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Mechanism of Head Assembly and DNA Encapsulation in *Salmonella* Phage P22

II. Morphogenetic Pathway

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We have identified and characterized structural intermediates in phage P22 assembly. Three classes of particles can be isolated from P22-infected cells: 500 S full heads or phage, 170 S empty heads, and 240 S "proheads". One or more of these classes are missing from cells infected with mutants defective in the genes for phage head assembly. By determining the protein composition of all classes of particles from wild type and mutant-infected cells, and examining the time-course of particle assembly, we have been able to define many steps in the pathway of P22 morphogenesis.

In pulse–chase experiments, the earliest structural intermediate we find is a 240 S prohead; it contains two major protein species, the products of genes *5* and *8*. Gene *5* protein (p5) is the major phage coat protein. Gene *8* protein is not found in mature phage. The proheads contain, in addition, four minor protein species, P1, P16, P20 and PX. Similar prohead structures accumulate in lysates made with mutants of three genes, *1*, *2* and *3*, which accumulate uncut DNA. The second intermediate, which we identify indirectly, is a newly filled (with DNA) head that breaks down on isolation to 170 S empty heads. This form contains no P8, but does contain five of the six protein species of complete heads. Such structures accumulate in lysates made with mutants of two genes, *10* and *26*.

Experiments with a temperature-sensitive mutant in gene *3* show that proheads from such 3^- infected cells are convertible to mature phage *in vivo*, with concomitant loss of P8. The molecules of P8 are not cleaved during this process and the data suggest that they may be re-used to form further proheads.

Detailed examination of 8^- lysates revealed aberrant aggregates of P5. Since P8 is required for phage morphogenesis, but is removed from proheads during DNA encapsulation, we have termed it a scaffolding protein, though it may have DNA encapsulation functions as well.

All the experimental observations of this and the accompanying paper can be accounted for by an assembly pathway, in which the scaffolding protein P8 complexes with the major coat protein P5 to form a properly dimensioned prohead. With the function of the products of genes *1*, *2* and *3*, the prohead encapsulates and cuts a headful of DNA from the concatemer. Coupled with this process is the exit of the P8 molecules, which may then recycle to form further proheads. The newly filled heads are then stabilized by the action of P26 and gene *10* product to give complete phage heads.

1. Introduction

Caspar & Klug (1962) described the favorable geometries for the packing of similar subunits into virus capsids; their prediction of icosahedral symmetry for spherical viruses has been amply borne out (Eiserling & Dickson, 1972). With small spherical

viruses containing chromosomes of single-stranded RNA, the actual assembly into particles of the protein subunits and nucleic acid proceeds *in vitro* and has been extensively studied (Bancroft, 1970; Hohn & Hohn, 1970). However, the mechanism of the formation of large spherical viruses containing chromosomes of double-stranded DNA remains a considerable mystery: how are shells initiated? How do subunits actually come together to form a closed capsid? How are extended double helical DNA molecules condensed into tight packages? That the geometry of the structure is not a sufficient description of the formation process is clear from genetic characterization of bacteriophage assembly, which has revealed that many gene products are needed for head morphogenesis and DNA packaging (Epstein *et al.*, 1963; Kellenberger, 1966; Frankel, 1968; Mount *et al.*, 1968; Laemmli *et al.*, 1970*a*,*b*; Kellenberger & Edgar, 1971). For coliphage T4, λ and P2, the proteins of the mature virus head differ from the precursor proteins that interact to form the head (Laemmli, 1970; Dickson *et al.*, 1970; Hosoda & Cone, 1970; Kellenberger & Kellenberger-Van der Kamp, 1970; Murialdo & Siminovitch, 1972; Lengyel *et al.*, 1973)

A great deal about virus assembly has been learned by analyzing cells infected with mutants defective in assembly. This approach has been most valuable in those cases in which true precursor structures accumulate in mutant-infected cells, as with the tail fibers and tail of phage T4 (Edgar & Wood, 1966; Edgar & Lielausis, 1968; King, 1968; King & Wood, 1969). Head assembly, in contrast, has been much more refractory to such analysis, since in general the structures accumulating in mutant-infected cells have not been true precursors (Kellenberger, 1966; Mount *et al.*, 1968; Laemmli *et al.*, 1970*b*).

In the accompanying paper (Botstein *et al.*, 1973) we identified: (1) the genes of phage P22 that are involved in head morphogenesis; (2) the protein products of these genes; and (3) the nucleic acid and protein structures that accumulate in cells infected with mutants defective in the various morphogenetic genes. These experiments established that with phage P22, as with T4 and λ, a head shell is needed for the cutting and maturation of the chromosome from the replicating DNA concatemer (Dove, 1966; Frankel, 1968; MacKinlay & Kaiser, 1969). In this paper we characterize the head-related structures in mutant and wild type infected cells and show that some of them are true intermediates in phage assembly. This has allowed us to define the pathway for the formation of this icosahedral virus in considerable detail, including identification of the phage P22 gene products that are explicitly involved in the DNA encapsulation and cutting process. Our experiments were originally stimulated by the discovery by Laemmli and others of the intimate association between the cleavage of T4 head proteins and their assembly into a head that could package a chromosome. Our results indicate, however, that the isometric P22 particles are assembled by a mechanism which differs from that of T4 and λ; the dimensions of the phage head are determined by a scaffolding protein, which is removed intact from a precursor particle on entry of the phage chromosome into the precursor head.

2. Materials and Methods

These are as described in the preceding paper except as noted below. Note that the term wild type is used to describe the reference phage that grows normally with respect to the conditions of our experiments. Strictly speaking these phage are not wild type, since they contain a *c*1 mutation to ensure lytic growth and either a lysozyme or gene *13* mutation to prevent spontaneous lysis.

(a) *Preparation of lysates*

In all experiments *Salmonella typhimurium* DB21 was grown to 2×10^8 cells/ml and infected with 5 phage/cell.

