight variations in the methods of labeling and lysing the cells. One of them lacked band 19.0 and showed instead a new band with a slightly higher mobility corresponding to that of the band found on gels of purified C half fibers (which are also prepared from a 36-defective lysate). The non-reproducibility of this effect suggests that it may be an artifact of lysate preparation, but we have been unable to determine the variable that is responsible.

(e) The effect of gene 36 amber mutations on P37 synthesis

The tail fiber genes 34 to 38 are located in numerical order in a cluster on the genetic map (Epstein et al., 1963). Amber mutations in gene 34 have been shown to impair the expression of gene 35 (Nakata & Stahl, 1966). Such a polar effect implies that these genes are included in the same unit of transcription, and are translated in the order 34 to 35 from a single polycistronic messenger RNA (see Zipser, 1969 for review). An earlier report that gene 36 amber mutations have a similar polar effect on gene 37 expression (Russell, 1967) was brought to our attention by J. King & U. Laemmli. As a test for this and other similar effects, we have examined the influence of various amber mutations on the synthesis of P37, as indicated by the relative

Plate V. SDS gels of lysates.

Samples were prepared in parallel to those for Plate 4. (++) wild type lysate; (35-) *am* B252 defective lysate; (36-) *am* E1 defective lysate; (38-) *am* C290 defective lysate; (57-) *am* E198 defective lysate.


++
35-
36-
38-
57-
21.0
19.0

levels of radioactivity in bands 19.0 and 21.0 on gels of defective lysates as above. To obtain quantitative results, gel fractionation and liquid scintillation counting of the gel fractions were employed in place of autoradiography. A typical fractionation profile from the upper portion of a gel of a wild type lysate is shown in Figure 1. Similar experiments were carried out with 35-, 38-, 57-, and 2 different 36-defective lysates. The results are presented in Table 2 as the ratio of total counts in the P37 peak to total counts in the P34 peak to normalize for variations in total label incorporated in the various lysates. For wild type, 35-, 38- and 57-defective lysates, the ratio varies from 1.22 to 1.46. By contrast the ratios for the two 36-defective lysates are 0.69 and 0.84. These results indicate that amber mutations in gene 36 reduce the amount of P37 synthesized during the labeling period by approximately 40%. The three gene 36 mutations used for this experiment were mapped by three factor crosses as described by Nakata & Stahl (1966). They were found to map two recombination units apart, with am302 and am229 at the same site, and closer to gene 37 than amE1. We conclude that there is a polar effect of gene 36 amber mutations on gene 37 expression, but no significant gradient in polarity between the two amber sites tested.

(f) The effect of gene 38 and 57 amber mutations on P34 and P37

Earlier studies have shown that production of A antigen and the corresponding half fiber requires the function of gene 57 as well as gene 34, and that production of the C antigen and its corresponding half fiber requires the functions of genes 38 and 57 as well as gene 37

FIG. 1. Fractionation and counting of an SDS gel of a wild-type lysate.

A ^{14}C-labeled lysate was dissociated and run on an SDS-gel as described in the legend to Plate 2. After electrophoresis the top 1.3 cm of the gel was cut off and discarded then twenty fractions were collected starting at the top of the remaining gel. These were mixed with scintillation cocktail and counted in a Beckman LS 200 Liquid Scintillation Spectrophotometer and the results plotted (——o——). The identity of the peaks of counts corresponding to P34 and P37 was established by comparison with the counting of a parallel gel of a mixture of ^{14}C-labeled purified BC' (---Δ---) and ^{3}H-labeled purified A (---o---). The mobility of each of these peaks was identical within two fractions for many independent lysates and gels.

TABLE 2

Defective Gene	Amber Mutation	P37 cts/min / P34 cts/min	
		Average	Range
++	---	1.46 (4)	1.32 - 1.62
35	B252	1.36 (2)	1.31 - 1.42
38	C290	1.22 (3)	1.1 - 1.36
57	E198	1.29 (4)	1.18 - 1.48
36	E1	0.84 (6)	0.62 - 1.18
	E302, E229	0.69 (4)	0.64 - 0.73

Table 2. The effect of amber mutations in gene 36 on the synthesis of P37.

Various defective lysates were electrophoresed and fractionated as described in the legend to Figure 1. The cts/min in the P37 peak and the P34 peak were totaled and the ratio of P37 cts/min : P34 cts/min was calculated. The average for each defective mutant is shown in the third column; the number of gels used for computation is shown in parentheses. Two or more independent lysates were prepared for each mutant except B252. The range of values of the different gels is shown in the last column.

(Edgar & Lielausis, 1965; Eiserling, Bolle & Epstein, 1967; King & Wood, 1969). Since genes 38 and 57 are not required for the synthesis of P34 and P37, as shown in section (d) above, they must be involved in the conversion of these polypeptides to antigenically active structures in the early steps of tail fiber formation. Two kinds of experiments have shown differences in P34 and P37 properties resulting from the action of genes 57 and 38.

In the first, crude lysates of radioactively labeled mutant-infected cells were fractionated by centrifugation at 14,000 x g for 10 min. The amounts of P34 and P37 counts in the supernatant and pellet fractions were determined after dissociation and gel electrophoresis in SDS by fractionation and counting as in Figure 1. The results are presented in Table 3 as the percent of P34 and P37 counts found in the supernatant fractions of the various lysates. Most of the P34 and P37 counts remained in the supernatant except in 38-defective lysates where only 1% of the P37 counts were found in the supernatant, and in 57-defective lysates where less than 6% of both the P37 and P34 counts were found in the supernatant. Neither P34 nor P37 was removed from the pellet fraction of a 57-defective lysate by resuspension in 0.01 M phosphate buffer and recentrifugation. One attempt was made to remove the polypeptides by treating a freshly prepared 57-defective pellet with infected-cell extracts containing P57 and P38, but no significant solubilization of either P34 or P37 was observed after several hours incubation. This is not too surprising since neither P57 nor P38 have yet been shown to function <u>in vitro</u> (Edgar & Wood, 1966; King & Wood,

TABLE 3

Defective Gene	% P37 in Supernatant	% P34 in Supernatant
34	-	70
37	90	-
36	91	69
38	69	1
57	< 6	< 5

Table 3. Distribution of P34 and P37 in centrifuged lysates.

Various defective lysates were centrifuged at 14,000 x g for 10 min in a Sorvall refrigerated centrifuge. The supernatants were decanted and dialyzed against 0.01 M phosphate buffer, and the pellets were resuspended in this buffer. After dissociation in SDS and mercaptoethanol the two fractions and an aliquot of the unfractionated lysate were electrophoresed on SDS gels and fractionated as described in the legend to Figure 1. The counts in the P34 and P37 peaks were totaled for each lysate and the percentage of total counts in the supernatant fraction is shown.

1969). These results suggest that one effect of genes 57 and 38 action is the conversion of P34 and P37 from a rapidly-sedimenting non-antigenic state to antigenic tail fiber components, with P34 conversion requiring only gene 57 function, and P37 conversion requiring both 57 and 38 function.

A second effect of gene 57 and 38 defects is to make P34 and P37 soluble in SDS at 37°C. This observation was reported to us by J. King & U. Laemmli (personal communication), and we have repeated their findings as shown in Plates 6 and 7. Plate 6 compares autoradiographs of SDS gels made from wild type, 36-, and 35-defective lysates after either heating to 100°C as before or heating to only 37°C. Bands 19.0 and 21.0 are faint in the samples heated to only 37°C, and other higher molecular weight components are observed. Identical results are observed for P34 in a 37-defective lysate and for P37 in a 34-defective lysate. When purified tail fibers or tail fiber precursors are subjected to the same two dissociation conditions bands 19.0 and 21.0 appear only after 100°C treatment; after 37°C treatment bands with mobilities corresponding to molecular weights greater than 250,000 are found. Plate 7 shows the results of similar experiments with 38- and 57-defective lysates. In the 38-defective lysate the P37 band is present after 37°C treatment; in the 57-defective lysate both P34 and P37 bands are present after 37°C treatment. Thus a second effect of gene 57 and 38 action is to convert P34 and P37 from forms which dissociate in SDS at 37°C to structures which are not dissociable to free polypeptides under these conditions. Again P34 conversion requires gene 57 function alone, while P37 conversion requires both 57 and 38 functions.

Plate VI. SDS gels of lysates treated at 37°C or 100°C.

Lysates were treated with 1% SDS and 1% mercaptoethanol at 100°C for 2 min, or 37°C for 30 min. (++) wild type lysate; (36-) *am* E1 lysate; (35-) *am* B252 lysate.

