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P22 MORPHOGENESIS I: CATALYTIC SCAFFOLDING PROTEIN IN CAPSID ASSEMBLY

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About 250 molecules of the 42,000 molecular weight gene 8 product catalyze the polymerization of the major phage coat protein into a precursor shell temporarily containing both proteins. The resulting prohead appears to be a shell structure with the P8, or scaffolding protein, on the inside, and the coat protein on the outside. In concert with DNA condensation inside the shell, all 250 scaffolding molecules exit from the prohead, without proteolytic cleavage. These molecules then recycle and catalyze the formation of more proheads from newly synthesized coat protein. Such proteins, which catalyze assembly by temporarily associating with an intermediate stage, may represent a general mechanism of macromolecular assembly.

INTRODUCTION

Though mature virus particles often consist of a relatively simple icosahedral shell packed with condensed nucleic acid, it is becoming clear that the *formation* of such structures is not a simple process, particularly in viruses containing double stranded DNA. This was initially suggested by the discovery of Epstein et al. (1) that the formation of the head of phage T4 required the products of more than ten phage genes, and by the complex nature of the defective structures that accumulated in cells infected with mutants in these genes as shown by Kellenberger and Laemmli (2, 3). In addition we now know that the formation of bacteriophage heads is coupled with the maturation of the virus chromosome; cutting of a mature length chromosome from the overlength replicating DNA does not happen in solution but only in the presence of a head precursor shell (4–8). Furthermore, in many viruses the proteins forming the head are proteolytically cleaved during the shell formation and DNA packaging steps (9–17).

We have been concerned with trying to understand the exact mechanism of the assembly of subunits into shell structures. Here we show that in fact the polymerization of the major coat protein into a shell requires another protein which aids the major coat protein in the formation of a correctly dimensioned isometric shell and is then removed from this precursor shell in concert with DNA condensation. The protein is not degraded but instead takes part in further rounds of prohead assembly. Some of these experiments have been previously described by King and Casjens (52). The function of scaffolding protein may be analogous to that of the T4 assembly core described by Showe and Black (44).

P22 is a temperate phage which infects Salmonella typhimurium. It was discovered by Zinder and Lederberg (18), who found that it could transfer bacterial genes from one host to another. The molecular basis of this generalized transducing capacity is intimately involved with the mechanisms of DNA packaging and is described in the accompanying paper by Tye and Botstein (19). The P22 virus particle is an isometric hexagon in outline, about 550 Å in diameter with a small spiked tail plate (20) (Fig. 1b), and contains a double stranded DNA molecule of molecular weight 27 million. The growth and DNA metabolism of phage infected cells has been studied in detail by Botstein and Levine (21). Genetic analysis of amber and temperature sensitive mutants by Botstein and coworkers (22) has revealed ten viral genes whose products are necessary for the formation of a complete virus particle (23). The pathway for the assembly of these proteins into a phage particle is reasonably well understood (24); however, before reviewing the assembly pathway we will briefly describe the methods employed for those who are not familiar with bacteriophage experiments.

By growing infected cells in the presence of radioactive amino acids, virus proteins can be efficiently labeled. P22 infection terminates the replication of the host cell DNA, and host specific protein synthesis is substantially decreased. The entire culture can be heated in the detergent sodium dodecyl sulfate (SDS) in the presence of disulfide reducing agent to dissociate all the proteins into their constituent polypeptide chains. These then are separated according to molecular weight by electrophoresis through an acrylamide gel, in the presence of SDS (10). After electrophoresis the gel is dried and applied to an x-ray film to detect the radioactive protein bands (30). Comparison of the radioactive proteins of infected and uninfected cells identifies those proteins which are virus specific. Electrophoresis of purified virus identifies those proteins of infected cells which are incorporated into mature virus particles.

To match viral proteins with the genes that specify them, cells are infected with phage carrying amber mutations. In the restrictive host, this results in the synthesis of only a fragment of the mutant protein; thus, the absence of a protein band identifies it as the product of the mutant gene. Conversely a new smaller molecular weight band, the amber fragment, should appear, unless it is degraded in the cell.

Cells infected with a phage carrying a mutation in a particular gene affecting morphogenesis generally lack only the product of that gene. A general experimental question we can ask is what is the state of aggregation of those proteins which are synthesized in the absence of the gene product in question. Our analysis has been initially by electron microscopy to detect large phage specific structures, then sucrose gradient centrifugation to better characterize phage related structures, followed by gel analysis of the protein composition of the structures and EM studies on the isolated structures.

Structures that accumulate in mutant infected cells are in many cases likely candidates for intermediates in the normal assembly process. With an idea of what to look for, we can then proceed to study the assembly process in wild-type infected cells. It is often difficult to begin the study with the normal assembly process, since 1) assembly is rapid and intermediate states are present only transiently, and 2) assembly is not synchronous and all intermediates in assembly are present simultaneously.

Figure 2 summarizes the pathway for the assembly of phage P22. Three kinds of structures can be isolated from wild-type cells: proheads, empty heads, and phage (23, 24) (see also Fig. 1). Proheads lack DNA, sediment at about 240S, and appear as round structures with some internal organization. These structures accumulate in cells infected with mutants in genes 1, 2, or 3. Pulse chase experiments indicate that they are the first

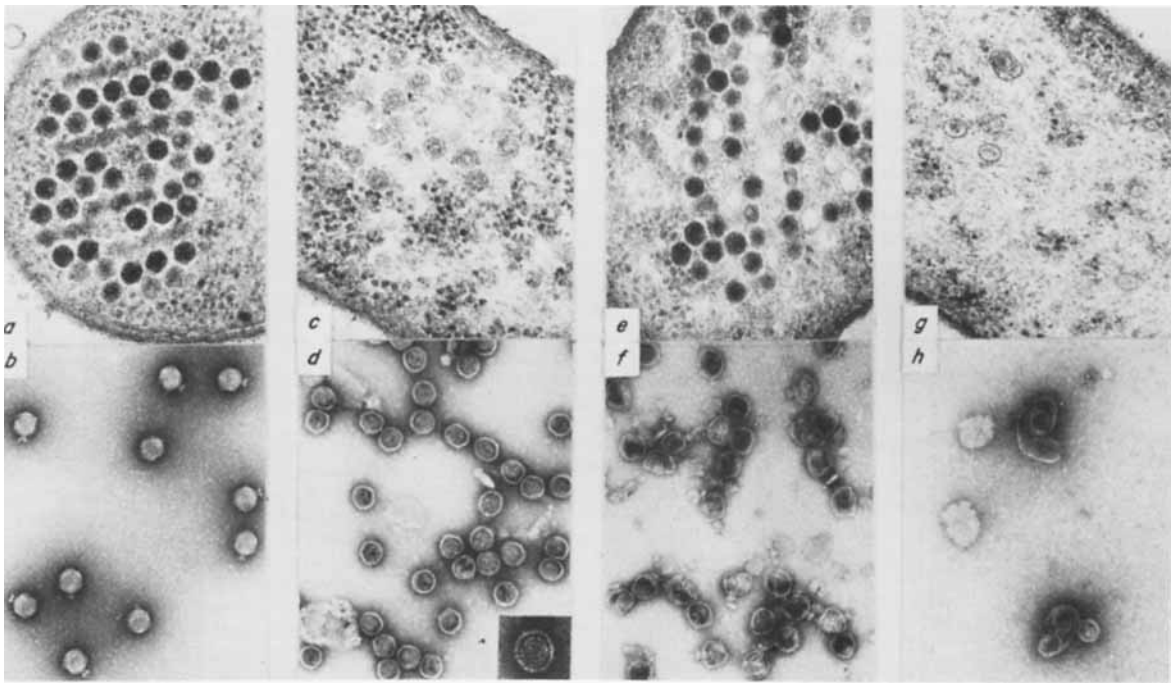

Fig. 1. Morphology of phage head structures. The upper panels are electron micrographs of ultrathin sections of P22 infected cells. Each lower panel is an electron micrograph of particles (negatively stained) isolated from cells infected with the same phage as the panel above it. (a, b) Normal phage particles; (c, d) proheads from cells infected with phage carrying an amber mutation in gene 2. These structures contain both the coat protein and the scaffolding protein; (e, f) empty heads from cells infected with phage carrying an amber mutation in gene 26. Both full heads and heads which have lost DNA (23, 24) are seen within the cell; after cell lysis loss of DNA is nearly complete and all particles appear empty. These particles lack the scaffolding protein (24); (g, h) aberrant structures from cells infected with a phage carrying an amber mutation in gene 8. A more detailed account of the in vivo morphology of the various head-related structures formed during P22 infection will be presented later (36).

The magnification of all panels is 48,000 ×, and the magnification of the insert in panel (d) is 110,000 ×. [Reproduced from King and Casjens (52) with permission of Nature.]

intermediate in head assembly and are the true precursors to phage particles. They are composed of two major proteins, the products of genes 5 and 8. The gene 5 product is the major coat protein of P22. The gene 8 product is not in mature virus. Since this protein which is required for virus assembly is found in a precursor of the virus, but is not in the mature particle, we termed it a scaffolding protein.

Empty phage heads can be isolated from wild-type infected cells and accumulate in cells infected with mutants defective in genes 10 and 26. A variety of experiments has shown that the empty heads from the 10^- and 26^- infected cells were derived from particles that were initially full of DNA, but subsequently lost it after cell lysis. The gene 10 and 26 products appear, therefore, to somehow stabilize heads which have just packaged DNA. These empty heads lack scaffolding protein (24).