For isolation of structures (Figs 1 and 2), the phage strains used in the experiments all contained, in addition to the amber mutant of interest, a temperature-sensitive mutant in the lysozyme gene, to aid recovery of infected cells. Lysates were prepared at 40°C. The cultures (10 ml) received 0·4 μCi of ^{14}C-labeled amino acids/ml at 5 min after infection, and the same amount again at 20 min after infection. Incorporation was stopped at 35 min by addition of excess Casamino acids. At 45 min the cells were collected by centrifugation and the pellet resuspended in 1 ml of a 2-fold dilution of phosphate-buffered saline (BS/2) containing 0·01 M-EDTA and 100 μg lysozyme/ml. After 30 min incubation at room temperature the cells were shaken with chloroform to complete lysis and then made up to 0·01 M-$MgSO_4$, 10 μg DNase/ml. The lysate was centrifuged at 4000 revs/min to remove large debris and unlysed cells, and then 0·1-ml samples were layered on 5% to 20% sucrose gradients, as described below.

For pulse–chase experiments, the infecting phage in these experiments carried, in addition to the *c*1 allele, an amber mutation in gene *13*, which extends phage synthesis past the normal lysis time. The cells do not lyse spontaneously, but do accumulate lysozyme and can be efficiently lysed by shaking with chloroform. Cultures (10 ml) received 2 μCi of ^{14}C-labeled amino acids/ml at the times shown in Figures 8 and 9. The incorporation was stopped by addition of 0·5 ml of 10% Casamino acids. Samples were taken at various times thereafter and lysed immediately with chloroform. No further treatment was given to the samples before layering 0·25-ml samples on sucrose gradients for centrifugation. Re-centrifugation of such samples after weeks in the refrigerator gave identical distributions of radioactivity.

For temperature-shift experiments, the infecting phage carried in addition to the temperature-sensitive allele in gene *3* (*ts*3.1, Botstein *et al.*, 1972), the *c*1 allele and the amber mutation in gene *13*, as described above. The control infection was with *c*1 13$^-$ phage. The cells were infected at 40°C. At 25 min after infection, 2 μCi of ^{14}C-labeled amino acids/ml were added to each culture. Incorporation was stopped at 35 min by addition of excess Casamino acids. At 55 min portions of the temperature-sensitive and control lysates were shifted to 25°C. At the same time samples were taken and lysed by shaking vigorously with chloroform. At 80 min after infection, samples from the cultures that had been shifted to low temperature, and also those which had remained at high temperature were lysed with chloroform. Lysate samples (0·25 ml) were layered directly on sucrose gradients and centrifuged as described below.

(b) *Sedimentation of head structures through sucrose density gradients*

Linear 5-ml 5% to 20% sucrose density gradients containing 0·033 M-phosphate buffer (pH 7·0), 0·075 M-NaCl and 0·01 M-$MgSO_4$ were prepared on top of 0·3-ml "shelves" containing either 50% sucrose in the same buffer or 80% iothalamic acid (Angio-Conray, Mallinckrodt) and 20% sucrose. Samples (0·1 to 0·3 ml) were layered on the top and the gradients centrifuged for 30 min in a Spinco SW50.1 rotor at 20°C at either 20,000 revs/min (to resolve phage particles (530 S) and other DNA-containing particles) or at 30,000 revs/min (to resolve other head-related structures). About 20 fractions were collected through a hole punctured in the bottom of the tube; samples (20 to 50 μl) were taken directly into scintillation fluor (4·8 g butyl PBD (New England Nuclear) per l of 2:3 2-methoxyethanol toluene) in order to estimate total radioactivity in each fraction. The gradient fractions were stored at 4°C; electron microscope grids were made immediately, although the appearance of the particles remained the same even if the grids were prepared as much as 2 weeks later.

Fractions containing sodium iothalamate were dialyzed against the phosphate buffer used to make the gradient solutions before electrophoresis.

The sedimentation coefficient of phage P22 was measured by mixing P22 with bacteriophage T7 and centrifuging through a 5% to 20% sucrose gradient prepared as described above. The viable phage titers of the fractions were determined by plating. Using the value $s_{20,w} = 487$ S reported by Davison & Freifelder (1962) for T7, we calculated a

sedimentation coefficient for phage P22 of $s_{20,w} = 526$ S. We have not carefully measured the sedimentation coefficient of defective particles which sediment similarly to phage. In the text, therefore, we speak of a 500 S class, since the actual value could differ by a few per cent from the sedimentation coefficient of mature phage. The sedimentation coefficients of particles sedimenting more slowly than phage were determined with respect to mature P22 phage. They are only nominal values, which we used to identify and classify the particles. In most centrifugation experiments on "240 S" proheads and "170 S" empty heads the gradients did not contain internal sedimentation markers. Instead, we compared the position of the peaks from other mutant lysates with the single well-defined peaks of proheads from the 1^-, 2^- and 3^- lysates, in gradients centrifuged together. Therefore, small differences that probably exist between different but closely related classes of defective particles would not have been detected.

(c) *Sodium dodecyl sulfate gel electrophoresis*

Discontinuous gel electrophoresis in the presence of sodium dodecyl sulfate (Laemmli, 1970) was carried out as described in the preceding paper on both cylindrical and vertical slab gels. The stacking gels for the slab gels were cast using a well-former making 24 $\frac{1}{8}$-in slots. 10 μl samples were applied to each slot.

Tracings of autoradiograms were made with a Joyce–Loebl microdensitometer using optical wedges with cutoffs of 1·5 density units or less. Thus all peaks not truncated in the tracings are within the linear range of the film sensitivity. Tracings in each Figure derive from a single film of samples analyzed by electrophoresis through the same slab gel, or from the same set of cylindrical gels. Though the major bands in autoradiograms of isolated particles could generally be seen after a few days of exposure, 2 weeks to a month of exposure was generally required to detect the minor bands with certainty. In all experiments, films were also exposed to the gels for 2 months or more, but little further information was gained. For quantitation of the P5 and P8 bands in pulse–chase experiments, films were chosen in which the density of the band was in the linear range of the film, tracings were made, and the peaks cut out and weighed. To calibrate densities measured from films exposed for different lengths of time, a band was measured whose density was within the linear range of both films. The ratio of the densities for this peak was then used to calibrate other bands, when comparing measurements from the two films.