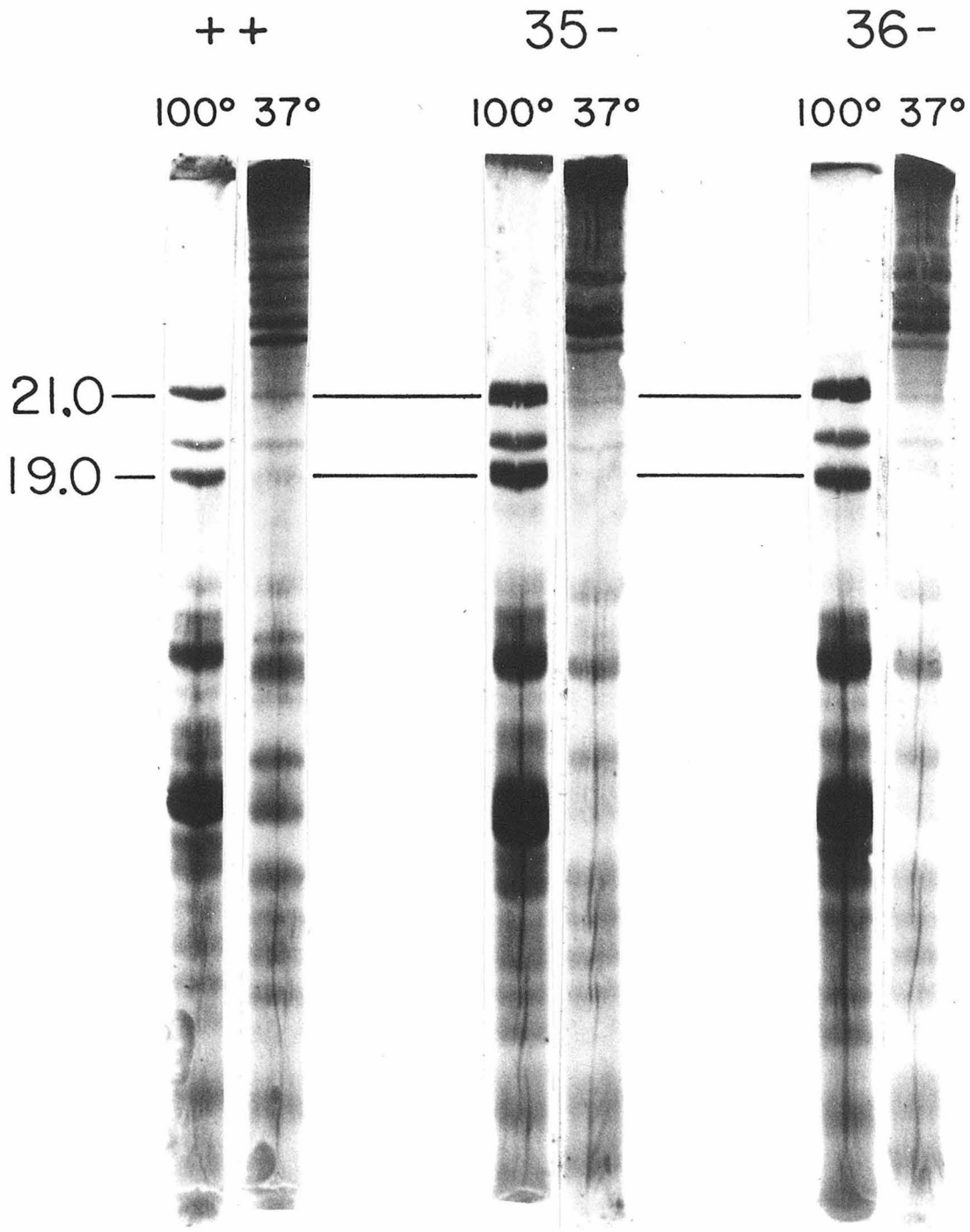
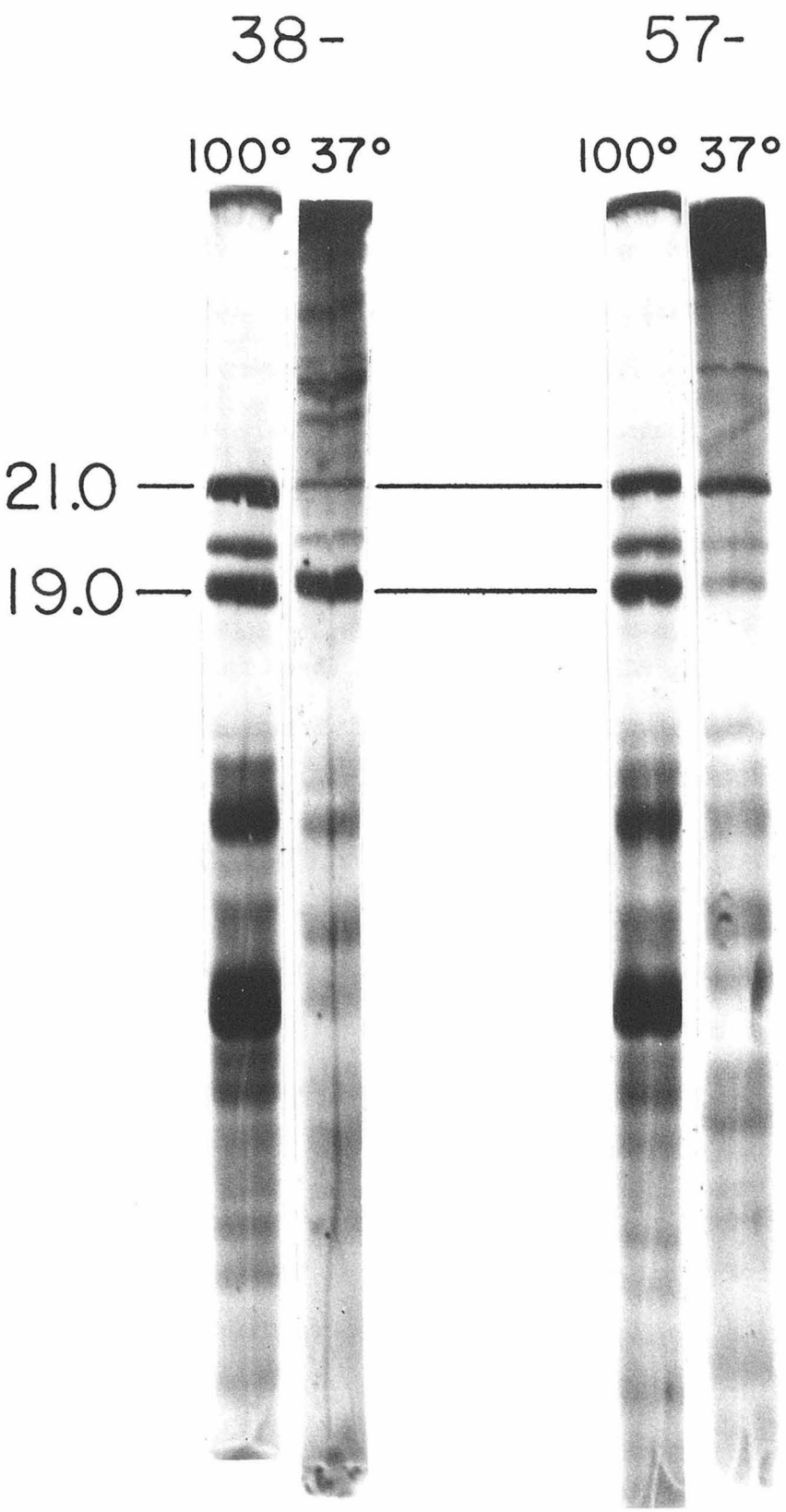

++
35-
36-
100° 37°
100° 37°
100° 37°
21.0
19.0

Plate VII. SDS gels of lysates treated at 37°C or 100°C. Samples were prepared in parallel to those for Plate VI. (57-) am E198 lysate; (38-) am C290 lysate. In two other preparations of 57-defective lysates the band 19.0 was as strong after treatment at 37°C as at 100°C.


38-
57-
100° 37°
100° 37°
21.0
19.0

In order to test the effect of the two dissociation conditions on the A and C antigens, the lysates and isolated fiber precursors used for the previous experiment were assayed before and after SDS treatment for A and C serum-blocking-power as described by Ward et al. (1970a). Heating to 37°C did not effect the amount of either antigen in any of the preparations, but heating to 100°C destroyed more than 99.9% of the antigen in all of them. Thus a condition which dissociates the P34 and P37 also destroy the A and C antigens.

4. Discussion

The presence of bands 19.0 and 21.0 on SDS gel patterns of phage and isolated tail fibers and their absence on SDS gel patterns of fiber-less particles indicates that these bands represent structural polypeptides of the tail fiber. The absence of bands 19.0 and 21.0 from SDS gels of 37- and 34-defective lysates suggests that genes 34 and 37 direct the synthesis of these structural polypeptides. The structural contribution of P34 was predicted by Edgar & Lielausis (1965) from their observation that temperature sensitive mutations in this gene alter the tail fiber A antigen. The structural contribution of the gene 37 product was also predicted by King & Wood (1969) from the observation that some mutations which affect the ability of T4 to adsorb to host cells map in this gene.

The decreased amount of P37 synthesized in cells infected with amber mutants in gene 36 suggests that these two gene products are translated from the same operon starting from gene 36 (Zipser, 1969). The transcription unit probably includes gene 38 as well because the

physical mapping of these genes by Gisela Mosig (1968) suggestthat genes 37 and 38 may share the same initiation point of transcription. The two amber mutation sites in gene 36 differed insignificantly in the extent of polarity, indicating that any gradient in polarity within gene 36 is small. Together with the data of Nakata & Stahl (1967) our results suggest that the tail fiber genes 34 to 38 are synthesized from two operons, one with a promoter at the rIII end of gene 34 extending through gene 35; the other with a promoter at the rIII end of gene 36 extending through gene 38.