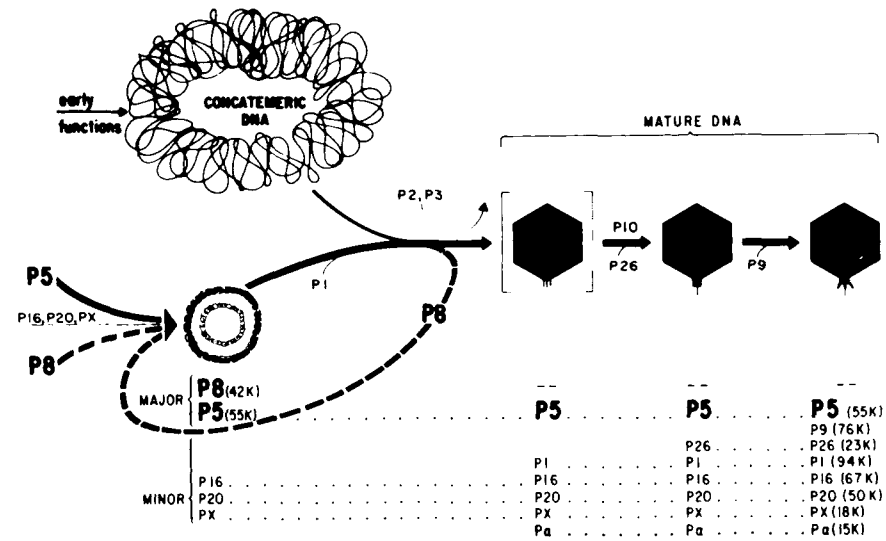

Fig. 2. Pathway of phage P22 head assembly and DNA encapsulation. This diagram summarizes results from Botstein et al. (23), King et al. (24), and this article. A prohead shell lacking DNA is the first identifiable structure in the pathway. It contains two major protein species, the products of gene 5 and gene 8. With the incorporation of the product of gene 1, and the function of the gene 2 and 3 products, the prohead encapsulates and cuts a headful of DNA from the concatemer DNA molecule, and concurrently p8 exits from the particle without proteolytic cleavage. The newly filled head is unstable (it loses the DNA) until acted upon by the gene 10 and 26 products, after which the complete head is converted to a phage particle by addition of the tail parts (gene 9 protein), as shown by Israel et al. (25).

In the figure "p" before a gene number refers to the polypeptide of that gene. The polypeptide chains found in each structure are listed below it, with their molecular weights shown in the last column. Brackets around the first filled head structure designate that it is unstable and loses DNA upon isolation.

The nature of the replicating DNA in P22 infected cells has been described by Botstein and Levine (21); Tye et al. (37) have shown conclusively that P22 chromosome maturation proceeds by a "sequential headful cutting" mechanism (38).

The last structures are stable full heads and phage. Full heads accumulate in cells infected with mutants in gene 9, the tail protein cistron (25). They are stable and very similar to complete phage particles, which differ from full heads only by the presence of the gene 9 protein.

Botstein et al. (23) showed that overlength replicating DNA accumulated in cells infected with mutants blocked in the formation of proheads (5^- and 8^- lysates) and in cells infected with mutants of genes 1, 2, and 3, in which proheads accumulate. These results indicate that the prohead is the shell precursor in the cutting and packaging of a mature length DNA molecule from the overlength concatemer. Since all phage structures containing DNA, or which have contained DNA at some time in their history, lack scaffolding protein, we presume that the exit of the scaffolding protein is coupled with the entry of the DNA into precursor shells.

METHODS

Bacterial Strains

The bacterial strains used are derivatives of Salmonella typhimurium LT2. Strain DB21 (prototroph, su^-) was used in all experiments, except where host protein synthesis was inhibited with ultraviolet irradiation, where strain TA1530 (deleted for the uvrB gene) (23) was used. Phage stocks were grown on the permissive (su^+) strain DB74 (23).

Phage Strains

The isolation and characterization of the phage P22 mutants used have been previously described in Botstein et al. (22, 23). A representative amber allele was chosen for each of the phage genes. These are described in detail by Botstein et al. (23). In all cases the amber allele was combined with a clear-plaque mutation (cl^7) to block lysogeny and insure a lytic response upon infection. In addition, most strains carried a mutation in gene 13 (H101) to block cell lysis. During infection 13^- phage continue to multiply within the cell for several hours beyond the normal lysis time.

Radioactive Labeling of Proteins During P22 Infection

Preparation of radioactive phage and related particles. Strain DB21 was grown in M-9 minimal medium (23) at 37°C to 2×10^8 cells per ml and infected with the desired phage strain at 5–10 phage/cell. ^{14}C-mixed amino acids were added at the desired time after infection. Cells were lysed with chloroform and the cell debris removed by a low speed centrifugation. The particulate structures were isolated by sedimentation through 5–20% sucrose gradients as follows: phage were separated from other particles by centrifuging for 20 min at 20,000 rpm in a Beckman SW 50.1 at 20°C. Proheads and empty heads could be separated by a similar centrifugation for 30 min at 30,000 rpm; all three types of particles could be displayed in one gradient if a high density (1.6 gm/cc) solution of CsCl is layered beneath the sucrose gradient and the tube centrifuged as above for proheads and empty heads.

Suppression of host protein synthesis with ultraviolet light irradiation. Although synthesis of the major phage proteins can be visualized against the background of host proteins in SDS electrophoresis gels (see Fig. 9), in order to see the phage proteins made in small amounts it is necessary to further suppress host protein synthesis. This can be accomplished by using a method analogous to that developed by Ptashne (26) and Hendrix (27) for E. coli. They found that if bacteria carrying a UV sensitive mutation were irradiated with UV light, protein synthesis was almost totally abolished. Furthermore, if unirradiated phage were allowed to infect these irradiated bacteria only phage coded proteins were synthesized. Salmonella mutants in the urvB gene have been used for the host in our infections where host protein synthesis had to be suppressed. The details of the method have been described by Botstein et al. (23).

Purification of Large Amounts of Unlabeled Particles

Proheads and empty heads were purified in large amounts after concentrating DB21

cells (infected with P22 13^-, carrying any additional mutations desired and grown at 37°C for several hr after infection) 100-fold before lysing with chloroform, reducing the viscosity with DNAse, and removing the debris with a low speed centrifugation. The particles were further concentrated by centrifuging them onto a cushion of CsCl solution (1.6 gm/cc). After the removal of the CsCl by dialysis, the particles were purified by centrifuging through a 10–30% sucrose gradient at 20,000 rpm for 3 hr in a Beckman SW 27.1 rotor at 20°C. The opalescent bands were removed from the side of the tube with a syringe. Phage were purified in a similar manner except that the second centrifugation was into a preformed CsCl gradient (1.2 to 1.6 gm/cc) and centrifugation was carried out at 25,000 rpm for 5 hr.

Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate (SDS)

Discontinuous SDS gel electrophoresis was carried out in slabs of 10 or 12% acrylamide as described by O'Farrell et al. (28). The gels were dried and subjected to autoradiography according to the methods of Maizel (29) and Fairbanks et al. (30), respectively. Gels of unlabeled proteins were stained with Coomassie Brilliant Blue (31) or Fast Green (32). All samples were heated to 100°C for 1.5 min before electrophoresis.

Electron Microscopy

Negatively stained preparations were prepared by allowing a drop of solution (about 10^{11} particles/ml) to stand on a carbon coated grid for about 1 min, washing the grid several times with distilled water, and staining by allowing a drop of 2% uranyl acetate to stand on the grid for about 1 min. Ultrathin sections of infected cells were prepared according to the method of Simon (33) and of purified particles by the method of Lenk and Penman (34).

RESULTS

Structure of the Prohead

Negatively stained proheads appear as spherical shells with scalloped edges whose centers partially exclude the stain, making it appear as if there were material of some sort within (Fig. 1d). In contrast, empty heads do not exclude stain and phage particles exclude the stain more strongly than do proheads (Figs. 1b and f). Shadowing experiments show that the appearance of the proheads is not due to a pool of stain in a pucker on the prohead as it lies on the electron microscope grid (W. Earnshaw, unpublished results) and so could be due either to internal material or to impermeability of the shell to stain. In order to separate these possibilities, ultrathin sections of pellets of purified phage, empty heads, and proheads were made and observed in the electron microscope (Fig. 3). The phage appear as uniform dark staining bodies and the empty heads as thin, empty shells. The proheads appear in the sections as round shells with some internal material which is less dense than that of the phage particles.