3. Results

(a) *Analysis of structures accumulating in mutant lysates*

(i) *Sedimentation*

As shown in the preceding paper, cells infected with phage P22 mutants defective in any of eight phage genes accumulate structures morphologically related to P22 particles, but non-infectious. Cells infected with mutants defective in gene *5*, which specifies the major coat protein, and gene *8*, accumulate very few phage-related structures.

In order to characterize the mutant structures in more detail we prepared ^{14}C-labeled lysates of these mutants and separated the larger structures from supernatant proteins by centrifuging through sucrose gradients. Label was added both early and late during the infection, to be sure that we would detect both early and late proteins in the structures of interest. Two centrifugations were done for each lysate; low speed to isolate particles with a sedimention rate similar to that of complete phages, and high speed to isolate particles that sediment at a rate resembling that of empty heads. Fractions from the gradient were collected and the ^{14}C radioactivity measured in a scintillation counter. Since P22 infection does not shut off host protein synthesis, much of the radioactivity at the top of such gradients is residual host protein. To ensure recovery of rapidly sedimenting materials a shelf of 50% sucrose was in the

bottom of the tubes used for the low-speed centrifugation, and a shelf of sodium iothalamate was in the bottom of the tubes in the high-speed centrifugation. The results of these experiments are presented in Figures 1 and 2. (Note that the radioactivity is plotted on a logarithmic scale.)

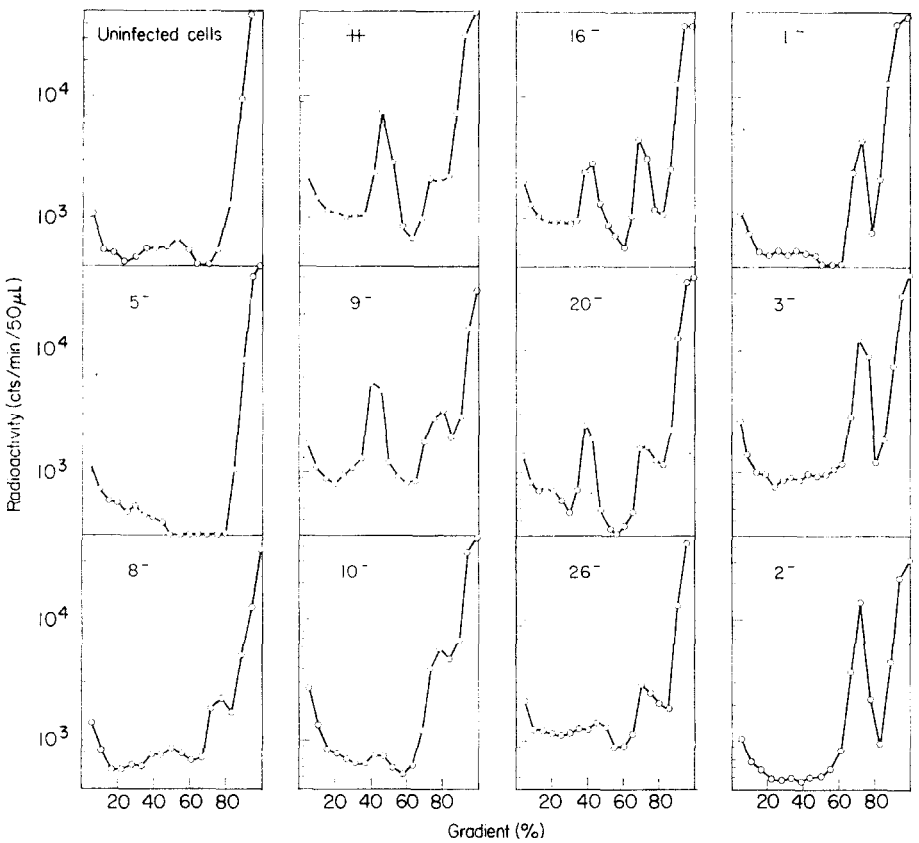

FIG. 1. Sucrose gradient centrifugation of phage-like particles accumulating in mutant lysates. The peak of radioactivity in the middle of the gradient of the wild type lysate (++) represents mature P22 phage particles; $s_{20,w} = 526$ S.

Lysates were prepared as described in Materials and Methods. The final lysate supernatants contained 25 to 50×10^6 cts/min/ml. Samples of 0·1 ml were layered on 5% to 20% sucrose gradients containing a shelf of 50% sucrose. The gradients were centrifuged at 20,000 revs/min for 30 min, at 20°C.

Figure 1 displays the results of the low-speed centrifugation. The gradient of the wild type control lysate contains the expected peak of P22 phage sedimenting at about 500 S. As expected, no peak of radioactivity is present in this region of the gradient of uninfected cells, nor in the gradient of the 5^- lysate (gene *5* specifies the major head structural protein). The peak from the 9^- lysate presumably represents the complete but tailless heads, which accumulate in such lysates (Israel *et al.*, 1967), while the peaks present in this region of the gradients from 16^- and 20^- lysates represent the morphologically normal but inviable particles that accumulate in those lysates (preceding paper). Gradients of the remaining lysates, 8^-, 1^-, 2^-, 3^-, 10^- and 26^-, did not display well-defined peaks in the 500 S region. Thus, only the lysates

that accumulated particles which appeared full of DNA in the electron microscope, also displayed a structure sedimenting similarly to intact phage at about 500 S.

In addition to the peaks of radioactivity at the phage position, a more slowly sedimenting peak can be seen near the tops of most of the gradients in Figure 1. This peak is missing in the gradients derived from uninfected cells and 5⁻ lysates; it is particularly strong in the gradients of lysates that did not accumulate particles sedimenting like phage.