The role of genes 57 and 38 in the assembly of the tail fibers is not accessible to direct study because 57- and 38-defective extracts do not complement other tail fiber defective extracts *in vitro* (Edgar & Wood, 1966; King & Wood, 1969). Since we have shown that genes 57 and 38 do not regulate the synthesis of the major tail fiber polypeptides, P34 and P37, the role of genes 57 and 38 must be to promote the assembly of P34 and P37 into functional fibers. Our comparison of P34 and P37 before and after the action of genes 57 and 38 shows two effects that must be under the control of these genes and the earlier serological studies of Edgar & Lielausis (1965) show a third: in the absence of gene 57 function the P34 cosediments with the cell debris, it is dissociable by SDS at 37°C and little A antigen is formed; similarly, in the absence of either gene 57 or 38 function the P37 cosediments with the cell debris, it is dissociable in SDS at 37°C and little C antigen is formed.

A plausible interpretation of these observations is that genes 57 and 38 control the dimerization of P37 to form the C half fiber and

gene 57 controls the dimerization of P34 to form the A antigen and subsequently the A half fiber. The determination of the half fiber molecular weights indicated that P34 and P37 are present in two copies in the half fibers. That these gene products are not monomers in their functional form has also been shown by the finding of Bernstein, Edgar & Denhardt (1965) that temperature-sensitive mutations in these genes exhibit efficient intragenic (interallelic) complementation. This demands that the gene products form multimers in order that the two mutationally altered polypeptides can interact and complement each other (McGavin, 1968; Fincham, 1966). The resistance of P34 and P37 to dissociation in SDS at 37°C after gene 57 and 38 action and the appearance of high molecular weight components (>250,000) on SDS gel patterns of isolated C or A half fibers is consistent with dimer formation resulting from gene 57 and 38 action.

Both the C and A antigens are stable in SDS at 37°C but are destroyed by heating to 100°C. Electron microscopic studies of antibodies bound to tail fibers (Yanagida & Ahmad-Zadeh, 1970) indicate that both C and A antigenic determinants are distributed along the length of their half fibers, but the chemical nature of these antigens has not been determined. These antigens could be specific conformations of P34 and P37 or they could be haptens added to these polypeptides in the course of assembly. The former is more likely for the A antigen because Edgar & Lielausis (1965) have shown that temperature-sensitive mutations in gene 34, which presumably cause misfolding of P34, alter the amount and apparent

affinity of the A antigen(s) as if the conformation of P34 determined the antigen. If this is so the P34 must change conformation in the course of dimerization in order to explain the lack of antigenicity of the P34 monomer. An alternative explanation, that P34 has the A antigen as a monomer, but the antigenicity is not detectable because the monomer interacts with the cell debris, is ruled out by the observation that treatment of a 57-defective lysate with SDS at 37°C, which removes the monomer from the debris, does not increase the amount of antigen.

The interpretation that the role of P57 and P38 is to assemble P34 and P37 into dimeric fibers does not predict whether these gene products act stoichiometrically or catalytically. Snustad (1968) has approached this question for most of the genes of T4 by determining the dependence of phage yield on levels of various gene products which are varied by multiply infecting cells with different ratios of amber mutant to wild type phage. He found that the yield is not as dependent on the amount of P38 as it is on the amount of the other tail fiber gene products, suggesting that P38 is made in several-fold excess or used catalytically. Since gene 38 is probably on the same operon as genes 36 and 37 and is distal to them, P38 cannot be made in larger numbers than P37 (Zipser, 1969). Since P37 is present in only two copies per fiber, the P38 cannot be in more than two-fold excess; thus Snustad's result suggests that it acts catalytically in

Snustad finds that
assembly. In contrast, the yield of phage is proportional to the amount of P57 to nearly the same extent that it is proportional to P34 and P37 as if P57 is used stoiciometrically. If P57 does bind to both P34 and P37 in the course of assembly it must have a different conformation in the two half-fibers or be non-antigenic because there are no antigens in common between the A and BC' half fibers (Edgar & Lielausis, 1965; Ward et al., 1970a).

That gene 57 is under different transcriptional control than the other tail fiber genes is consistent with the proposed role of P57 in controlling the association of P34 and P37. Its synthesis begins early after infection and increases following DNA replication (Hosoda & Levinthal, 1968; Wilhelm & Haselkorn, 1970); thus a pool of P57 accumulates in the cell before the synthesis of P34 and P37 begins so that P57 would be immediately available to interact with these polypeptides as they are synthesized. In the absence of P57, P34 and P37 cosediment with the cell debris as if they aggregate. A similar aggregation is observed in the pathway of head assembly. In the absence of P31 (an early gene) the major structural component of the head, P23, aggregates into "lumps" which cosediment with the cell debris (Laemmli, Beguin & Gujer-Kellenberger, 1970). These authors showed that the aggregation into "lumps" was reversible by doing temperature shift experiments with temperature sensitive mutations in gene 31. Unfortunately, temperature sensitive mutations are not available in genes 57 or 38 so the reversibility of the P34 and P37 aggregation is not easily tested.

The large sizesof P34 and P37 provide a simple mechanism for

length determination of the halves of the tail fiber; the length is determined by polypeptides which extend the length of the fiber. The 150,000 molecular weight P34 should have about 1200 amino acid residues; if it were fully extended the polypeptide would be 3100 A° long, if it were 100% alpha-helical it would be 1800 A° long (Dickerson & Geis, 1970, p. 28). Since the assembled A half fiber, containing two copies of P34 is only 690 A° long, (Ward et al., 1970a) the polypeptides must be folded back on themselves like a hairpin or not in a fully extended or alpha-helical conformation. If the two polypeptides were entwined about one another this could shorten them and would provide a large surface of contact between them explaining the strength of the interaction which prevents their dissociation in SDS at 37°C. Similar calculations with the 123,000 molecular weight P37 indicate a maximum length of 2500 A° and an alpha-helical length of 1400 A°. Since the length of fiber precursor, C, containing two copies of P37, is only 560 A° (Ward et al., 1970a) these polypeptides must also be folded back on themselves or else not in a fully extended or alpha-helical conformation.

Two observations suggest that P37 does not fold back on itself like a hairpin. Temperature sensitive mutations in gene 37 which allow formation of normal C antigen but prevent formation of the B antigen map near the N-terminus of the gene (Edgar & Lielausis, 1965). The B antigen, which results from the action of gene 36 on the C fiber, is located at the end of the C fiber which interacts with the A half fiber (Yanagida & Ahmad-Zadeh, 1970). Since temperature-sensitive alterations in P37 which prevent formation of the B antigen should be near the site of B antigen formation, P37 must be folded in the C fiber so that its

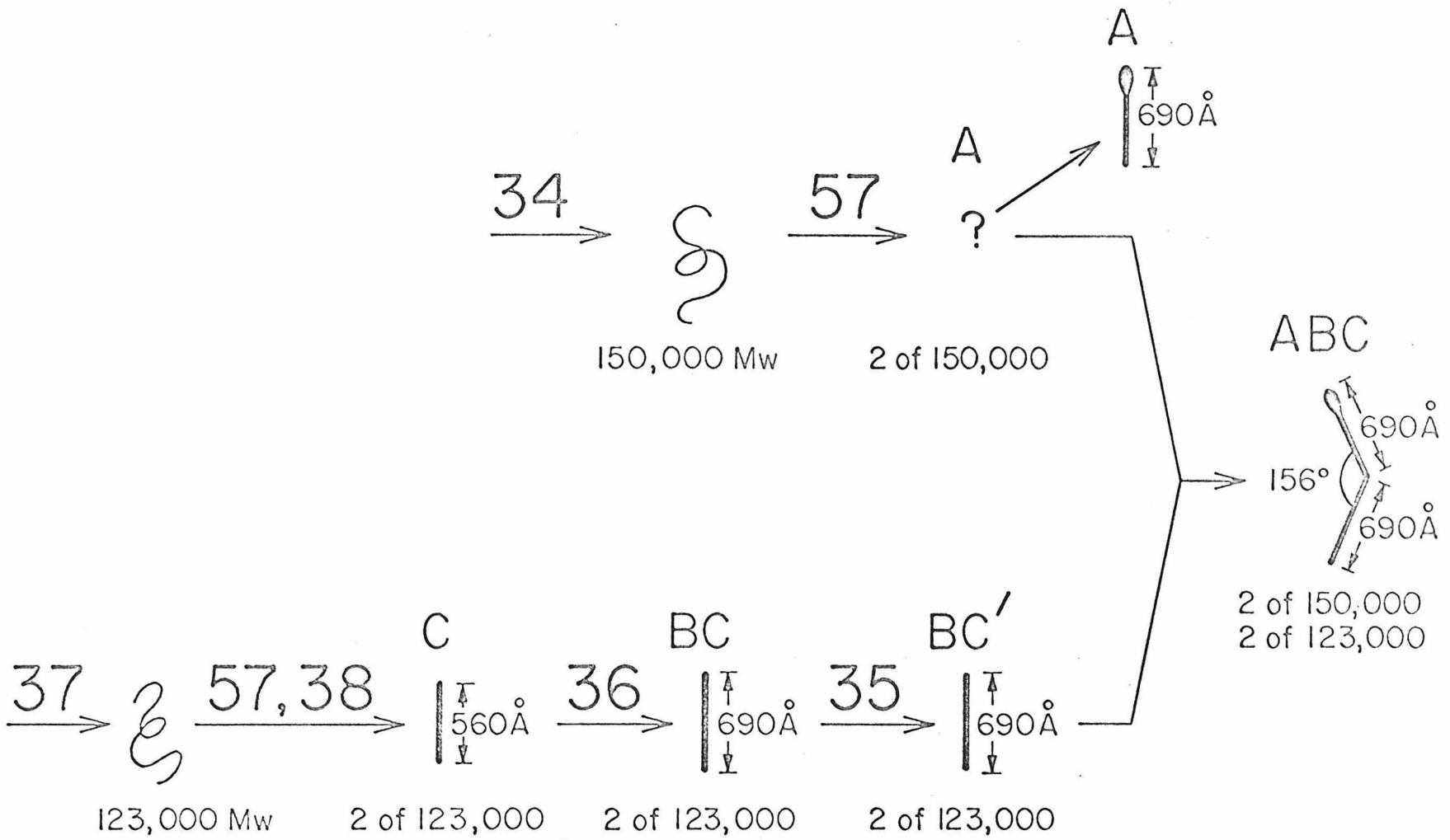
N-terminal end is at the B end of the C fiber. On the other hand, host range mutations in gene 37 map near the C-terminus of the gene (S. Beckendorf, personal communication). Since the site of interaction of the C fiber with the E. coli host is the distal tip of the fiber, opposite from the site of B antigen formation (Simon & Anderson, 1968; Wilson, Luftig & Wood, 1970) P37 must be folded so that its C-terminal is at the distal end of the C half fiber.