What is this internal material? Several additional experiments have substantially answered this question. The protein composition of the particles (see below) suggests that, since there are only two major protein components in proheads (p5 and p8), one of them

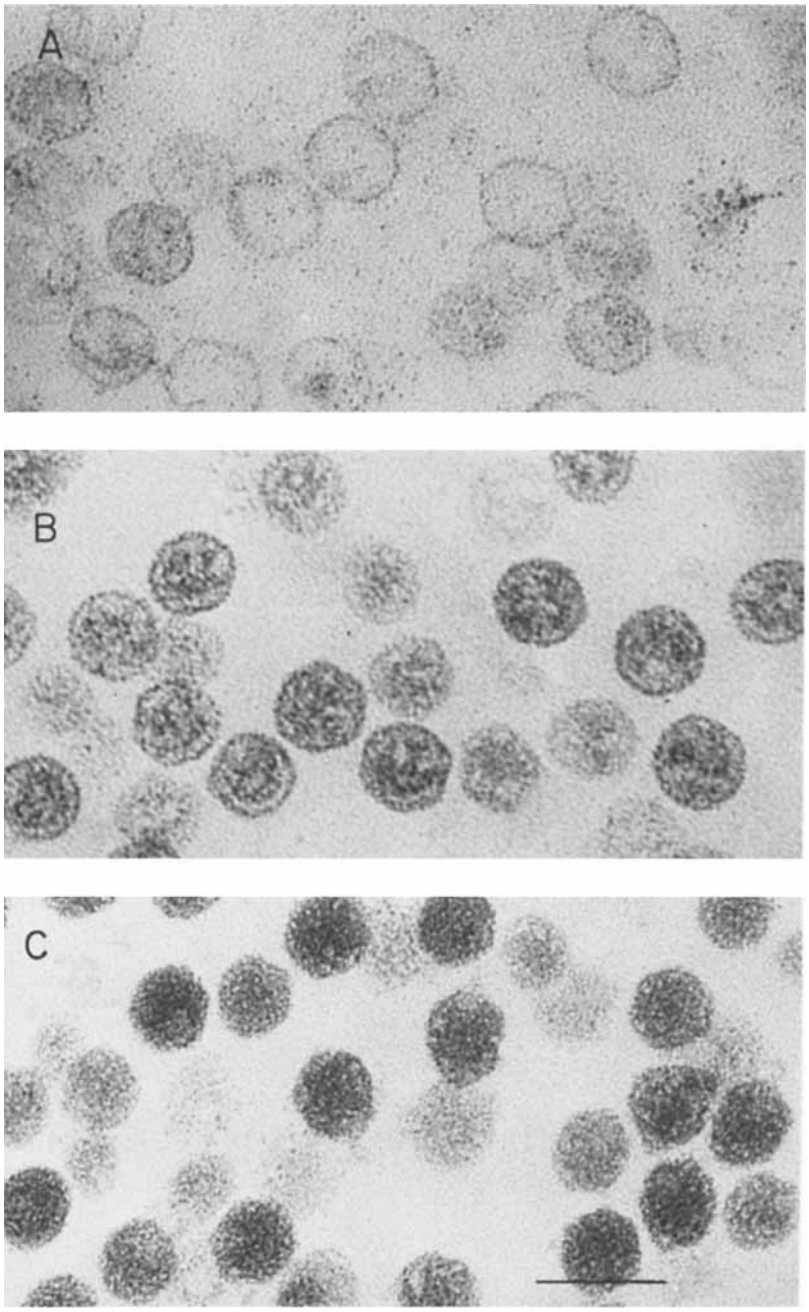

Fig. 3. Electron micrographs of ultrathin sections of pellets of purified a) empty heads, b) proheads, and c) phage. The particles were purified as described in the Methods section from 10^-, 3^-, and wild-type P22 infected cell extracts, respectively. The purified particles were pelleted by centrifugation at 17,000 rpm for 4 hr at 10° C in a Beckman SW 50.1 rotor, and the resulting pellets were fixed, embedded, and sectioned according to the method of Lenk and Penman (34). The magnification bar represents 1000 Å.

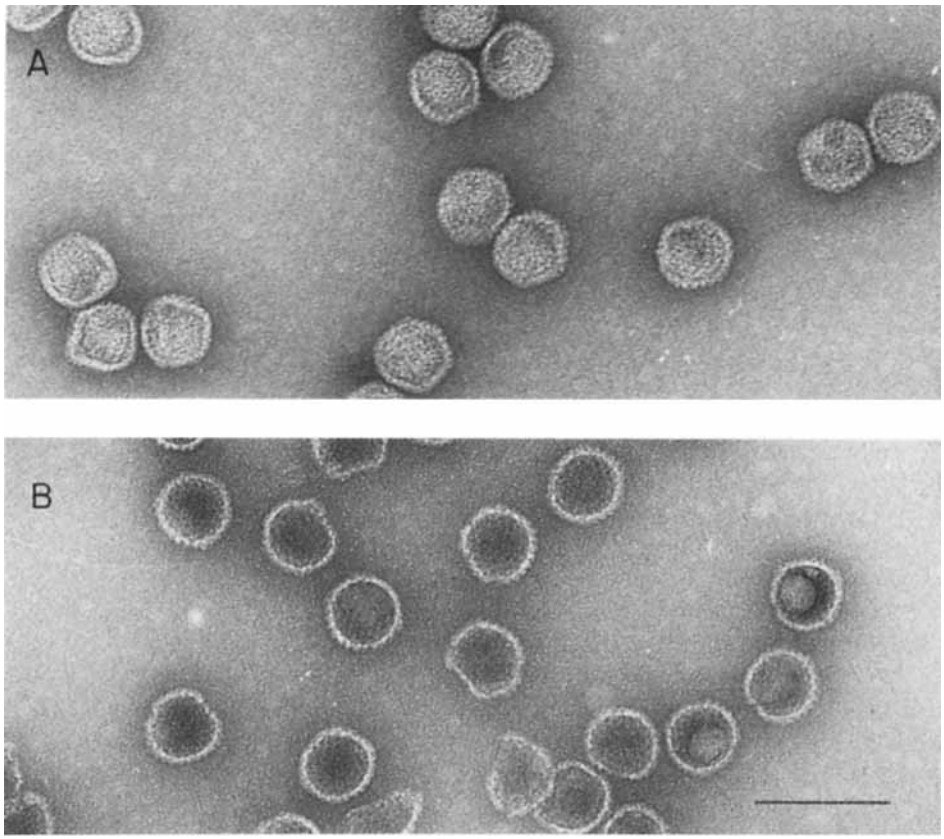

Fig. 4. Electron micrographs of negatively stained a) proheads and b) SDS treated proheads. Proheads were purified from 3^- infected cells as described in the Methods section. SDS treated proheads were prepared by making the solution of concentrated (10^{13}/ml) proheads 0.8% SDS (in 10^{-2} M Tris Cl, 10^{-3} M $MgCl_2$, pH 7.5) and allowing the mixture to stand at room temperature for 2 hr. The remaining empty proheads were separated from the solubilized proteins by sucrose gradient centrifugation, followed by extensive dialysis at room temperature to remove any residual SDS. The magnification bar represents 1000 A.

may be the internal material. Alternatively, it could be that some DNA is associated with proheads in subphage amounts and is seen in the micrographs as the internal substance. This last possibility is unlikely since analysis of proheads (purified from P22 3^- infected cells) for organic phosphate by the method of Chen et al. (35) gave only about 650 phosphorous atoms per prohead. If this represents phosphorous in DNA it is less than 1% of the DNA content of the phage. It is unlikely that such a small amount of DNA could be responsible for the appearance of the proheads. In an attempt to determine which protein is responsible for the internal material, conditions were searched for that would specifically release the internal material from the proheads. Such conditions were found when proheads were treated with SDS at room temperature. Figure 4 is an electron micrograph of 3^- proheads before and after the SDS treatment. It is clear that the SDS treated proheads do not exclude the stain and appear empty by this criterion. The outside shell retains its scalloped edges and does not change greatly in diameter. SDS gel analysis of the empty proheads revealed that p8 as well as the minor proteins pl, p16, and p20 were quantitatively removed by the SDS treatment leaving a shell of only the coat protein (p5) (gels not shown). We conclude that p8 is responsible for the "partially filled" "

appearance of proheads in the negatively stained preparations and it is therefore most likely the internal material seen in the thin sections. We do not know the exact positioning of the p8 molecules within the prohead; thin sections of infected cells (see Fig. 1c) suggest that the internal material may be arranged in an internal spherical shell. However, it remains for this to be shown more clearly.

Gene 8 Product Performs a Scaffolding Function for Coat Protein Assembly

The gene 8 product has been termed a "scaffolding" protein because it is necessary for proper assembly, but is absent from the mature phage (24). Since it is found associated with the prohead structure its scaffolding function is presumably associated with the formation of this precursor shell from unassembled coat protein. Several recent experiments clarify this.

If the gene 8 protein is absent from infected cells (due to mutation) several types of aberrant particles are produced: spherical shells of diameter similar to proheads; smaller spherical shells about 2/3 this diameter; and spiral appearing "monster" structures (Fig. 1g–h). These particles are not produced in large numbers. Although coat protein is synthesized in normal amounts during an 8^- infection, less than one-fourth of the coat protein assembles into rapidly sedimenting structures after a radioactive pulse and long chase. In a parallel wild-type infection essentially all of the coat protein is assembled into phage, proheads, and empty heads.