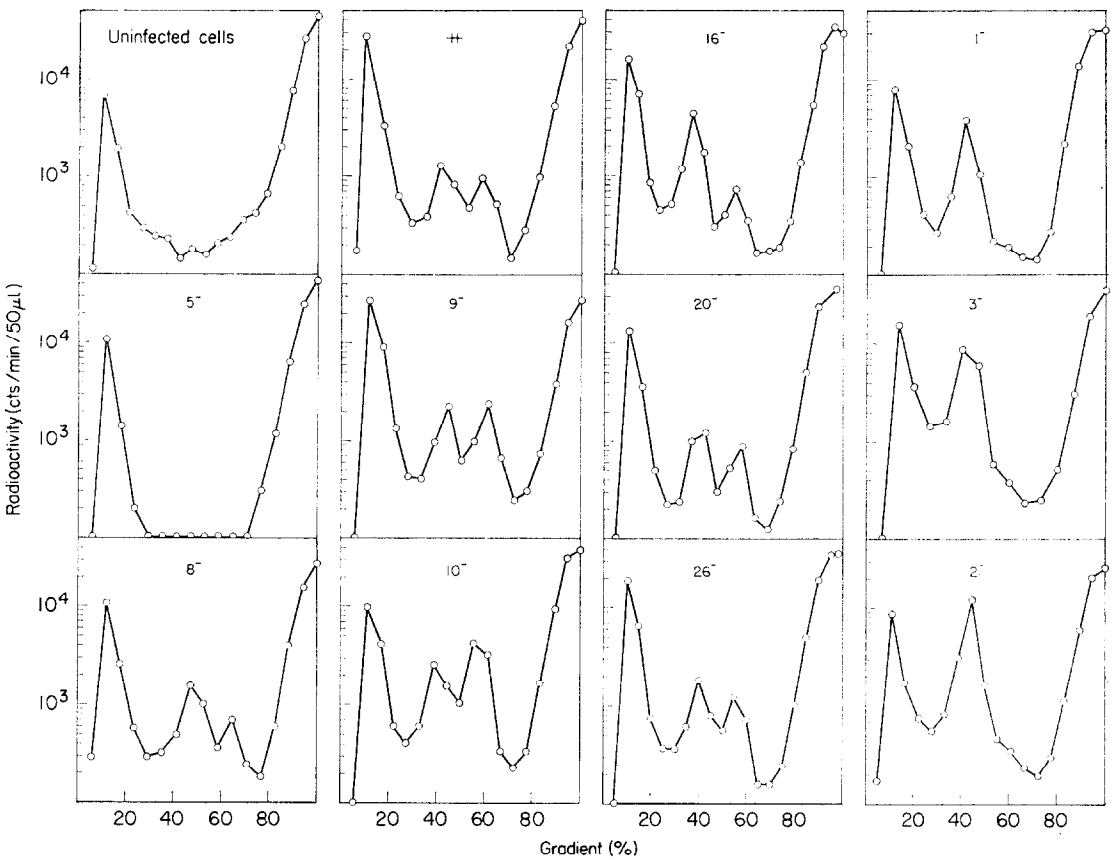

FIG. 2. Sucrose gradient centrifugation of lysate structures sedimenting slower than phage particles. Samples (0·1 ml) of the same lysates described in the legend to Fig. 1 were centrifuged at 30,000 revs/min for 30 min, at 20°C, to resolve peaks sedimenting slower than phage. These gradients contained a shelf of sodium iothalamate. This very efficiently catches material sedimenting to the bottom of the gradient, forming a peak there. Electron microscopic examination of the material in the shelf fraction from the gradients of uninfected cells and from 5⁻ lysate revealed cell debris and membrane vesicles of various sizes. The peaks in the middle of the wild type (++) and other gradients have sedimentation coefficients of about 240 S and 170 S. The two peaks in the 8⁻ gradient sediment more slowly, at about 195 S and 125 S.

Figure 2 shows sucrose gradient profiles of the radioactivity from the same lysates, which were centrifuged at high speed in order to resolve the slower peak. Particles sedimenting with the rate of complete phage have sedimented onto the shelf at the bottom of these gradients. The gradients derived from uninfected cells or the 5⁻ lysate show no peak of radioactivity between the top of the gradient and the pellet. All

the other lysates display one or two peaks of radioactivity. Two peaks are present in the gradient of the wild-type lysate, and the gradients from 9$^-$, 16$^-$, 20$^-$, 26$^-$ and 10$^-$ lysates. The faster peak sediments at about 240 S, while the slower peak sediments at about 170 S. In contrast, the 1$^-$, 2$^-$ and 3$^-$ lysates accumulated all their particulate radioactivity in the 240 S structure. These were the lysates that contained particles which we have called prohead structures. The gradient of the 8$^-$ lysate shows two peaks, but these two peaks sedimented more slowly than the 240 S and 170 S peaks. (The sedimentation coefficients are about 195 S and 125 S.) This difference in sedimentation coefficient between the peaks from the 8$^-$ lysate and those from all the other mutant lysates is reproducible. Furthermore, a smaller fraction of the total 8$^-$ lysate radioactivity sediments into these gradients than is the case with the other particle-containing lysates.

Comparing both sets of centrifugations, we see that all lysates that accumulate a 500 S structure, that is a particle filled with DNA, also contain both 240 S and 170 S structures. The 10$^-$ and 26$^-$ lysates accumulate just 240 S and 170 S structures, whereas the 1$^-$, 2$^-$ and 3$^-$ lysates accumulate only 240 S structures. The 5$^-$ lysate accumulates no rapidly sedimenting structures, while the 8$^-$ lysate may contain some unusual forms.

(ii) *Electron microscopy of gradient fractions*

To characterize further the structures from the mutant lysates, the peak fractions from the sucrose gradients were examined with the electron microscope. These results are summarized in Table 1.