The results of this study and the results of the previous paper (Ward et al., 1970a) can be summarized by reformulating the pathway of tail fiber assembly (King & Wood, 1969) in greater detail as shown in Figure 2. In the top branch of the pathway the 150,000 molecular weight P34 dimerizes to form the A antigen under the control of gene 57. The knobbed A half fiber is shown as an aberrant structure because when purified it is inactive in vitro, and it is not observed in crude extracts which are active in vitro. Presumably the in vitro active A antigen is in a form we do not recognize with the electron microscope which becomes the knobbed A half fiber when it interacts with BC'.

In the lower branch of the pathway the 123,000 molecular weight P37 dimerizes to form the C antigen and the C half fiber under the control of genes 57 and 38. It is not certain that the short C half fiber is a normal intermediate in assembly. We found that the purified C which was used for length measurements seems to have P37 reduced in molecular weight. We tested whether this apparent

FIG. 2. The pathway of tail fiber assembly.

Arrows indicate the steps of assembly under control of the numbered genes shown above them. The structures shown as intermediates are drawn to approximate their appearance in the electron microscope and the letters above them designate their antigens. The lengths of these intermediates and the number and molecular weight of their major polypeptides are also shown.


A
690Å
34
57
A
?
150,000 Mw
2 of 150,000
ABC
690Å
156°
690Å
2 of 150,000
2 of 123,000
37
57,38
C
560Å
36
BC
690Å
35
BC′
690Å
123,000 Mw
2 of 123,000
2 of 123,000
2 of 123,000

cleavage in P37 accounts for the decreased length of C by measuring the length of C in a crude extract. The length was 560 A° in agreement with the length measurement on the purified C (R. Luftig, personal communication). Thus C may be a normal intermediate in fiber assembly. Gene 36 controls the introduction of the $_{A}$B antigen at one end of the C fiber and an increase in length to 690 A°. Gene 35 does not change the antigenicity or the electron microscope appearance of the fiber, but is necessary to convert it to an active form which can interact with the A antigen to form the whole fiber with its characteristic kink in the middle and knob at one end.

This pathway does not specify whether or not genes 35, 36, 38 and 57 contribute structural polypeptides to the fiber. Indirect arguments presented above suggest that P57 may be a structural polypeptide and P38 may not. The role of these genes and genes 35 and 36 is being examined more directly by studying the minor bands on SDS gels of the isolated fibers.

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General Discussion

The results and discussion presented in parts I and II of this thesis provide partial answers to the three questions posed in the introduction and point the way to experiments which will give more complete answers. These answers and suggestions for further research are considered below.

(1) Does each gene product required for tail fiber assembly become incorporated into the completed fiber or do some of them act as catalysts of assembly?

The results in Part II show that P34 and P37 are the major polypeptides of the complete fiber. Indirect arguments in the discussion of part II suggest that P57 is also structural but P38 is not. From Part I an effect of P36 action is to lengthen the C half fiber. This could occur most simply if P36 is incorporated at one end of the C fiber, adding the B antigen and increasing the length, but there is not direct evidence for this. By electron microscopy and serology P35 makes no detectable change in the BC fiber so if it adds a structural polypeptide to the fiber, it is probably a small one that does not change fiber morphology.

The structural contribution of P35, P36, P38 and P57 could be tested directly if bands corresponding to their gene products could be identified on SDS gels of lysates and purified phage. This would require much higher

resolution of small molecular weight phage components than we have demonstrated using the standard Maizel SDS gel technique. It is now possible to obtain such resolution using a discontinuous buffer system containing SDS (1). This system might allow resolution of the minor components on SDS gels of isolated fibers and precursors permitting identification of the genes synthesizing these polypeptides after autoradiography of gels of crude lysates of mutant-infected cells. One problem with analysis of minor components of the isolated precursor structures is that C, BC and A are biologically inactive after purification. Thus one has to be careful in interpreting the subunit structure of these precursors because the inactivity could be due to a missing structural component. A simple way to avoid this problem and to avoid the difficult purification of the precursors is to label each precursor with radioactive amino acids in a crude extract then mix it <u>in vitro</u> with an unlabeled extract containing all of the gene products necessary to convert the precursor to complete fibers and attach these to phage. The phage can be easily purified from unattached labelled phage proteins by centrifugation. The labelled polypeptides which are attached to the phage can be analyzed after dissociation and gel electrophoresis in SDS by autoradiography or scintillation counting. Preliminary results of these kinds of experiments demonstrate their feasibility.

(2) What kind of bonds hold the structural polypeptides of the tail fiber together?

All the dissociation studies on the isolated fibers and precursors were done in SDS and mercaptoethanol. We showed in part II that heating to 37°C in these reagents was not sufficient to dissociate P34 and P37 to their subunit weights. This indicates that the bonds holding these polypeptides together are relatively strong because many protein complexes are dissociated under these conditions. The observation that heating to 100° in SDS and mercaptoethanol dissociates the P34 or P37 dimers suggests that these complexes are not held together by covalent bonds other than disulfides. It has not been determined whether disulfide bonds exist between the polypeptides because nearly all dissociations were carried out in the presence of mercaptoethanol. In a few experiments with isolated BC', omitting mercaptoethanol from the SDS did not prevent the P37 from dissociating indicating that disulfide bonds probably do not hold the gene 37 polypeptides together. A second class of covalent bonds that might be labile in SDS at 100° and thus could cross link the P34 or P37 polypeptides are esters between carboxyl groups of acidic amino acids and hydroxyl groups of amino acids such as serine. These would not ordinarily be hydrolyzed at neutral pH at 100°, but they could be under unusual stress due to protein conformation in the assembled fiber and thus be labile. However, treatment of isolated

BC' with 1 M hydroxylamine at pH 10 does not increase the susceptibility of P37 to dissociation in SDS at 37°, so esters are not involved in the P37 association. It appears at present that covalent bonds do not play a role in the associations of P34 and P37 and that these polypeptides are held together by non-covalent interactions.

(3) What imposes the obligate ordering of the steps of fiber assembly?

This question is difficult to answer in the absence of exact knowledge of the contributions of the P57, P38, P36, and P35, but some speculations can be made. It was argued in part II that the formation of the A and the C antigen might involve conformational changes in P34 and P37. If such changes in conformation occur commonly, they could impose the ordering of the pathway of assembly by having the action of each gene product form the specific sites with which successive gene products interact. Thus the gene products act in their proper sequence because their sites of interaction are not folded properly or are inaccessible before the previous gene has acted.

Comparative studies of the conformations of the polypeptides in the structures C, BC and BC' could be made to test this. Circular dichroism could be used to detect gross changes in conformation but low angle X-ray diffraction would provide more information. Studies of oriented gels of the BC' half-fiber by X-ray diffraction are presently

being undertaken by Dr. Michael Moody at the Rockefeller Institute.

Another direction of research on these fiber structures which has not been described in this thesis is determination of their chemical composition. This work is being done in other laboratories and some analyses are presently in progress on the isolated BC' structure. Isolation of large quantities of the other structures would allow comparative studies of their chemistry which could give insight into the contributions of genes 35 and 36.

The work presented in this thesis indicates that it is possible to take advantage of the availability of mutants in phage T4 to study the molecular details of phage assembly. The methods developed in this work should help to determine the role of the genes involved in the assembly of structures other than the tail fiber. So far no new mechanisms of assembly have been found but detailed assignment of gene functions is just beginning. As this work proceeds our understanding of mechanisms of assembly will grow and the work with model systems such as T4 will be extended to the more complex structural elements of higher organisms.