The p5 that does assemble into aberrant aggregates in 8^- infected cells does so more slowly than p5 assembles into large structures in wild-type infected cells. Figure 5 shows the fate of radioactive pulse labeled proteins in 5^-, 8^-, and wild-type infected cells. At 2 min after the end of the pulse of radioactive amino acids (administered at 28 min after infection) some of the radioactivity sediments as proheads and empty heads, and some is already assembled into phage. In the 8^- infected cells no particulate material is seen. By 10 min most of the label in the wild-type infected cells has chased into phage particles; however, in the 8^- infection only a small amount of the label is found in the particulate fraction. The 5^- control experiment shows that the rapidly sedimenting structures formed in the $P22^+$ infected Salmonella are aggregates of p5, and that no stable p8 aggregates are detected. Similar results were obtained at 20 min after the pulse of label (results not shown). From these data it can be seen that the coat protein assembles at less than one-tenth the wild-type rate in the absence of the p8 scaffolding protein (and that which does assemble forms aberrant aggregates).

We have also observed that p8 apparently does not form aggregates in the absence of capsid protein. Electron microscopic searches, using visualization both by negative staining of infected cell extracts (24) and by making ultrathin sections of infected cells (36), have failed to reveal any kind of particles in 5^- infected cells. Experiments designed to display structures in sucrose gradients of labeled 5^- infected cell extracts (as described above for 8^- extracts) have similarly failed to show any large aggregates of p8. In fact the p8 was recovered at a position in the gradient expected of a protein with a sedimentation coefficient of 2–3S (results not shown).

Protein Composition of Particles

In order to detemine the amounts of the various proteins in the particles, we purified large amounts of phage, empty heads, and proheads by centrifugation and then

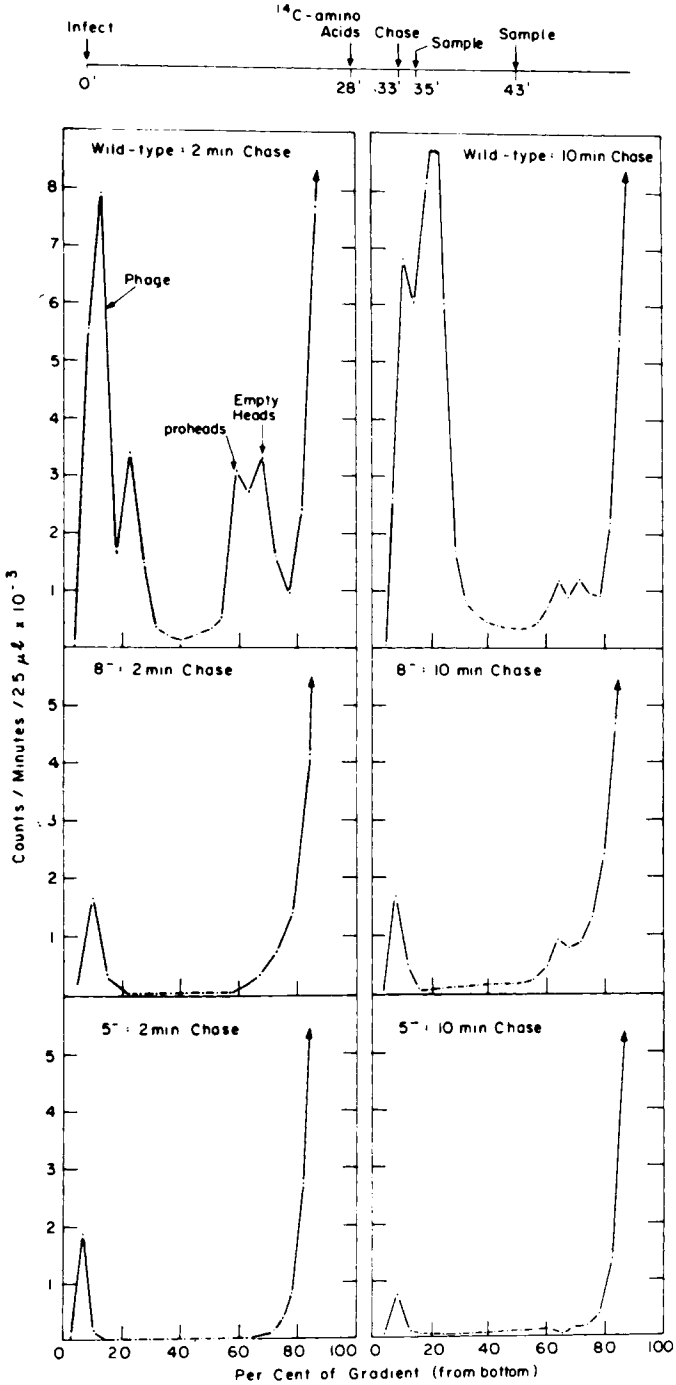

Fig. 5. Coat protein assembles slowly in the absence of scaffolding protein. Strain DB21 was infected with P22 cl^{7} am13 H101 at 30° C, and chased with excess nonradioactive amino acids at 33 min. Samples were taken at 35, 43, and 53 min and after lysis with chloroform were centrifuged through a sucrose gradient with a CsCl cushion to display phage, proheads, and empty heads (see Methods).

dissolved and separated the proteins by electrophoresis through SDS polyacrylamide gels. We determined the mass ratios of the proteins by staining with the dye Coomassie Brilliant Blue. To easily obtain the amounts of particles necessary to perform such an analysis we isolated proheads first from cells infected with amber mutants defective in genes 1, 2, or 3, since proheads are the only particulate structures (240S) formed in such cells. Similarly, empty heads were purified from cells infected with an amber mutant in gene 10. Previous experiments had shown that proheads and empty heads from mutant infected cells are qualitatively indistinguishable from the precursor particles isolated from wild-type infected cells (23, 24). The particles were isolated from the crude lysates by centrifugation through a sucrose gradient onto a CsCl step, and further purified on a second sucrose gradient as described in the Methods. Figure 6h shows the proteins of 2^- proheads (proheads purified from nonpermissive cells infected with an amber mutant in gene 2) separated in an SDS gel and stained with Coomassie Brilliant Blue with a densitometer tracing above. It is clear that p5 and p8 make up the bulk of the protein mass of the proheads. Proheads isolated from 1^-, 2^-, and 3^- infected cells were very similar in protein composition, except that the 1^- proheads lacked p1 as expected (24). Nearly identical results were obtained if the gels were stained with Fast Green instead of Coomassie Brilliant Blue.

The results of many such gel analyses are summarized in Table I. The relative number of each type of protein molecule has been calculated in Table I assuming that there are 420 coat protein subunits (p5) in each type of particle. We calculate that the proheads accumulating in 1^-, 2^-, and 3^- infected cells contain about 250 ± 20 subunits of p8, the "scaffolding protein." In these lysates more than 90% of the total p5 and p8 were recovered in proheads.

To determine the ratio of scaffolding protein to coat protein in proheads from productively infected cells, we infected a culture with P22 and isolated proheads from samples lysed at 22, 51, 90, and 109 min after infection. The separated and stained proteins from these proheads are shown in Fig. 6a–d with their respective densitometer tracings. p8 is a major component of these proheads; however, wild-type infected cells contain in addition to phage and proheads, empty heads which have just encapsulated DNA, but which have not been stabilized by the actions of p10 and p26. At lysis these structures lose their DNA (22–24). These structures which contain no p8, sediment at 170S and contaminate the prohead fractions. We determined the extent of this contamination by electron microscopic analysis of the prohead fractions; it was about 30% for these samples. Table II gives the number of p8 molecules in these wild-type proheads, corrected for the contamination by empty heads. The table also shows that the empty heads and phage contain no detectable p8. The ratio of scaffolding protein to coat protein in these wild-type precursor proheads did not change significantly throughout the course of infection, and is similar to the p8/p5 ratio in the mutant proheads. The slightly higher p8/p5 ratio in the wild-type particles may be due to overestimation of the contamination of these fractions by empty heads in this experiment. In fact other identical experiments did not show any reproducible differences between the mutant (1^-, 2^-, and 3^-) and wild-type proheads. The protein composition data therefore agree with the earlier sedimentation and electron microscopic data showing that proheads are a discrete homogeneous population of particles (23, 24).

Since the amino acid composition of these proteins is unknown, we don't know if p8 and p5 bind the same amount of dye per mg protein. Published results indicate only small differences in binding for different proteins (31, 32). We therefore conclude that