The 500 S peak fractions from the gradients of the wild type lysates and the 16$^-$ and 20$^-$ lysates contained, as expected, large numbers of morphologically intact phage particles. The 500 S peak fraction from the 9$^-$ gradient contained P22 heads lacking base-plates. The 500 S fraction from the 26$^-$ lysate contained some phage ghosts, that is particles lacking DNA, but with tails, many of which were attached to the membrane debris present in the fractions. These particles may have been sedimenting at this position because of their attachment to the rapidly sedimenting cell envelope fragments; more likely, they had DNA at the beginning of centrifugation and lost it during the course of the experiment (see preceding paper).

The 500 S regions from gradients of the 5$^-$ lysate, and the uninfected cells, lysates which do not contain particles, displayed in the electron microscope the expected cell envelope debris and vesicles. The vesicles occurred in a range of sizes, which overlapped the size of P22, and formed a background level in the electron microscopic analysis corresponding to about 5% of the number of phage particles in this region of the wild type gradient.

All 240 S peak fractions from the mutant lysates contained particles corresponding to the prohead structures described for 1$^-$, 2$^-$ and 3$^-$ lysates in the preceding paper. The particles from the fractions derived from the different lysates could not be distinguished from each other on morphological grounds. They excluded the negative stain to a considerable degree and looked as if they had material within them. Typical electron micrographs of these fractions are shown in Plate I. The periphery of these particles typically exhibited a crenelated pattern. In contrast, the 170 S peak fractions contained predominantly empty phage heads, in the sense that they were penetrated by the stain, and showed the creasing and collapsing typical of such

structures. In contrast to the 240 S structures, the shell itself excluded the stain. The 170 S fractions contained some prohead particles, and the 240 S fractions some empty heads, due presumably to the cross-contamination of these two closely sedimenting distributions. Examples of the 170 S and 240 S particles derived from a gradient of 10^- lysate are shown in Plate II. The morphology of the particles seen in the 170 S fraction from all the lysates that yielded 170 S peaks was essentially the same, except for the presence of base-plates on 26^- empty heads. Both peaks were also present in the wild type gradient, and matched the above description. The dimensions of the 240 S particles were slightly smaller than those of the 170 S particles, but this was probably due to the flattening of the empty heads with respect to the proheads. The difference in sedimentation may well be due to the presence of material within the 240 S particles. Note that the lysates (1^-, 2^- and 3^-) which accumulated all their rapidly sedimenting radioactivity in the form of 240 S prohead particles also accumulate uncut DNA (preceding paper).

The two peaks from the 8^- gradient had considerably less radioactivity than the peaks from the other particle-containing lysates, and there was a corresponding dearth of particles. We therefore repeated the experiment, but using mutants that also contained an amber mutant in gene *13*, which extends phage synthesis past the usual lysis time, as described in Materials and Methods. This increases the yield of phage particles with respect to the background of cellular materials. Besides 8^- 13^-, the experiment also contained as controls, 3^- 13^-, 10^- 13^- and 13^- lysates. The distributions of radioactivity in the gradient centrifugations from these lysates was just like those shown in Figure 2, and the morphology of the particles present in the 3^- 13^-, 10^- 13^- and 13^- gradients were as described above.

The peaks from the gradient of this more concentrated 8^- preparation contained good numbers of what were clearly phage-related particles. The particles present in the 195 S front peak resembled prohead particles, in having a visible subunit structure in the edges, and containing some inner material (Plate III(b)). However, many of these particles were irregular in the distribution of stain within them. The 125 S peak fraction (Plate III(c)) contained spherical particles smaller than proheads, perhaps analogous to the petit λ particles found in phage λ-infected cells (Kemp *et al.*, 1968). These petit P22 particles, however, were rarely penetrated by the stain and had the round uncollapsed morphology of proheads, rather than empty heads. A second, less frequent, class of particles was present in the 125 S fraction. These were particles with the dimensions of proheads but fully penetrated by the stain. Such a particle can be seen in Plate III(c).

As noted in the preceding paper, we had occasionally seen spiral structures in 8^- lysates, but we were not certain that they were phage-related structures. In the sucrose gradient of the 8^- 13^- lysate described above, there was a broad peak of radioactivity in the 300 to 400 S region of the gradient. When we examined these fractions in the electron microscope we found large spiral structures (Plate III(a)). The shells or arms of the spiral structures often displayed a subunit pattern very similar to that seen in proheads. Fractions between 195 S and 300 S displayed less extensive spirals, including particles that looked like proheads whose edges overlapped rather than joined up. Spiral structures were rare in the equivalent regions of the gradients of the 3^- 13^-, 10^- 13^- and 13^- lysates. On one pair of grids that we examined particularly carefully, we counted both spirals and bacterial membrane vesicles, for reference; the ratio of spirals to membrane vesicles on the grid of the 300 S