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An Improved Transverse Destaining Apparatus for Acrylamide Gels

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Of the several methods used to destain acrylamide gels, the most common is lengthwise electrophoresis (1). This method requires careful handling of individual gels and takes several hours to remove excess stain completely. The handling of gels can be reduced by simply allowing excess stain to diffuse out of the gels; however, this requires 12–24 hours to destain well. I have found that it is difficult to remove the sen-

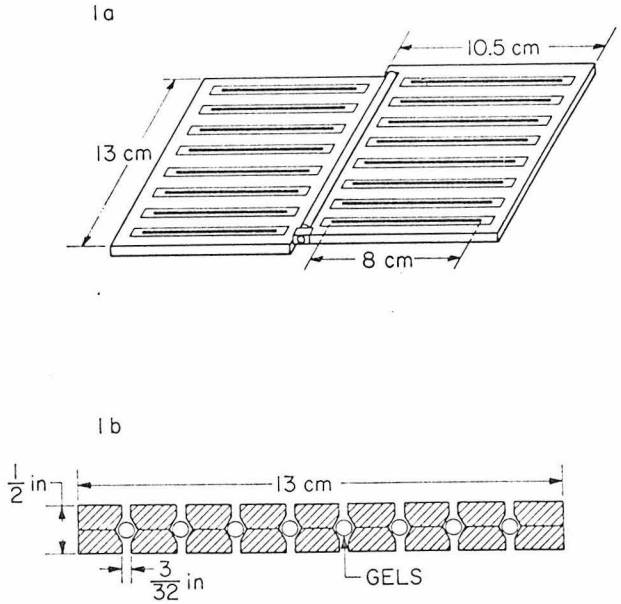

FIG. 1. Destaining rack. (1a) Rack open. It is constructed of two ¼ inch pieces of Plexiglas. The two halves are hinged together by a pin. Eight parallel slots 8 cm long are cut in each sheet of plastic, and the inside edges are then grooved with a 60° included angle mill cutter to ½ their depth. (1b) Cross-section of closed rack with gels in place.

sitive stain Coomassie Brilliant Blue (CBB) with either of these methods. As an alternative procedure, transverse electrophoresis is a simple way to destain CBB stained gels. This paper describes a transverse destaining apparatus similar to those of Schwabe (2), Maurer (3), and Peterson (4) but with a new gel supporting rack that allows staining, rapid destaining, and photography of the gels without removal from the rack.

4% and 5% acrylamide gels, 6.5 cm long and 0.5 cm in diameter, are used. The presence of 8 *M* urea or 1% sodium dodecyl sulfate (SDS) in the gels does not affect the staining or destaining procedures.

After electrophoresis is complete the gels are removed from their tubes and placed in the rack shown in Figure 1. The rack is closed by a clamp and placed in a container of staining solution: 0.25% CBB (Mann) in a mixture of water, acetic acid, and methanol 5:1:5 (5). Staining requires 2 hours, but can also be carried out overnight.

To destain, the rack is rinsed and placed in the destaining box (Fig. 2) with freshly mixed destaining solution: 10% methanol and 10% acetic acid by volume. Current is applied from a simple DC power supply con-

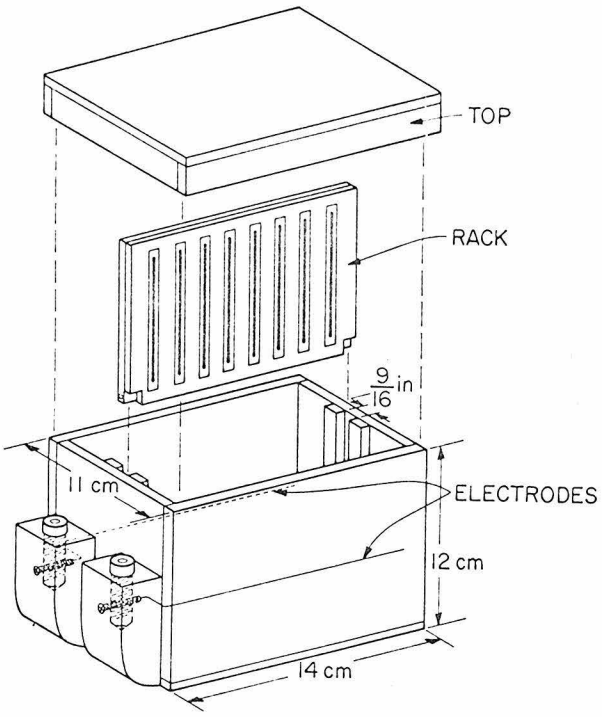

FIG. 2. Complete destainer. The destaining box is made of ¼ inch Plexiglas. The electrodes are platinum wire.

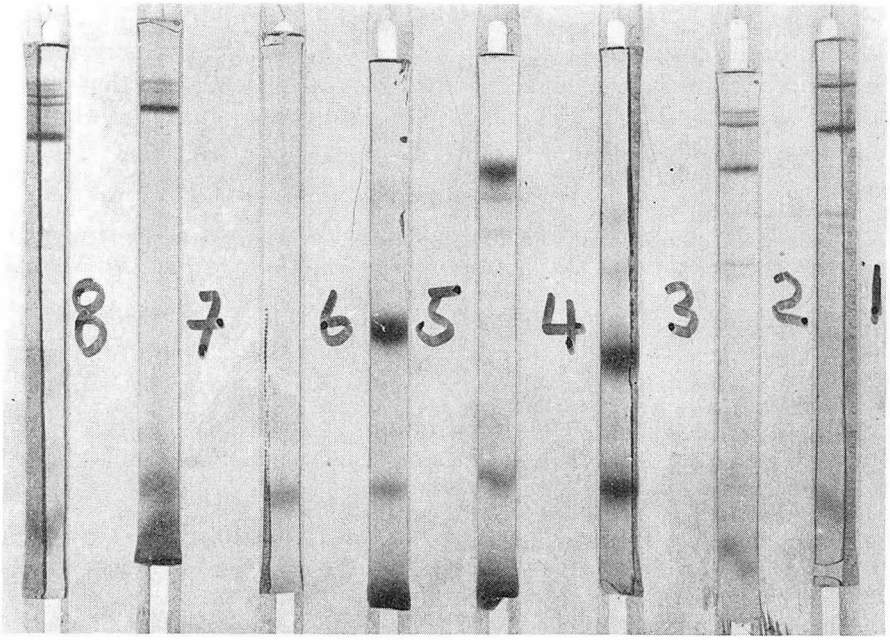

FIG. 3. Destained gels in rack. The samples are partially purified T4 proteins treated with SDS and separated on 5% acrylamide gels with 0.1% SDS (6). They were photographed with fluorescent back lighting using a Wratten 25 filter and Polaroid type 55 P/N film.

sisting of a bridge rectifier (Motorola MDA 962-5) connected to the AC line voltage. This 110 V source gives 0.25 A in the destaining device described. The current can be adjusted by varying the concentration of acetic acid in the destaining solution. Gels stained with CBB are destained in about 15 minutes; gels stained with amido black are destained slightly faster.

In contrast to Schwabe (2) and in agreement with Peterson (4), I have found that it is not necessary for the gels and the rack to make watertight seals between cathode and anode. This is because the resistivity of the gels is only slightly greater than that of the destaining solution. Thus the bulk of the current will pass through the gels and carry out the stain even though there are leaks around the sides of the gels and the rack.

After destaining, gels can be photographed directly in the rack. Figure 3 shows a photograph of bacteriophage T4 proteins which have been run on 0.1% SDS gels, stained, and destained as above.

SUMMARY

This paper describes an apparatus for rapid destaining of acrylamide gels by transverse electrophoresis. A removable gel supporting rack allows staining, rapid destaining, and photography of gels without handling the individual gels.

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Methods for Fractionation and Scintillation Counting of Radioisotope-Labeled Polyacrylamide Gels

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It is often convenient to be able to fractionate analytical polyacrylamide gels for radioisotope or enzymic determinations. This paper describes a gel fractionator patterned after the extrusion fractionator of Maizel (1), which is convenient for standard, 0.5 cm diameter, gels. The fractionator is inexpensive and easy to construct and to use. For miniature gels, which are useful when the amount of available labeled material is small, an alternative apparatus for extruding and slicing gels with a minimum of handling is described.

A simplified procedure for liquid scintillation counting of gel fractions is also presented. This counting procedure allows high efficiency counting of both ^{3}H and ^{14}C, but does not require degradation of the gel prior to counting.

Material and Methods

Preparation of Acrylamide Gels

Gels 6.5 by 0.5 cm were prepared by the methods of Ornstein (2) and Davis (3) with minor modifications. Acrylamide concentrations of 4, 5, and 10% were used with a 1:38 ratio of N, N'-methylenebisacrylamide (Eastman) as the cross-linking agent. Uniformly labeled gels were prepared by mixing either a ^{3}H- or ^{14}C-labeled acid-insoluble Escherichia coli protein extract with the gels before