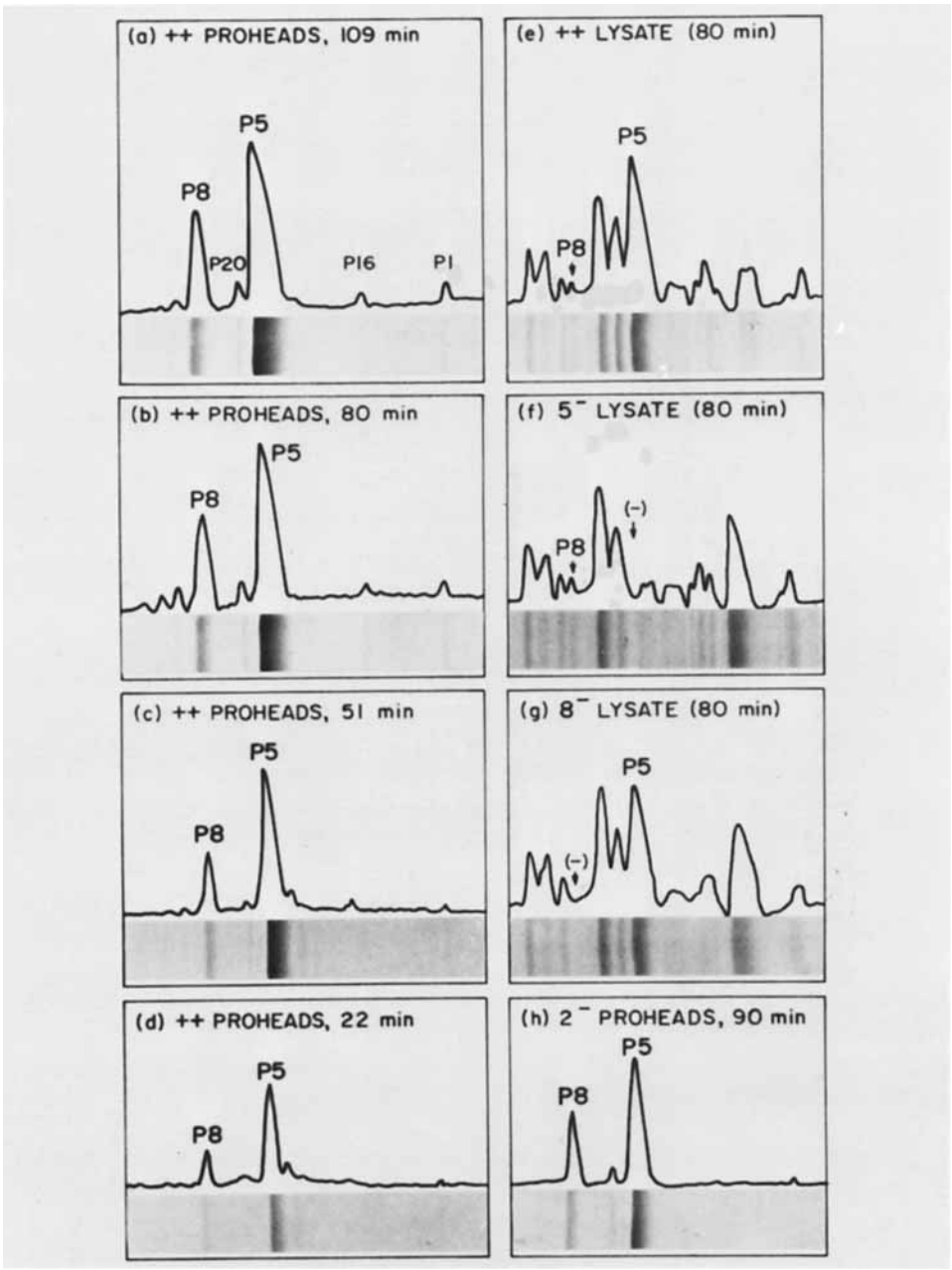

Fig. 6. SDS gel electrophoresis of the proteins in isolated proheads and in infected cells.

p8 is a major protein of proheads, though it is much less predominant in whole lysates. Infected cells were prepared and proheads were purified as described in the Methods section. The proteins were separated by SDS gel electrophoresis and the gels stained with Coomassie Brilliant Blue. The stained gels are shown in the lower portion of each panel, with Joyce-Loebl microdensitometer tracings of the same gels above them.

The high molecular weight regions of the gel are to the right. The band which is present in increased amounts in the 5^- and 8^- lysates is p9, the tail protein (25). Gene 9 is subject to a control system different from the other late proteins (Lew and Botstein, unpublished experiments); it is overproduced in cells infected with mutants blocked in DNA encapsulation. [Reproduced from King and Casjens (52) with permission of Nature.]

TABLE I. Protein Composition of Phage P22 and Precursor Particles*

Gene Product	Copies per Prohead	Copies per Empty Head	Copies per Phage
p1	10 ± 3	9 ± 2	10 ± 2
p2	0	0	0
p3**	0	0	0
p8	250 ± 20	20 ± 10‡	<2
p5†	420	420	420
p10	?	?	?
p26#	0	0	10 ± 4
p20	18 ± 3	14 ± 4	15 ± 4
p16	10 ± 4	7 ± 3	6 ± 2
p9	0	<2‡	16 ± 4
pα#	0	10 ± 5	10 ± 5
pX#	20 ± 10	20 ± 10	20 ± 10

*Proheads, empty heads and phage were purified from 3^-, 10^-, and wild-type infected cells, respectively, as described in Methods section and in King and Casjens (52). The values were determined by staining SDS gels of disrupted particles with Coomassie Brilliant Blue and scanning with a Joyce-Loebl microdensitometer. The ratio of copies of p5 to the various proteins was calculated by comparing the area of the various peaks with the area of the p5 peak in each gel and correcting for the molecular weight differences.

**Because of its low molecular weight, small numbers of p3 (< 6–8) would be very difficult to detect.

†Each type of particle was assumed to have 420 copies of p5 (23, 24).

‡Empty heads were always contaminated with small numbers of proheads and broken phage particles, hence small amounts of p8 and p9 were present.

#The low molecular weight bands p26, pα, and pX are difficult to detect by staining. These values are estimates from the ^{14}C-amino acid labeled particles shown in Fig. 12. This assumes that the amino acid compositions of p5, p26, pα, and pX are similar.

the prohead intermediate in phage P22 assembly contains 200–300 p8 scaffolding protein molecules for every 420 p5 coat protein molecules.

Recycling of p8 During Morphogenesis

Since particles containing DNA lack p8, the p8 must come out before or during DNA packaging. What happens to it after exiting? Pulse chase experiments described by King et al. (24) suggested that p8 might be reused; that is, after release from a prohead taking part in DNA encapsulation, the p8 molecules take part in further rounds of prohead assembly. If this is true, then p5 coat protein molecules should chase from proheads into phage, but the p8 scaffolding protein should remain associated with the prohead fraction, complexed with newly synthesized coat protein molecules; that is, scaffolding protein synthesized early in infection should be found in the prohead fraction much later during infection.

To test this prediction, infected cells were exposed to radioactive amino acids continuously from 12 min after infection (which is prior to the onset of late protein synthesis) to 28 min after infection, so that all the molecules of p8 and p5 synthesized up to that time would be radioactive. At 28 min excess unlabeled amino acids were added to stop the uptake of the labeled amino acids. At various times after the start of the chase (1, 2, 4, 7, 11, 16, and 22 min) samples were shaken with chloroform to lyse the infected cells,

TABLE II. Number of Molecules of p8 per Head Structure Isolated at Various Times After Infection*

	22 min	51 min	80 min	109 min
Proheads	295 ± 30	290 ± 25	315 ± 30	300 ± 25
Empty heads	< 10	< 10	< 10	< 10
Phage	< 10	< 5	< 2	< 2

*Errors represent the limits of at least two determinations. Particles were purified and values for p8 determined as described in the legend of Table I, and in the Methods section.

and aliquots were centrifuged through sucrose gradients to separate the particles from the soluble proteins. One centrifugation was done to separate phage particles (500S); a second higher speed centrifugation was done on a parallel aliquot of lysate to separate proheads and empty heads (240S and 170S, respectively), Examples of the distribution of radioactivity in these gradients are shown in Fig. 7. The total radioactivity in the prohead and empty head fractions (Figs. 7a–c) decreases with longer chase times while that in phage particles increases. To follow the fate of the individual protein species we dissociated the prohead, empty head, and phage fractions in hot SDS, and electrophoresed the samples through an SDS polyacrylamide gel which was dried and subjected to autoradiography. To quantitate these data, the p5 and p8 peaks from densitometer tracings of the autoradiograms of all the fractions were cut out and weighed, and the data plotted in Fig. 8. Radioactive p5 moves from the prohead and empty head fractions into phage particles with increasing time after the chase; however, all of the radioactive p8 found in proheads immediately after the chase remains associated with the prohead fraction. The dotted line in Fig. 8 shows the formation of viable phage in the culture during the course of the experiment; the dashed line at the top of the graph shows that the total amount of radioactive p5 remained constant. Not shown in the graph is the observation that even after just 1 min of chase, essentially all of the p5 and p8 was associated with proheads, empty heads, or phage; very little of these proteins remained at the top of the gradient. Since the ratio of p5 to p8 in proheads does not change during the course of infection (Table II) the radioactive p8 must be reassociating with nonradioactive, newly synthesized coat protein. The simplest interpretation of these results is that upon the encapsulation of DNA by the prohead, all the p8 molecules dissociate from the complex and rapidly reassociate with more coat protein to form new proheads. (These proheads would also be expected to contain newly synthesized scaffolding protein, which could not be detected in the experiment.)

Botstein et al. (23) showed that there was no detectable cleavage of the late proteins in P22 infected cells. We have since performed further pulse/chase experiments to look for possible proteolytic cleavage of p8 using shorter pulse times – 75 sec at 30° C. In this experiment, shown in Fig. 9, samples of cells infected with wild-type P22 were lysed quickly by shaking with chloroform, and the samples were quantitatively analyzed on SDS electrophoresis gels, as described above. The figure shows that the amounts of radioactivity in phage protein bands did not change with time after the pulse. We conclude that p8 does not undergo proteolytic cleavage during the phage life cycle, and thus must be removed from the prohead intact.