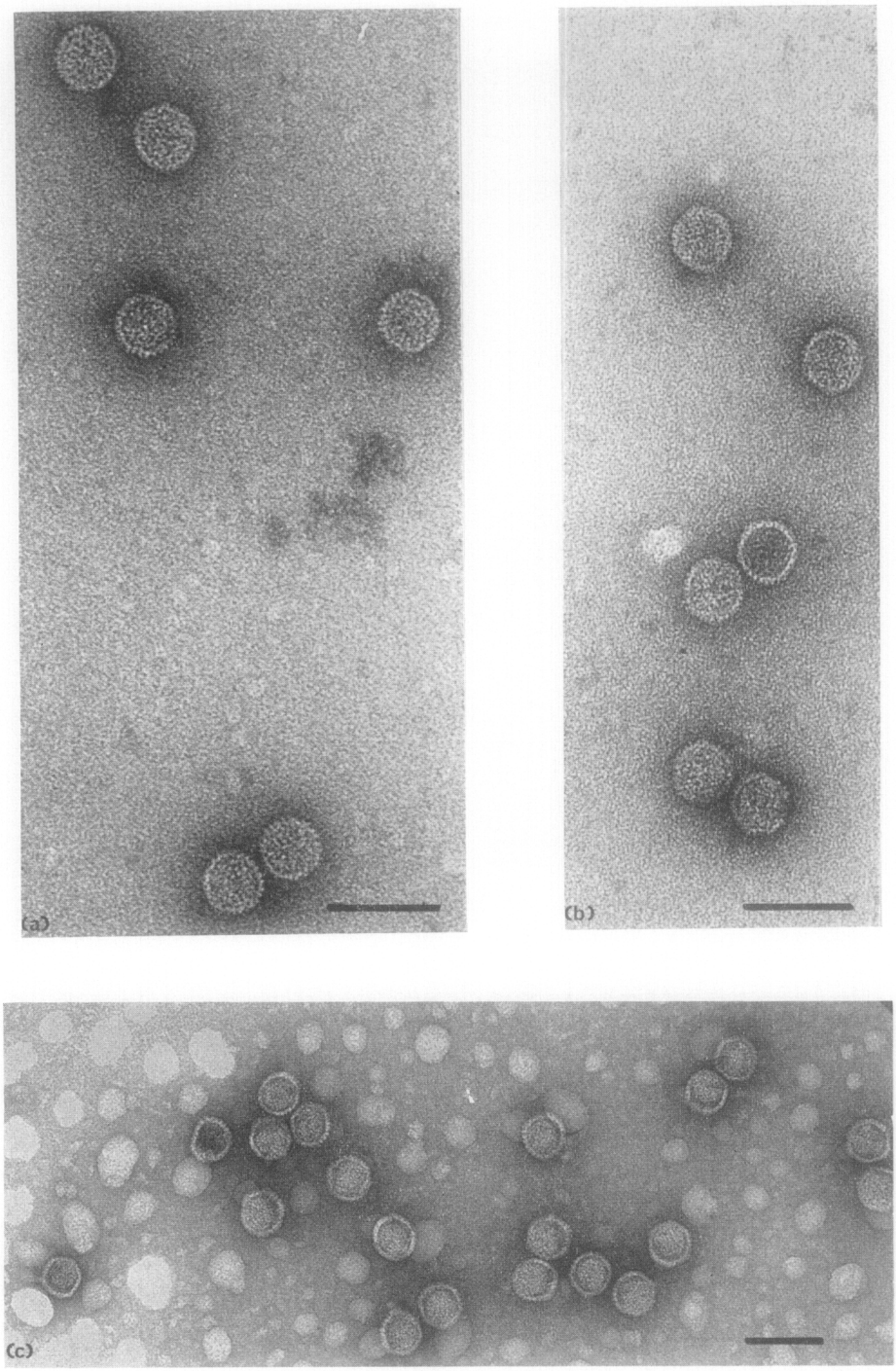

PLATE I. Particles in the 240 S fraction from 3⁻ lysate.

(a), (b) and (c) are typical fields of prohead particles isolated by sucrose gradient centrifugation from a 3⁻ lysate. Particles in the 240 S fractions from 1⁻ and 2⁻ lysates were not distinguishable by morphology from these particles. For direct comparison of proheads from the three lysates, see Plate II of the preceding paper. The particles in the 240 S fractions from wild type, 9⁻, 16⁻, 20⁻, 10⁻ and 26⁻ lysates were also similar to those above, except that they also contained some partially collapsed empty heads, presumably due to cross-contamination from the 170 S gradient fraction. Examples of 240 S proheads from 10⁻ lysate are shown in Plate II.

In thick stain the outer shell of the particles was more strongly differentiated from the inner material. In very thin stain the particles had a uniform density and it was difficult to distinguish an outer shell. The bar represents 100 nm. The two particles penetrated by stain represented a rare class.

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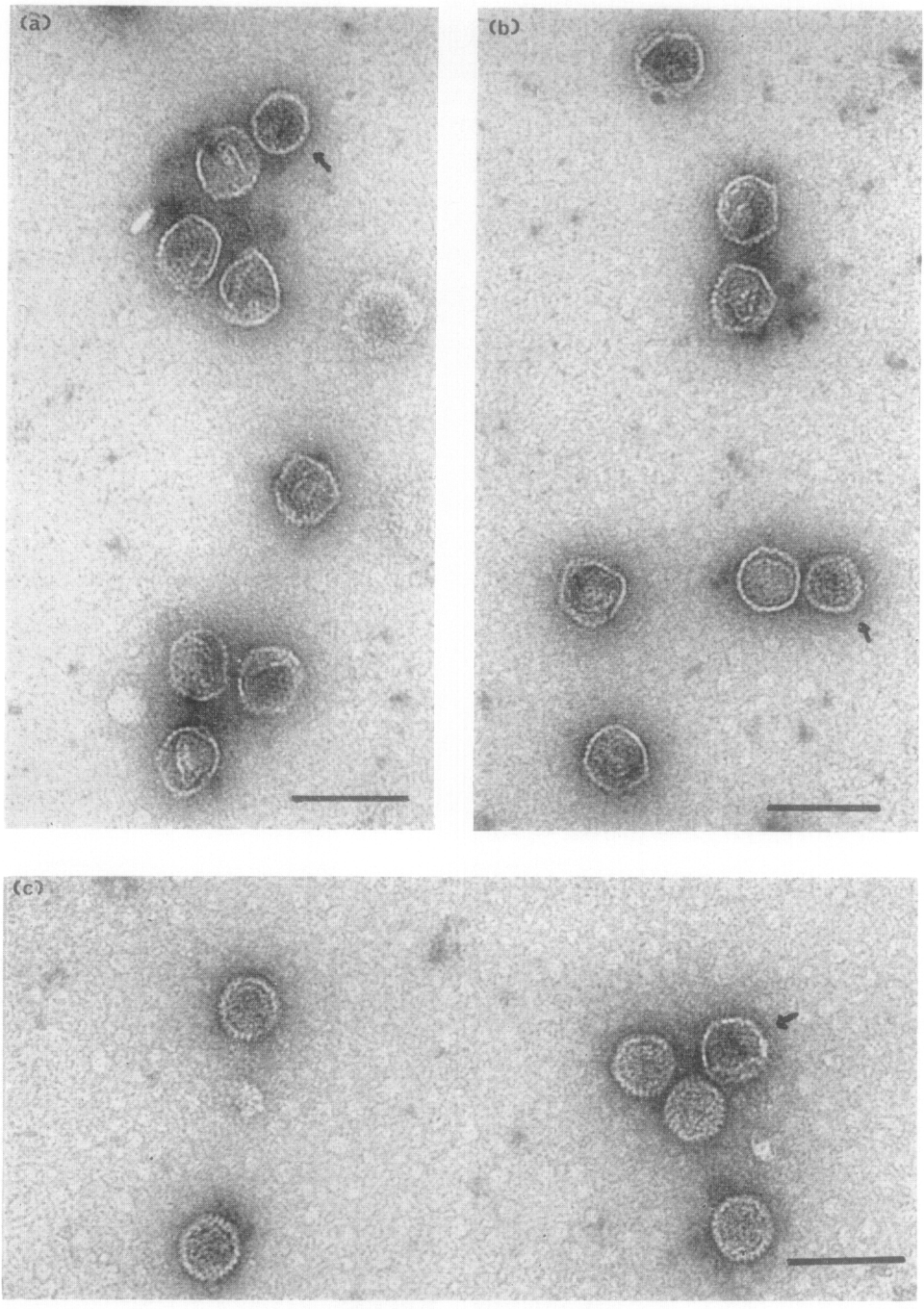