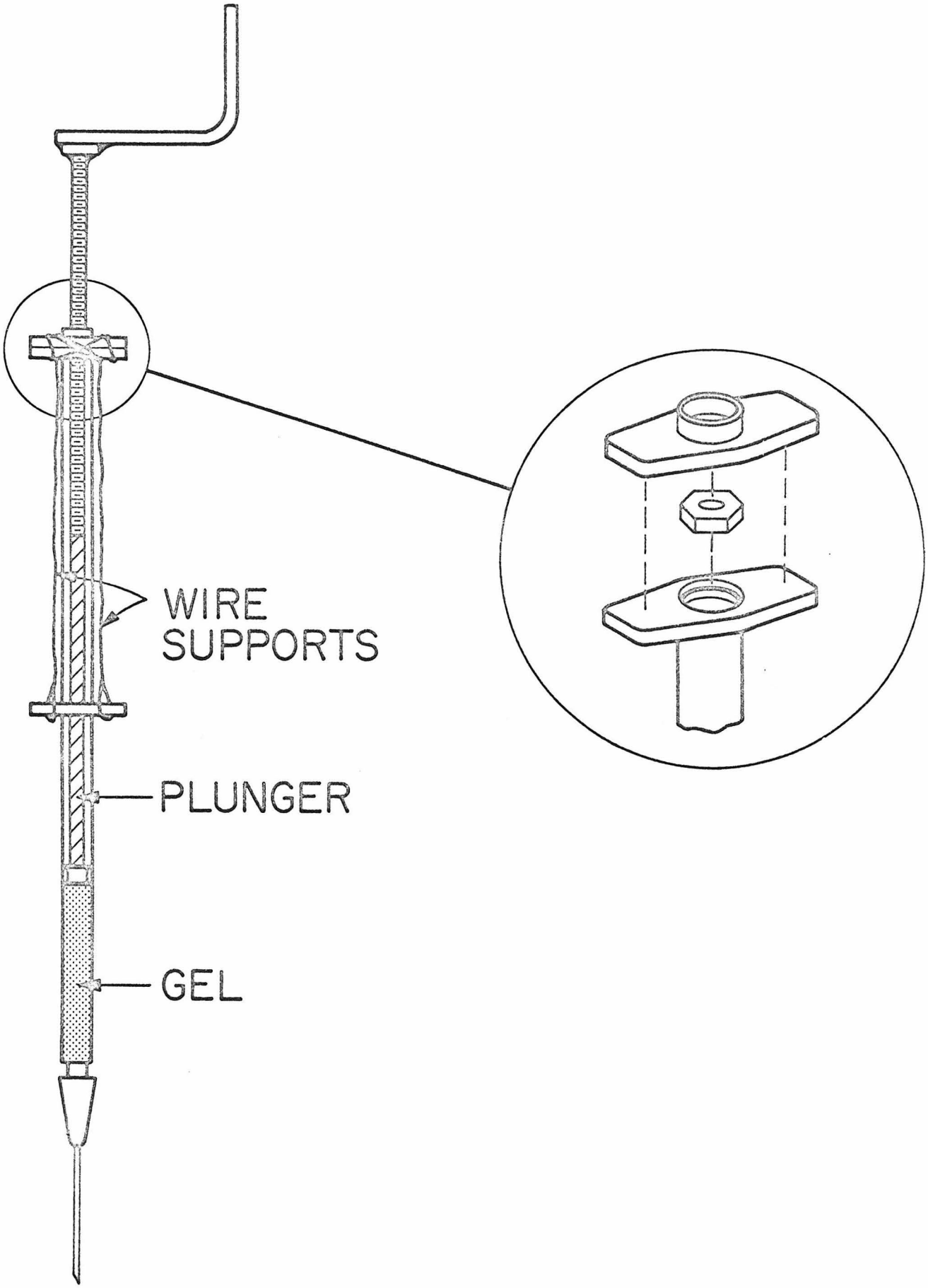
polymerization. Miniature polyacrylamide gel preparation is described elsewhere (4).

Standard-Size Gel Fractionation

The fractionator is described in Figure 1. It is designed for gels 6.5 cm by 0.5 cm, but longer gels can be accommodated by cutting them in half and fractionating the halves separately. The gel is inserted into the syringe barrel by sucking on the tip with a rubber tube fitted over the needle. When the gel is fully inserted a few drops of water are layered on top of the gel and the plunger is inserted. The loaded syringe is then fitted to the screw drive by the wire support loops.

Fractions are taken by turning the crank slowly and evenly by hand. As the gel is extruded through the needle it is broken into small beads. Every two turns of the crank the extruded gel is collected by hand in separate fractions. For radioactive counting, fractions are taken directly into scintillation vials as described in Radioisotope Determination below. Care must be taken to collect the extruding gel in the bottoms of the vials because it sticks tenaciously to surfaces. Collecting fractions every two turns of the crank gives 41-43 fractions from a 6.5 cm gel. Smaller fractions can be taken, but there will be larger variation in fraction size. It requires about 15 min to fractionate a gel.

FIG. 1. Gel fractionator. The gel holder is a 1 ml syringe (Plastipak, from Becton, Dickinson) with a 1 in. 20 gage needle secured with a drop of acetone. The screw drive is made by cutting the tip off a second syringe and then fastening an 8/32 hex nut in the indentation at the top of the syring as shown in the inset. The cut-off top of a third syringe is inverted over the nut and wired tightly to wedge the nut in place. The screw is 8/32 threaded brass with a crank soldered to the top. After the gel holder is loaded with a gel it is fastened to the screw drive by the wire support loops.


WIRE
SUPPORTS
PLUNGER
GEL

Miniature Gel Fractionation

The extruder and slicer for miniature gels is shown in Figure 2. It is designed for 0.14 cm diameter gels, but could be adapted for gel tubes of different diameter by use of interchangeable plungers. After electrophoresis, the gel, in its tube, is positioned in a V-shaped trough, and a tight-fitting plunger is run into the top of the gel tube. Fractions are then taken by turning the crank, which is connected to the plunger by a screw drive (1 revolution = 1.4 mm). After each revolution, a V-shaped knife blade is used to slice the extruded gel piece. The slice sticks to the knife edge, which is plunged into a scintillation vial containing counting fluid. The slice tends to remain in the fluid when the blade is removed. Less than 10 min is required to extrude and slice a 6 c mgel.

Radioisotope Determination

Radioactively labeled samples are counted by taking fractions directly into the following counting cocktail:

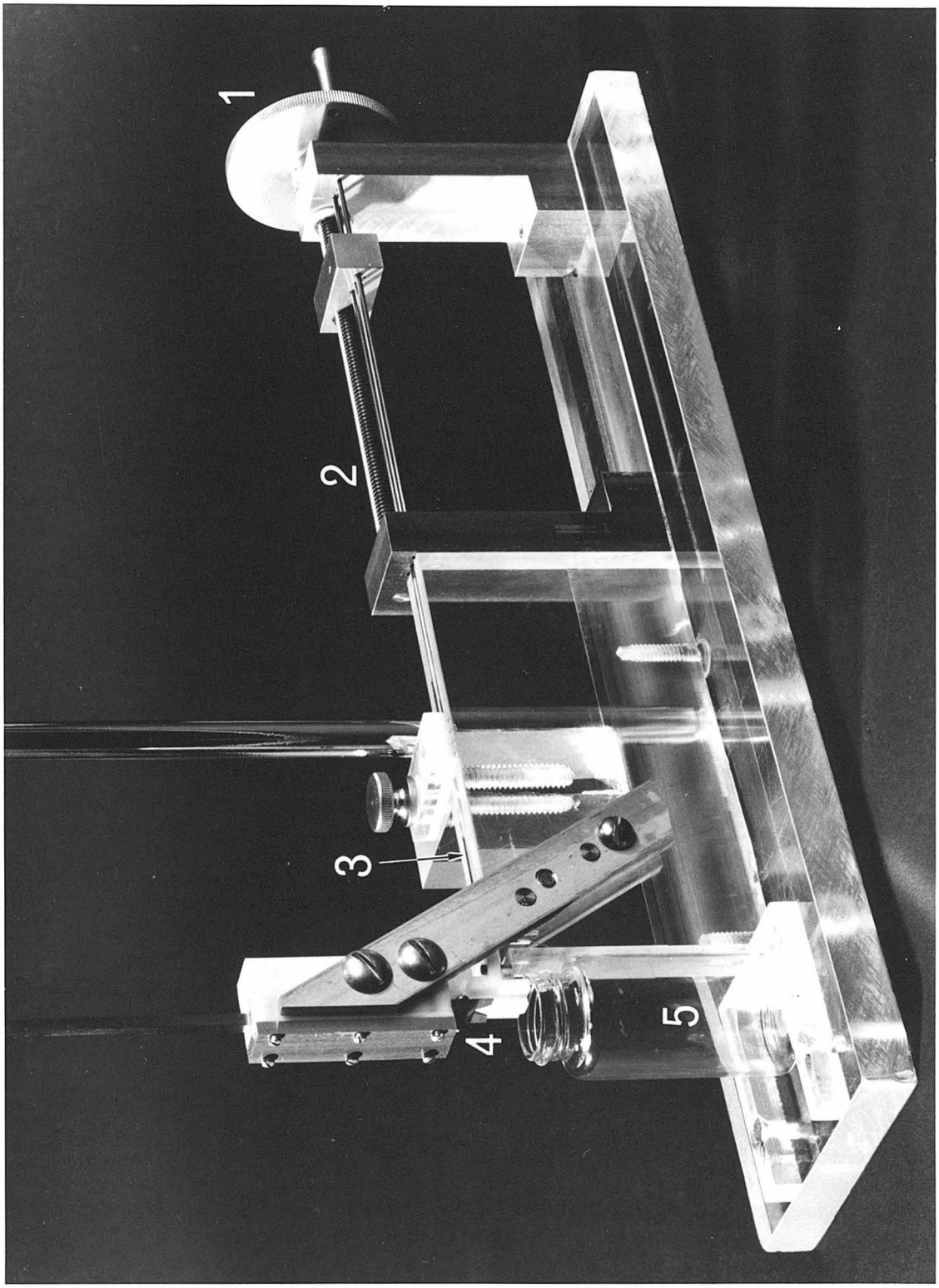
5 ml of toluene scintillation fluid (160 ml Liquifluor (NEN) per gal toluene)

0.5 ml of NCS solubilizer (Amersham/Searle)

0.1 ml of 4 M ammonium hydroxide

Large volumes of counting cocktail can be prepared by mixing the ammonium hydroxide with the NCS until the solution is clear, and then adding the mixture to the toluene scintillation

FIG. 2. Extruder and slicer apparatus for miniature polyacrylamide gel. (1) Crank. (2) Plunger attached to crank via a screw drive. (3) Miniature gel tube. (4) V-shaped knife edge attached to rod. (5) Scintillation vial.