The measurement of the average number of times each p8 molecule in the infected culture has taken part in a round of prohead assembly can be made in a straightforward manner when it is known that p8 is not destroyed during phage growth. The total p5 and

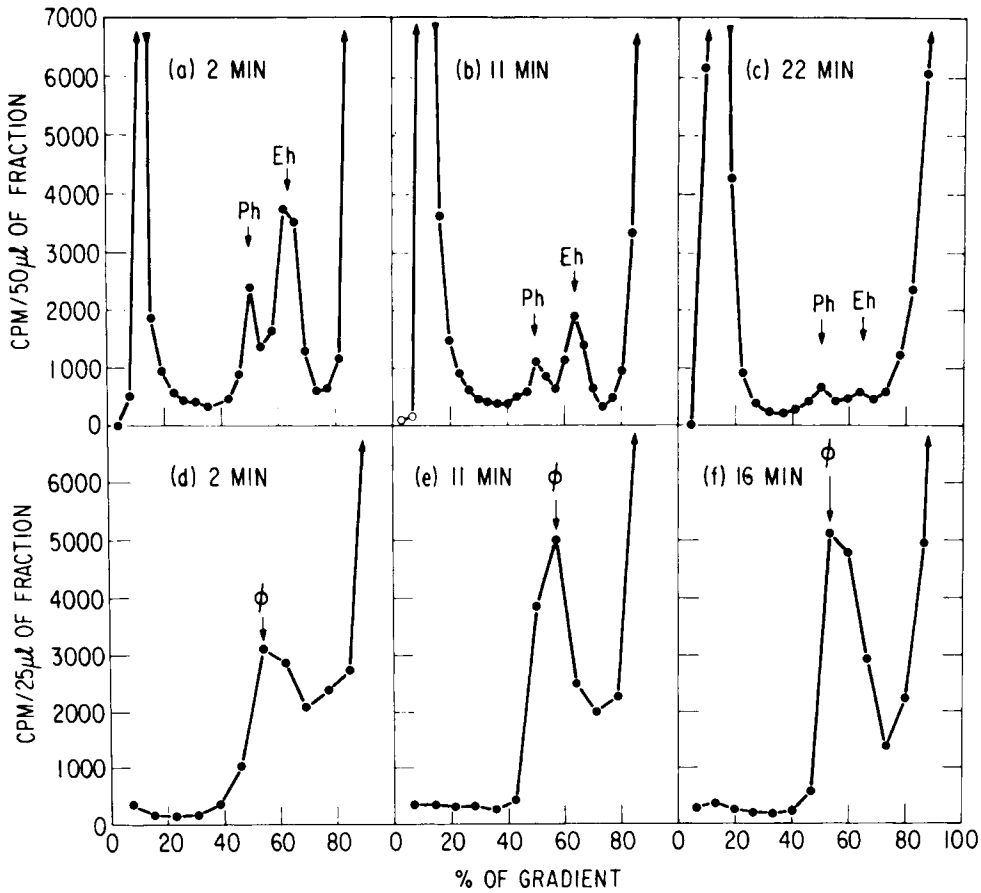

Fig. 7. Separation by sucrose gradient centrifugation of proheads (Ph) and empty heads (Eh) and phage (φ), from infected cells lysed at various times after exposure to radioactive amino acids.

The lower set of gradients have been centrifuged to separate phage particles (500S), while the upper gradients have been centrifuged longer to separate proheads (240S) and empty heads (170S). The experiment was performed as follows: D21 was grown in M9 medium at 30° C to 2 × 10^8 cells/ml and infected with 10 Cl^7 13^- phage per cell. At 12, 16, 20, and 24 min after infection 2.5 μCi of ^{14}C-labeled amino acid mixture was added per ml of culture (giving a final concentration of 10 μCi/ml). At 28 min excess casamino acids were added and at 1, 2, 4, 7, 11, 16, and 22 min thereafter, samples were lysed by shaking vigorously with chloroform. Incorporation of radioactivity into TCA insoluble material was linear during the labeling period. Formation of viable phage in the culture is shown in

p8 in the culture was determined by quantitation of Coomassie Brilliant Blue stained SDS gels of whole cells infected with wild-type P22 (see Fig. 6e–g) as was the amount of p5 and p8 in proheads, empty heads, and phage (Table I). Given that it takes about 250 molecules of p8 for the incorporation of 420 molecules of p5 coat protein into any particle, be it prohead, empty head, or phage, we simply calculated how many times the total amount of p8 present in the culture at the time of lysis would have to function in order to account for the coat protein assembled into particles (proheads + empty heads + phage) present in the culture at that time. The results of such experiments are shown in

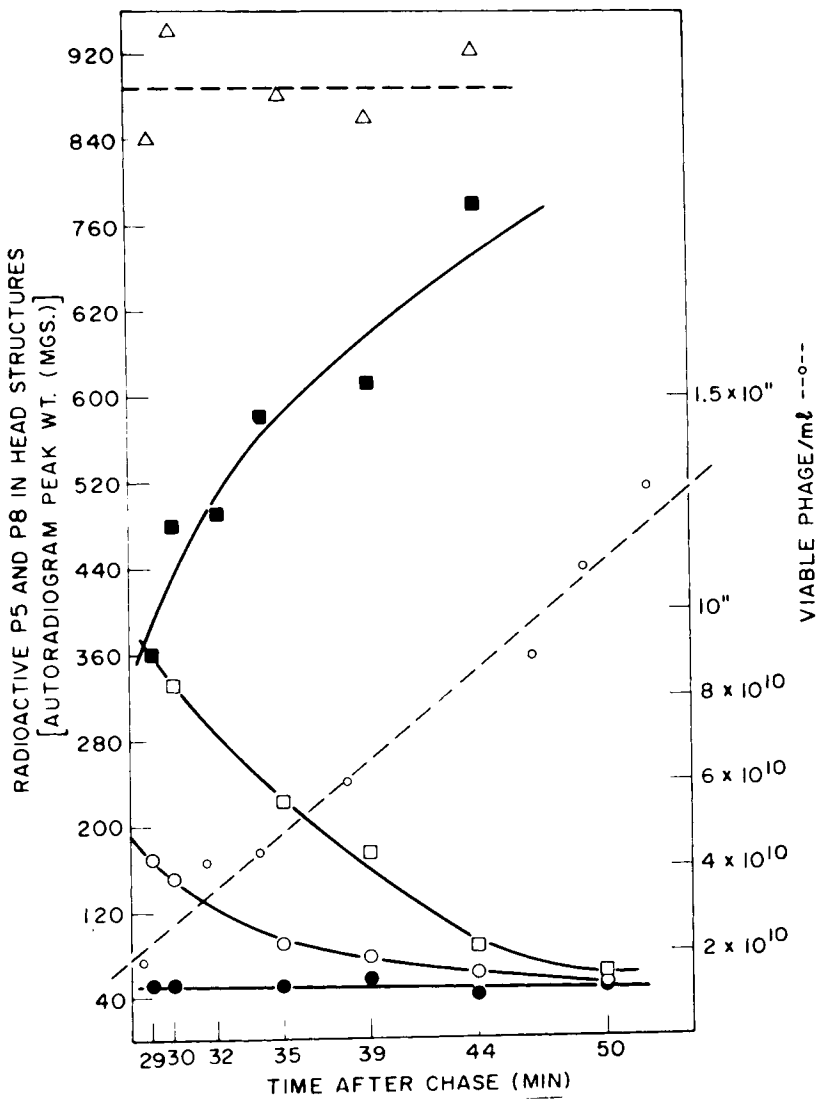

Fig. 8. Continued association of labeled scaffolding protein with proheads during phage growth. Summary of the fate of the labeled proteins from the continuous label and chase experiment described in the text and in Fig. 7. The figure shows the association of labeled coat protein and scaffolding protein with proheads, empty heads, and phage during the chase of radioactivity.

Densitometer tracings of the autoradiograms of gels were made, and the peaks cut out and weighed. The dashed line (△) shows the sum of the p.5 from the various structures, while the small open circles (○) show the formation of viable phage in the culture. The amount of radioactive p8 associated with the prohead fractions (●) remained essentially constant even though the proheads are serving as intermediates in head assembly, and the p5 associated with them is converted to phage during the experiment. The amount of labeled p5 in phage (■) increases concomitantly with the decrease of labeled p5 in proheads (○) and empty heads (□). [Reproduced from King and Casjens (52) with permission of Nature.]