PLATE II. Particles from the 170 S and 240 S fractions of 10^- lysate.

(a) and (b) show typical fields of the 170 S empty particles isolated by sucrose gradient centrifugation from 10^- lysate. The particles are somewhat irregular and often show a crease, as if partially collapsed. Many of the particles look as if they have distinct corners. The arrows mark particles that we would identify as contaminating proheads. (c) Particles from the 240 S fraction from the same gradient. They are rounder and less penetrated by the stain. The marked particle is probably a contaminating empty head.

The 170 S fractions derived from other mutant lysates displayed particles with morphology similar to those shown here. The 170 S particles from 26^- lysate however, often had tails on them.

The bar represents 100 nm.

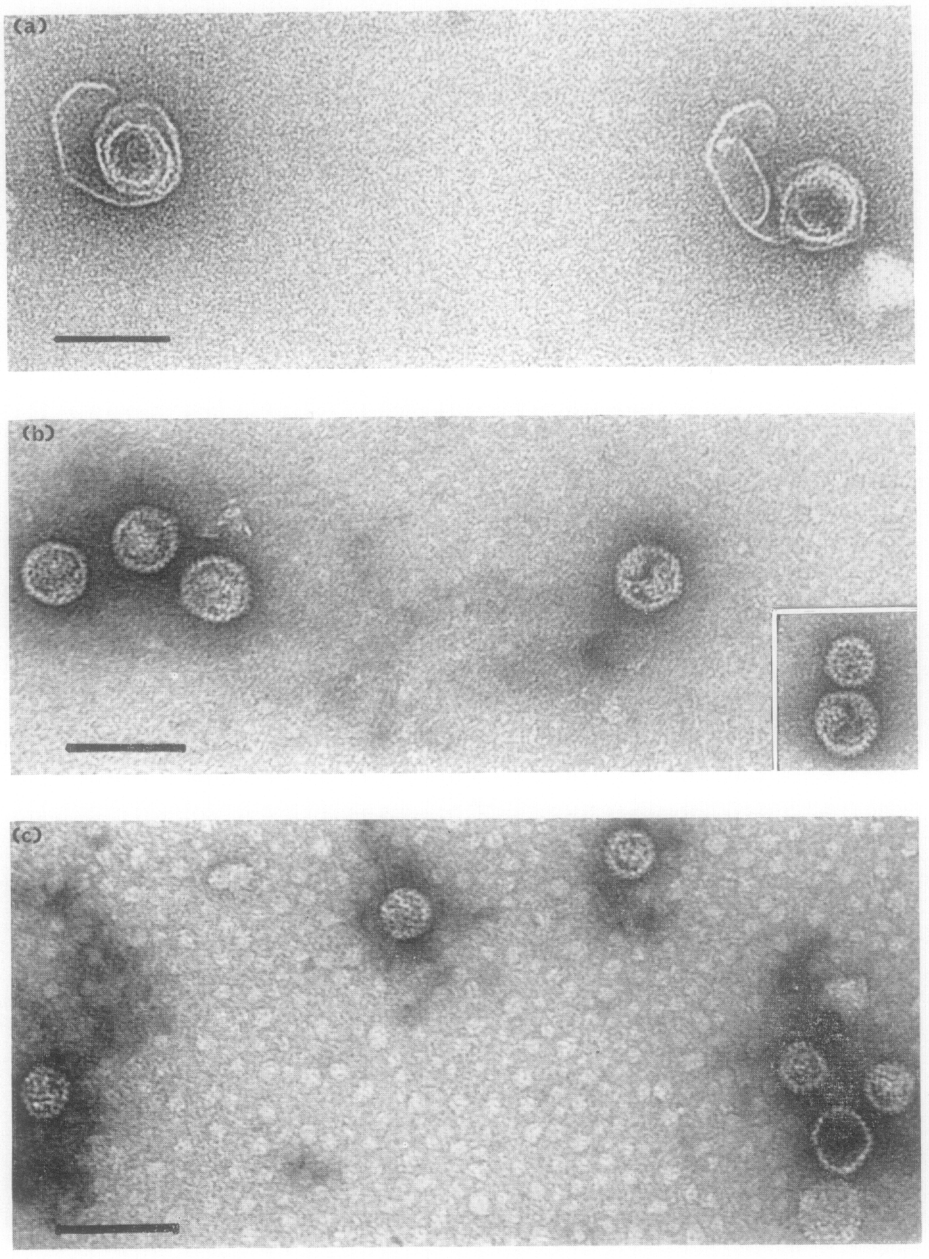

PLATE III. Particles isolated by sucrose gradient centrifugation from 8⁻ lysate.