1
2
3
4
5

fluid. The complete cocktail can be used for at least a week stored at room temperature, or preferably 4-6°, in the dark. The cocktail can be dispensed with a Repipet (L/I LabIndustries, Berkeley, Calif.).

After fractions are collected the vials are capped and allowed to stand at room temperature for 2 hr or overnight (see below) with occasional shaking. They are then counted in a Beckman LS 200 B liquid scintillation spectraphotometer. The efficiency of counting of tritium as determined by addition of a known number of ^{3}H-toluene counts (NEN) to the counting cocktail,was 38%.

Results

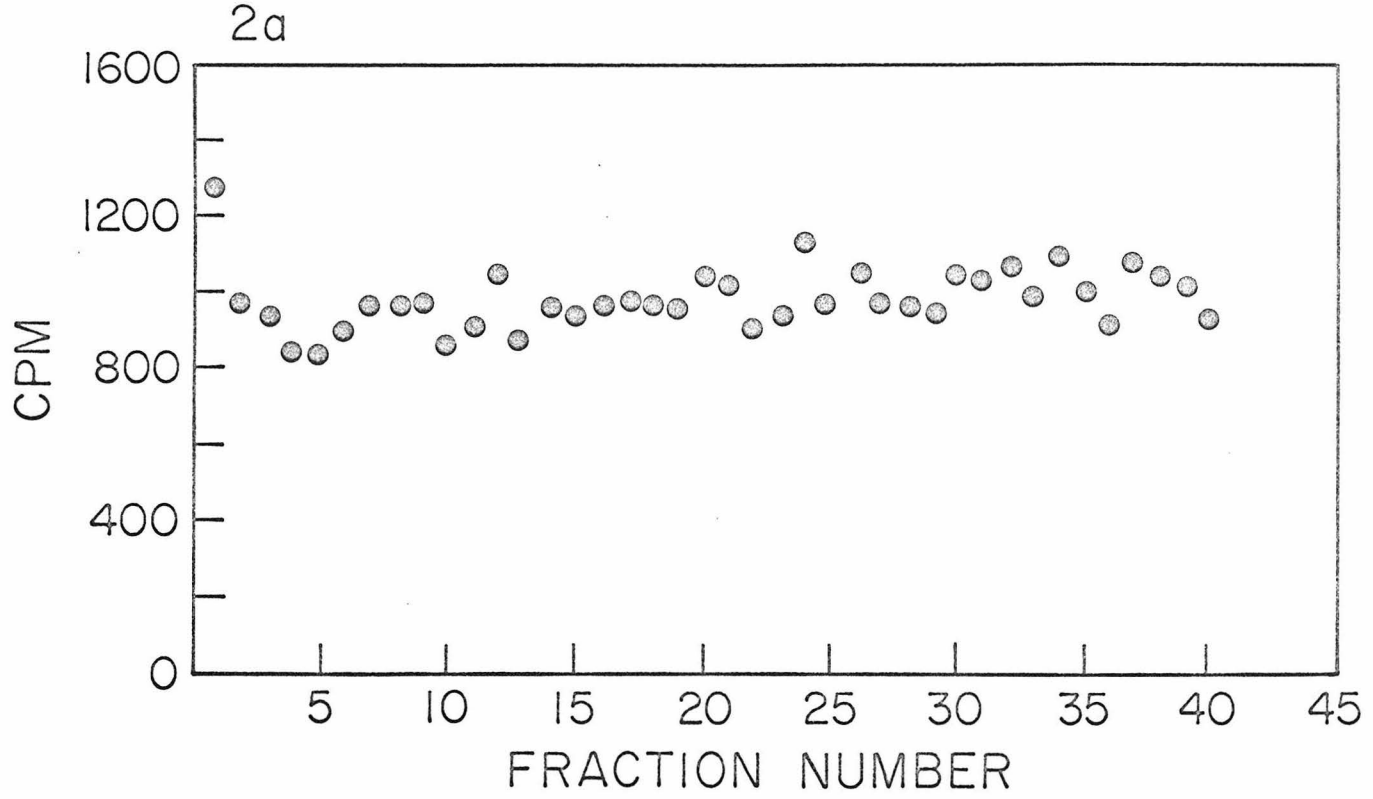
I. Precision of Gel Fractionation

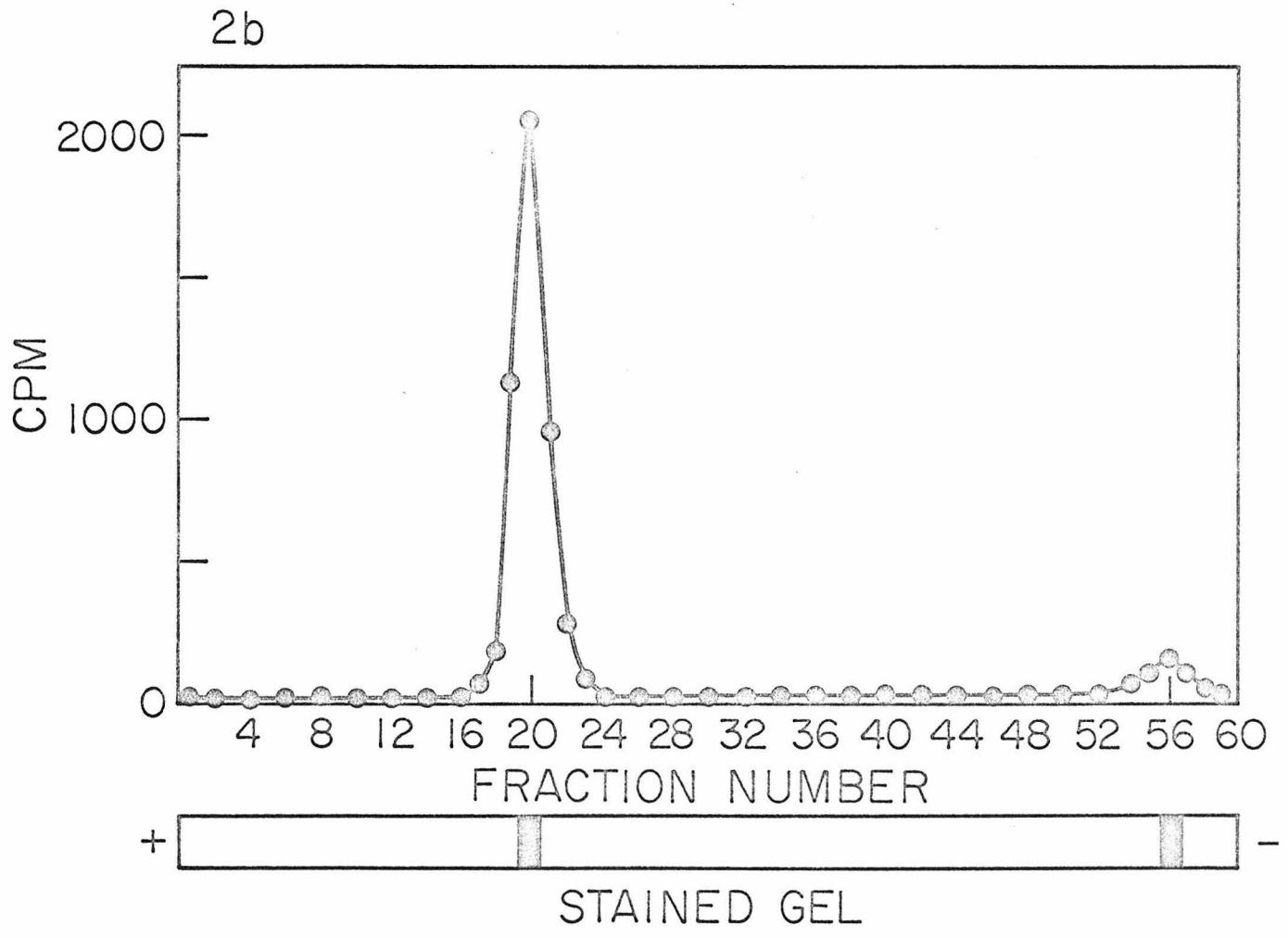
Figure 3a shows a uniformly labeled gel fractionated and counted as described above for standard-size gels. The standard deviations of the counts for eight ^{14}C- and ^{3}H-labeled gels ranged from 7 to 16%. Occassionally a deviation as large as 30% was observed.

The gel slices obtained with the miniature gel extruder and slicer showed little variation: slices from a uniformly ^{3}H-labeled gel gave 2% standard deviation.

Because the extrusion of standard-size gels through a needle could produce distortion of labeling patterns, the resolution of separate protein bands after fractionation was measured. A ^{14}C-labeled T4-tail-fiber-protein preparation was electrophoresed on a standard-size 4% polyacrylamide

FIG. 3. Fractionation of labeled gels. (a) Fractionation of a 5% acrylamide gel uniformly labeled with ^{14}C *E. coli* protein. The standard deviation about the mean of the counts per fraction is 8%. (b) Results of fractionating a 4% acrylamide gel, pH 4.3, after electrophoresis of a mixture of two ^{14}C-labeled T4 proteins. The gel was fixed and stained before fractionation, and a tracing of the stained gel is shown below the fractionation.