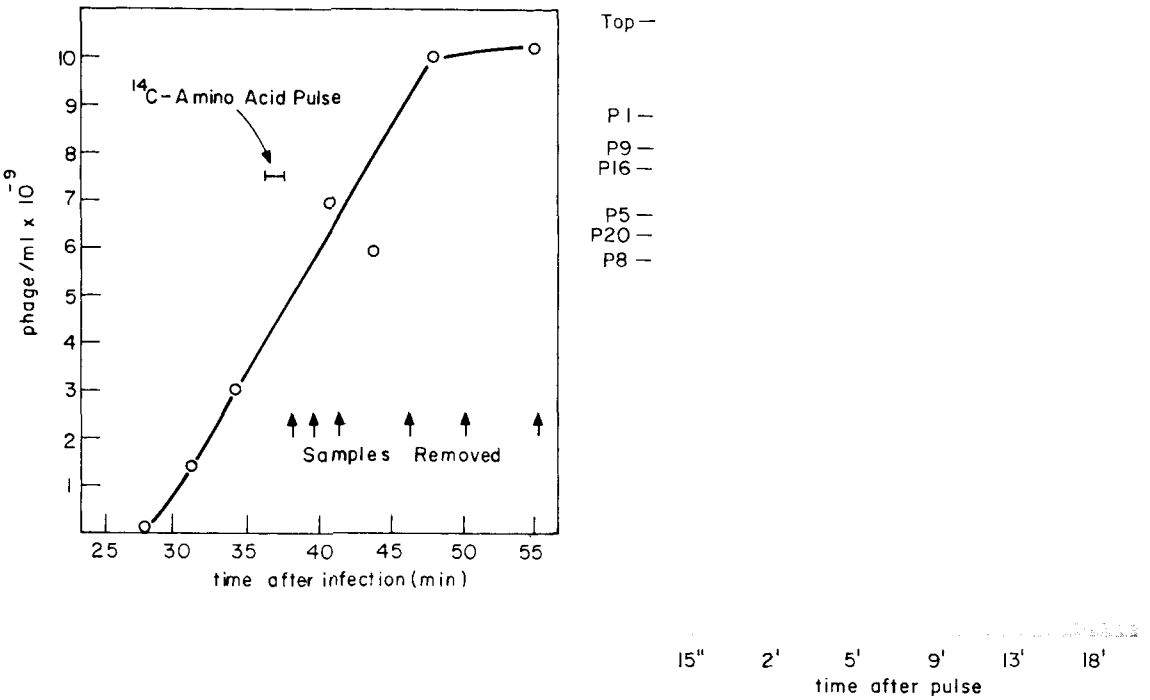

Fig. 9. P22 coded proteins are not destroyed during assembly. Strain DB21 was grown in M-9 minimal medium at 30°C to 2 X 10^8 cells/ml and infected with P22 cl^7 at 10 phage/cell. After growth at 30°C for 36 min, ^{14}C-amino acids were added to 2 μCi/ml; 75 seconds later a chase of excess non-radioactive amino acids was added and samples removed for SKS gel electrophoresis at the indicated times. The right-hand panel shows an autoradiogram of such a gel after electrophoresis of the samples. Phage production in the culture was also monitored and is shown in the left-hand panel.

Fig. 10, where the average number of times each p8 molecule must have acted is plotted vs. time during infection. It can be seen that at late times each p8 molecule must have taken part in from 4 to 8 rounds of prohead assembly.

Genes and Proteins

Botstein et al. (22, 23) found that at least 10 genes (1, 2, 3, 5, 8, 10, 26, 20, 16, 9) on the P22 chromosome are responsible for the assembly of a complete, infective phage; that is, if any of these gene products is missing, the remaining morphogenetic proteins are made, but infective phage particles are not formed. They were able to identify the protein products of 8 of these genes (all except p3 and p10) in SDS polyacrylamide gel electrophoresis patterns of the proteins made by P22 infected cells at late times after infection. The identifications were made by observing the bands specifically missing after infection by amber mutants in the various genes. We have since been able to identify p3 and p10 as proteins of molecular weights 9,500 and 56,000, respectively, in SDS gels. These data are shown in Fig. 11. Both are relatively minor species among the phage coded proteins. With these identifications, the products of all the known morphogenetic genes have been identified in the gel patterns. In these gels of the proteins made after P22 infection, one protein band is visible, which has the kinetics of synthesis expected of a morphogenetic gene product and which is not missing in any of the amber mutant infections (39). This protein, of molecular weight about 15,000, has been called pα. It is also made during in-

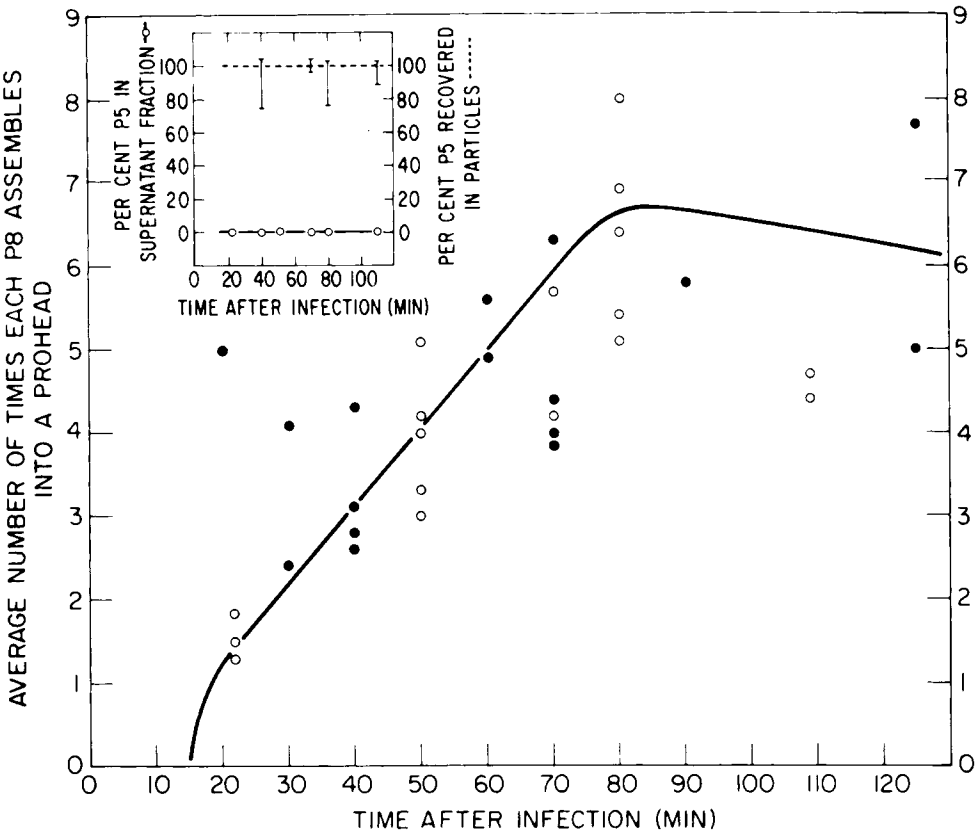

Fig. 10. Recycling of scaffolding protein molecules. The graph shows the average number of rounds of prohead assembly that each scaffolding molecule has taken part in during phage growth. The calculation was as follows:

Number of times p8 reused =

$$\frac{(\#p8 / \#p5)_{\text{proheads}}}{(\#p8 / \#p5 \times F)_{\text{cells}}}$$

Where "F" is the fraction of the total p5 molecules in the cell which is in phage or phage precursor structures. F was found to be very near 1.0 (see below). The open (○) and closed (●) circles represent two independent experiments.

S. typhimurium DB21 was grown in broth to 2×10^8 cells/ml and infected with P22 cl^7 13^- at a multiplicity of 10. At the times indicated samples of the infected cells were pelleted, resuspended in 0.1 volume of electrophoresis buffer, heated to 100° C for 2 min, and the proteins separated by SDS slab gel electrophoresis (see Fig. 6e–g). The protein bands were stained with Coomassie Brilliant Blue, and quantitated by tracing with a Joyce-Loebl microdensitometer, and the areas under the p8 and p5 peaks measured.

At various times after the infection described above, samples were also taken for analysis of the phage specific structures present. The infected cells were concentrated by centrifugation, lysed with $CHCl_3$, layered on the top of a sucrose gradient with a high density (1.6 gm/cc) CsCl cushion, and centrifuged for 45 min at 45,000 rev/min in a Beckman SW 50.1 rotor. The fraction containing all the particles (phage, proheads, and empty heads), that is, the top of the CsCl cushion, and the supernatant (top 1.5 ml) fraction were removed with a syringe, and the proteins separated and quantitated as above. The insert in the figure shows the results of such an analysis normalized to the total amount of p5 present. [Reproduced from King and Casjens (52) with permission of Nature.]

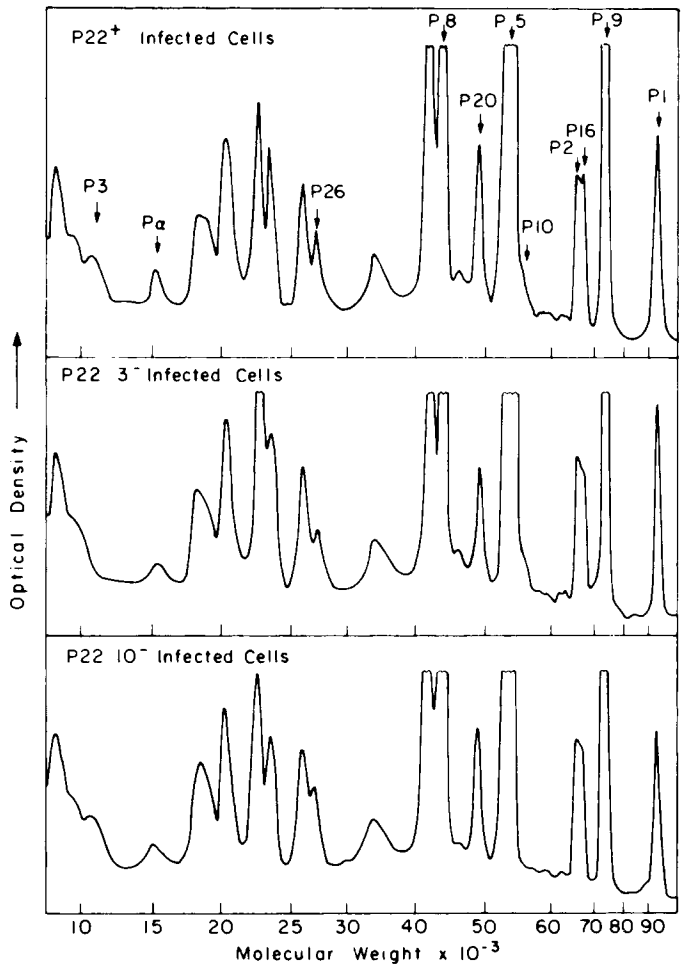

Fig. 11. SDS gel electrophoretic identification of the protein products of genes 3 and 10. Proteins synthesized in $P22^+$ and P22 3^- and 10^- amber mutant infected cells were labeled with ^{14}C-mixed amino acids according to the method of Botstein et al. (23). The host ($uyrB^-$) was irradiated with ultraviolet light before infection to suppress the background of host protein synthesis after phage infection. The genetically identified gene products are labeled above the wild-type P22 infected cell gel pattern (see 23). Migration is from right to left during electrophoresis. After electrophoresis the dried gels were subjected to autoradiography, scanned with a Joyce-Loebl microdensitometer, and the tracings smoothed by hand.

fections by amber mutants in gene 13 or 19. These genes, whose products are involved in cell lysis (23), are the only other known "late" genes. It thus appears that pα is the product of a late gene in which no mutants have been isolated. The remaining bands in Fig. 11 are proteins coded for by the "early region" of the P22 chromosome (39).

We have also re-examined the proteins which are found in proheads, empty heads, and phage to more accurately visualize the minor components. Particles labeled with ^{14}C-mixed amino acids were prepared as described in the Methods, disrupted by boiling in SDS for 1 min and subjected to electrophoresis in SDS. The results shown in Fig. 12 confirm and extend the observations of King et al. (24). The gene 5 protein is the largest

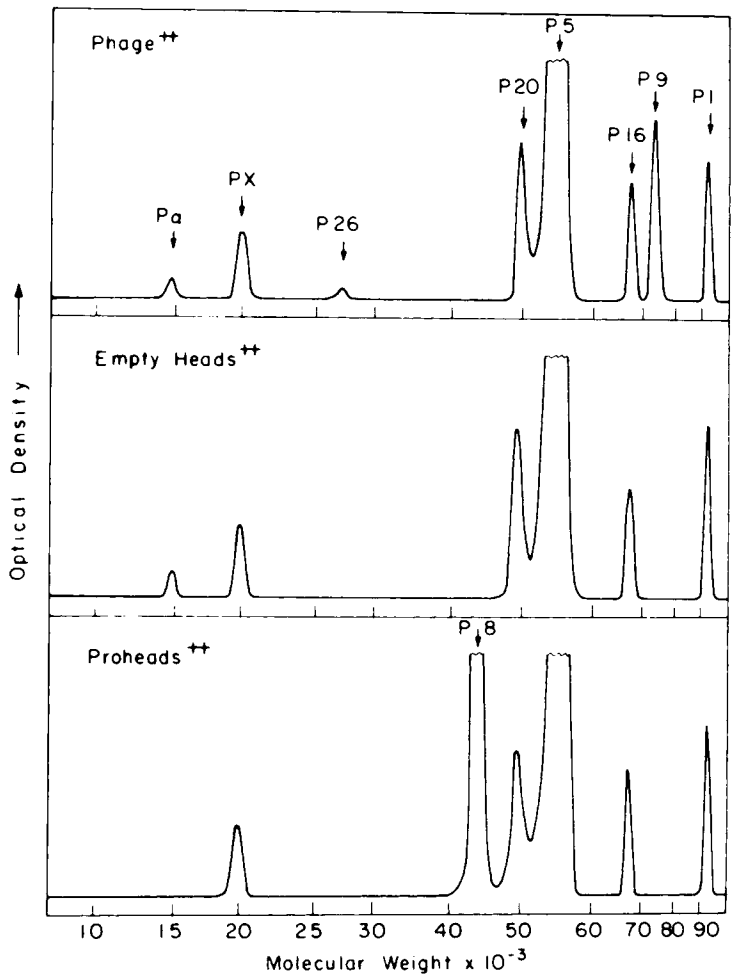

Fig. 12. SDS gel electrophoresis of phage and precursor particles. Phage, proheads, and empty heads labeled with ^{14}C-amino acids were prepared from P22^{+} infected cells, as described in the Methods section and in King et al. (24), disrupted by boiling in SDS, and the proteins separated by SDS gel electrophoresis. Autoradiograms of the gels were prepared and traced with a Joyce-Loebl microdensitometer. The resulting curves were smoothed by hand. The major peaks have been truncated to show the minor proteins more clearly. The known gene products are labeled above the tracings.

component of all three types of particles, and the gene 8 protein is a major component of the proheads and is absent from empty heads and phage particles. The minor proteins, p1, p16, and p20, are present in all three types of particles, and p26 and p9 are found only in phage. [It has been shown (23, 24) that although p16 and p20 are components of the prohead they are not required for normal prohead or phage assembly; instead they are required for proper infection by the finished particle.] The genetically unidentified protein pα mentioned above is a component of empty heads and phage. In addition, there is a second genetically unidentified protein (designated pX) (23) in all types of particles. This band is not unambiguously seen in the gel patterns of infected cells because several major

early proteins have very similar mobilities. Thus there are most likely two cistrons (X and α) in which no amber mutants have been isolated. p3 and p2 are not found in any of the particulate fractions, but are recovered in the low molecular weight fraction of a sucrose gradient. We were not able to unequivocally assess whether p10 is a structural component of phage since in these gels it runs extremely close to a major capsid protein. Our gel patterns of infected cells do not show the late protein called pY by Botstein et al. (23).

DISCUSSION

Since the capsid of P22 is isometric, the coat protein subunits are probably arranged into an icosahedral shell as described by Caspar and Klug (40).

However, our experiments show that the information for the correct assembly of the coat protein subunits is not completely contained in the subunits themselves, but requires the presence of a few hundred scaffolding protein molecules; the normal assembly process is apparently a copolymerization of coat and scaffolding proteins. Since the scaffolding protein does not form any detectable structure or aggregate in the absence of the coat protein, we suspect that the copolymerization involves the formation of an initial complex between coat protein and scaffolding protein, and then the polymerization of these intermediates into the acutal prohead shell. Our naive thinking about the reasons for such a process is that it may be difficult to specify the exact curvature or dimensions of the head when a large number of subunits and bonds are involved. Interaction of coat protein with scaffolding protein may result in a complex with an increased specific bonding surface, which can now polymerize into a shell of exactly defined dimensions. Alternatively, it is possible that the assembly function of the scaffolding protein is really secondary, and that the primary function may be in DNA encapsulation. If this process is coupled to the exit of the scaffolding molecules, then obviously there would have to be some specific assembly mechanism for getting it in in the first place.

The electron microscopic and x-ray evidence suggest that the scaffolding protein is on the inside of the prohead. This poses the problem of how 250 42,000 molecular weight protein molecules get out of the prohead shell during phage assembly. Since treatment of prohead with detergent removes the scaffolding protein but leaves the shell essentially intact, we suspect that the p8 may pass through gaps between coat subunits, or that in fact the coat protein subunits rearrange, expelling p8 in the process. Within the cell the dissociation of the p8 from the prohead is a complex reaction, which requires in addition to DNA, the products of genes 1, 2, and 3. The gene 1 product becomes part of the prohead, but the gene 2 and 3 products do not appear to be associated with either proheads or complete phage. They may interact with the DNA directly preparing it for packaging. The DNA substrate in the packaging reaction and the products of the section are described in detail in an accompanying paper (19). A DNA condensation role for the exiting p8 molecules can be formulated in terms of Laemmli's (41) model for DNA packaging. The exit of p8 exposes sites in the inner face of the coat protein that either bind or collapse the chromosome. When the coat protein is synthesized these sites are immediately covered up by scaffolding protein to prevent aberrant DNA binding. Only after the coat protein subunits are polymerized into a shell do the condensation sites get exposed.

Comparison between P22 and T4 is instructive. Both have permuted and terminally repetitious chromosomes (42, 43). In T4 head assembly the product of gene 22 serves as an assembly core (44), together with other phage proteins, for the correct polymerization of the major coat protein. During DNA packaging it is cleaved down to low molecular weight fragments (10, 44, 45). p8 of phage P22 is needed for correct assembly of the major coat protein also, but instead of being cleaved during condensation it is removed and recycled. There is also evidence for a possible scaffolding-type protein in phage T7 assembly and phage T3 assembly (46, 47). The need for such proteins may be quite general in the assembly of the larger icosahedral shells.

We would like to point out that without the availability of the P22 mutants of Botstein et al. (22) it would have been very difficult to detect the scaffolding protein function. Since the protein recycles it is not a major component of infected cells. It is not in the mature virion. Though it is a major component of the prohead precursors, these structures are only transient intermediates in wild-type infected cells and are not present in large numbers.

As is clear from a number of the contributions to this symposium, the assembly of many structures involves proteins that do not become incorporated in the final structure. Examples are the gene 57, 38, and 63 products in T4 tail fiber assembly (48–50) and the peptidases needed for collagen maturation (51). Proteins such as the scaffolding protein, which catalyze an assembly reaction by becoming temporarily associated with an intermediate stage in the assembly process, probably represent a further class of proteins which function in ensuring accurate and efficient morphogenesis.

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