2a
1600
1200
800
400
0
CPM
5
10
15
20
25
30
35
40
45
FRACTION NUMBER


2b
2000
1000
0
CPM
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
FRACTION NUMBER
+
-
STAINED GEL

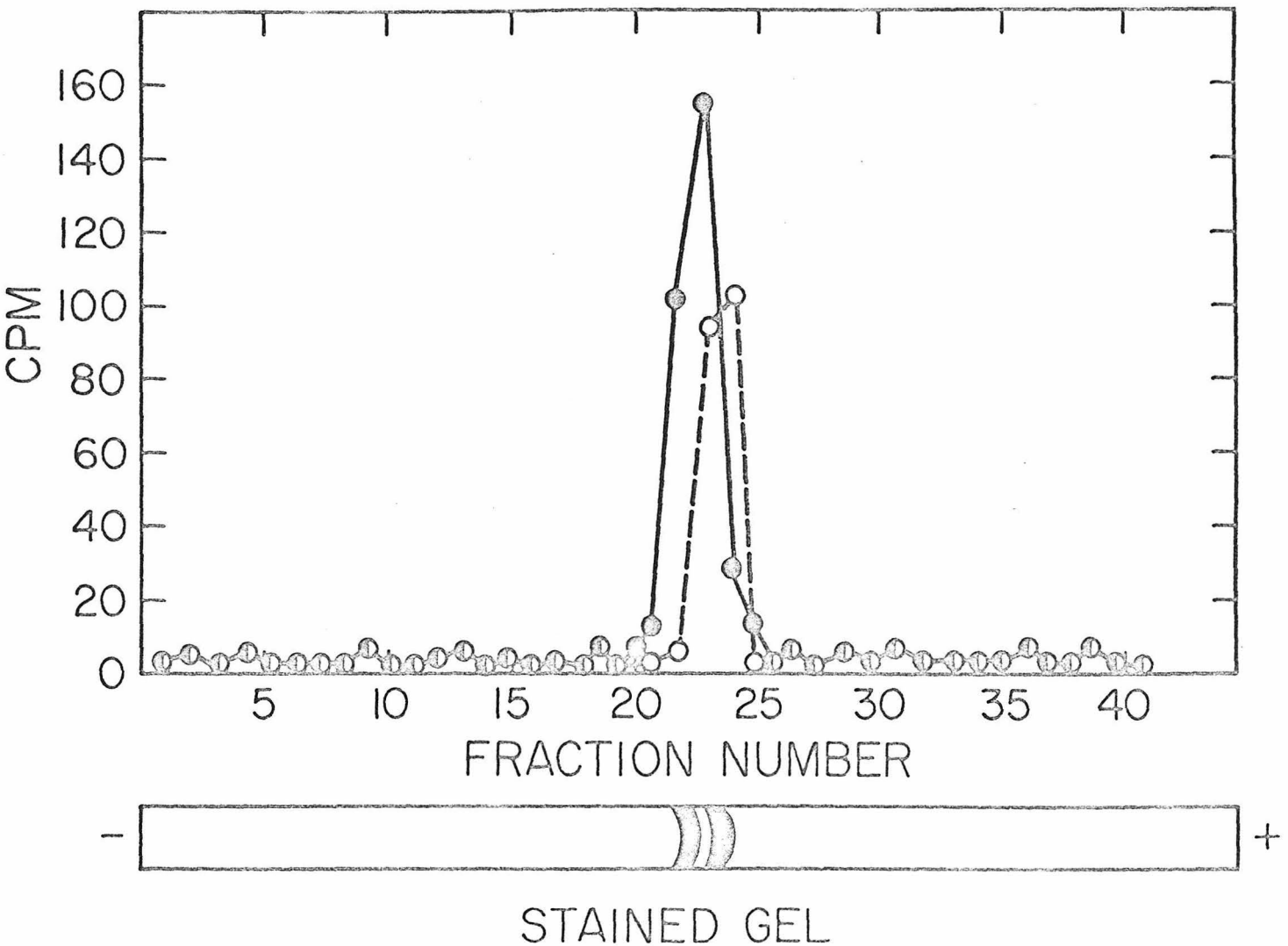
gel at pH 4.3 with no SDS. The gel was fixed, stained with Coomassic Brilliant Blue, destained by the method of Ward (5), fractionated, and counted. The results are shown by the graph in Figure 3b, with a tracing of the stained gel shown below. The protein peaks are not distorted by the fractionator, and there is little tailing of the counts. The fractionated peaks are slightly broader than the stained bands, however.

Figure 4 shows the fractionation of a standard-size 1% SDS gel after electrophoresis of a mixture of two similar viral proteins. One of the proteins was labeled with ^{3}H and one with ^{14}C. A tracing of an identical stained gel is shown below. The fractionation shows that the peak of ^{3}H counts migrates one fraction ahead of the peak of ^{14}C counts. This indicates that the ^{3}H-labeled protein has the smaller molecular weight (6). Molecular weight standards added to a similar gel indicated molecular weights of 114,000 and 123,000 for the two proteins. As in Figure 3, the peaks from the fractionator are slightly broader than the stained bands, and thus some resolution is lost by fractionation.

II. The Counting Cocktail

After a uniformly ^{14}C-labeled standard-size gel was fractionated as described above, the samples were immediately placed in a scintillation counter and counted repeatedly

FIG. 4. Fractionation of a doubly labeled gel. Two bacteriophage T4 proteins were mixed, boiled 2 min in 1% SDS, 0.01 M phosphate, pH 7.1, and 1% mercaptoethanol, then electrophoresed 4.5 hr at 40 V on 5% acrylamide gels containing 1% SDS. One of the proteins was labeled with ^{3}H (○) and the other with ^{14}C (●). The graph shows the results of fractionating and counting one such gel, the tracing below shows the results of staining a parallel gel.


160
140
120
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80
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CPM
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25
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35
40
FRACTION NUMBER
−
+
STAINED GEL

every 30 min. It required 2 hr for the counts to reach 99% of their maximum, and the maximum counts remained stable for several days. Slices of minature gels reach maximum counts more slowly and are routinely stored overnight at room temperature in a cocktail before counting. In both cases, decanting the scintillation cocktail from the gel and recounting revealed that the labeled protein had diffused out of the gel into the scintillation cocktail.

Recovery of counts from labeled gels was high. For the fractionation of standard-size uniformly labeled gels, the sum of the counts from all the fractions is 96 $\pm$ 3% of the counts in the gel before polymerization for both ^{14}C and ^{3}H. Approximately 0.5% of the counts remains unpolymerized under the water layer at the top of the gel, and 0.7% of the counts is washed out of the gel in the process of rimming it with a syringe needle to remove the gel from the glass tube. Thus the recovery of counts from these gels is quantitative within the counting error.

When labeled T4-phage proteins are treated with 1% SDS and applied to standard-size gels and electrophoresed, the recovery of either ^{3}H or ^{14}C counts is about 90%. The slight loss of counts probably represents material loss during sample application. The recovery of counts varies betwen 90 and 70% for gels which have stained before counting. This may be due to quenching of counts by the dye or to loss of protein during staining and destaining.

The use of NH_4OH in the counting cocktail reduces light-induced phosphorescence of the NCS-toluene scintillation mixture. The phosphorescence overlaps the scintillation spectrum of ^3H. Without NH_4OH, samples with less than a few hundred counts of ^3H must be stored overnight in the dark to allow the phosphorescence to decay. With NH_4OH in the cocktail, a small amount of phosphorescence decays to background in a few minutes.

Discussion

The results described above indicate that the extrusion gel fractionator for 0.5 cm diameter gels gives reasonably uniform fractions and does not distort protein bands. Resolution of two closely spaced bands is not as high after fractionation as it is if the protein bands are stained in the unfractionated gel. This loss of resolution is offset by the advantage of being able to identify bands with different isotope labels. The advantage of using double labels for SDS-gel determinations of molecular weights are discussed in Shapiro and Maizel (7). The results obtained with the sample hand-operated fractionator described here seem to be comparable to those obtained with Maizel's more complicated and expensive apparatus (1,7), although the resolution may be less. The miniature gel extruder and slicer is a more convenient apparatus for smaller diameter

gels. It allows rapid, uniform fractionation of miniature gels directly from gel tubes to counting vials. Resolution of bands should not be affected by the slicing procedure.

The many procedures for counting radioactive gel fractions have been reviewed by Tishler and Epstein (8). Nearly all the procedures require degradation of the gel in order to obtain good recoveries of counts. The results described in this paper show that the degradation step is not necessary to obtain quantitative liquid-scintillation counting of labeled gel proteins. We believe that this is due to the efficient diffusion of proteins and water out of the gels, perhaps aided by partial hydrolysis of peptide bonds in the strongly basic scintillation fluid.

The diffusion of proteins out of the gel is certainly speeded by having the gel macerated by extrusion fractionator, but the recovery of counts is also quantitative with standard-size or miniature gels fractionated by slicing, if the fractions are allowed to stand overnight before counting.

Summary

This paper describes two polyacrylamide gel fractionators. One can be easily constructed from disposable syringes and used for fractionating standard-size analytical gels. The second is more complex but can be used for fractionating miniature gels. A rapid method of preparing gel fractions

for liquid-scintillation counting is also presented. The method gives high counting efficiency, does not require hydrolysis of the gel prior to counting, and allows gel fractions to be collected directly in scintillation vials.

Acknowledgments

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