(c) The petit particles found in the 125 S fractions. This fraction also contains particles with the same dimensions as proheads, but fully penetrated by the stain, one of which can be seen here. (b) Particles found in the 195 S fraction. These are of the same dimensions as proheads, but often show an irregular internal structure. (a) The large spiral structure found in the 300 S fraction. Fractions between 195 S and 300 S contained smaller spirals.

The petit particles and the spiral structures were rare in the equivalent fractions derived from other mutant lysates.

The bar represents 100 nm.

region of the 8⁻ 13⁻ gradient was 55 : 120; the ratio on the grid of the 300 S region of the 10⁻ 13⁻ gradient was 1 : 112.

After finding the petit particles and spirals in the fractions from 8⁻ lysates, we re-examined our original electron micrographs of unfractionated mutant and wild type lysates. We did occasionally find spirals and petit particles in wild type and mutant lysates, but they were very rare, except in the 8⁻ lysate. The normal assembly process is apparently quite efficient, as it is with T4 and λ (Kemp *et al.*, 1968; Laemmli *et al.*, 1970*a*). The absence of tubular structures is unusual and suggests that the major coat protein of P22 may have considerable form-determining specificity. (We show below that the structures from the 8⁻ lysate are composed of P5 only.) However, P8 clearly functions in forming a properly dimensioned head, since in its absence the P5 forms, in addition to low numbers of normal-sized particles, petit particles and aberrant particles that look as if one edge of a growing shell has missed the opposite edge and continued polymerizing around.

TABLE 1

Particles accumulating in phage P22 lysates

Lysate	DNA state	240 S Proheads	170 S Empty heads	500 S Full heads or phage
Wild type	Cut	+	+	+++
9⁻	Cut	+	+	+++
16⁻	Cut	+	+	+++
20⁻	Cut	+	+	+++
10⁻	Cut	+	+++	−
26⁻	Cut	+	+++	−
1⁻	Concatemer	+++	−	−
2⁻	Concatemer	+++	−	−
3⁻	Concatemer	+++	−	−
5⁻	Concatemer	−	−	−
		195 S Prohead-like	125 S Petit	300 S Spiral
8⁻	Concatemer	±	±	±

See Table 3 of the preceding paper for descriptions of the various gene products. +, Present; −, absent; +++, the major component.

(iii) *Protein composition of head-related structures*

The electron microscopic examination of gradient fractions show that the three major classes of structures distinguished by sedimentation (500 S, 240 S (peak A) and 170 S (peak B)) correspond to the major morphological classes; full particles, proheads and empty heads. To identify the proteins of the three classes of structures, we mixed the appropriate peak gradient fractions with sample buffer, dissociated the proteins by heating, and carried out electrophoresis on the resulting samples through 10% polyacrylamide gels in sodium dodecyl sulfate. The gels were then sliced longitudi-

nally, dried and applied to X-ray film. Densitometer tracings of appropriately exposed autoradiograms are presented in Figures 3 to 6.

The gel analysis of protein composition was done for each structure from all the different mutant lysates which accumulated that structure. We expected this to indicate which phage proteins can or cannot assemble in the absence of each of the known phage P22 gene products.

(1) Particles containing DNA. Figure 3 displays the gel patterns of the 500 S peaks, the DNA-containing particles, from the wild type, 9^-, 16^-, 20^- and 26^- lysates shown in Figure 1. The top panel displays the wild type gel pattern, with the five largest structural polypeptide chains identified. We are uncertain about the identification of the peaks in the lower molecular weight region of the gel; electron microscopy revealed some co-sedimenting membrane material in these gradients: these could contain labeled host protein. The 9^- particles lack only P9, the 16^- particles lack only P16 and the 20^- particles lack only P20. In the tracing of this particular film, the minor bands of the 26^- particles are not clearly visible, but longer exposures reveal that these particles contain the above-mentioned proteins.

(2) Proheads-240 S. Figures 4 and 5 display densitometer tracings of the gel patterns of the 240 S peaks. The top panel of Figure 4 is the wild type phage pattern for reference. The striking feature of the gel patterns is the presence of a major protein component that is absent in mature phage. This band has the same mobility as the band identified as P8 in whole lysates (preceding paper). The rapidly sedimenting material in the gradient of the 8^- lysate is totally lacking this band, confirming that the band in question is indeed P8. The 240 S structure derived from all the other mutant lysates, and from the wild type lysate, contains P8 as a major component. This 240 S structure is the only fast sedimenting structure accumulating in 1^-, 2^- and 3^- lysates. Measurement of the optical density of the P8 band and P5 band in these autoradiograms, and division by the appropriate molecular weights, gave a molar ratio of radioactivity for P8 : P5 of about 1 : 1 in the 240 S structure from 1^-, 2^- and 3^- lysates. Since labeling of P8 and P5 may not be uniform, this estimate cannot be taken as definitive.

In addition to P8 and P5, the 240 S structures from wild type, 3^- and 2^- lysates also contain P1, P16 and P20, but not P9. However, as expected, the 240 S structure from 1^- lysate lacked P1, the 240 S structure from 20^- lysate lacked P20, and the 240 S peak from the 16^- lysate lacked P16, but contained the other bands. We are not certain about the presence or absence of the smaller proteins, PX and P26.

The results show that a structure containing P8 and P5 as major components, and sedimenting around 240 S can form in the absence of P1, P16, P20 and P9. This structure does not form in the absence of P5. The electron microscope analysis suggests that the P5 in 8^- lysates forms fast-sedimenting structures with a range of dimensions, many of which are not in fact closed shells. These structures did not contain any of the minor proteins which were found in the structures accumulating in other mutant lysates. The observations suggest that in the absence of P8, P5 polymerizes into aberrant aggregates.

(3) Empty heads-170 S. The 240 S prohead peak from all the mutants examined contained both P5 and P8, plus minor proteins. On the other hand, as shown in Figure 6, the 170 S empty heads never exhibited P8 regardless of the nature of the lysate. This is consistent with its empty appearance in the electron microscope. We are uncertain about the disposition of the minor high molecular weight proteins

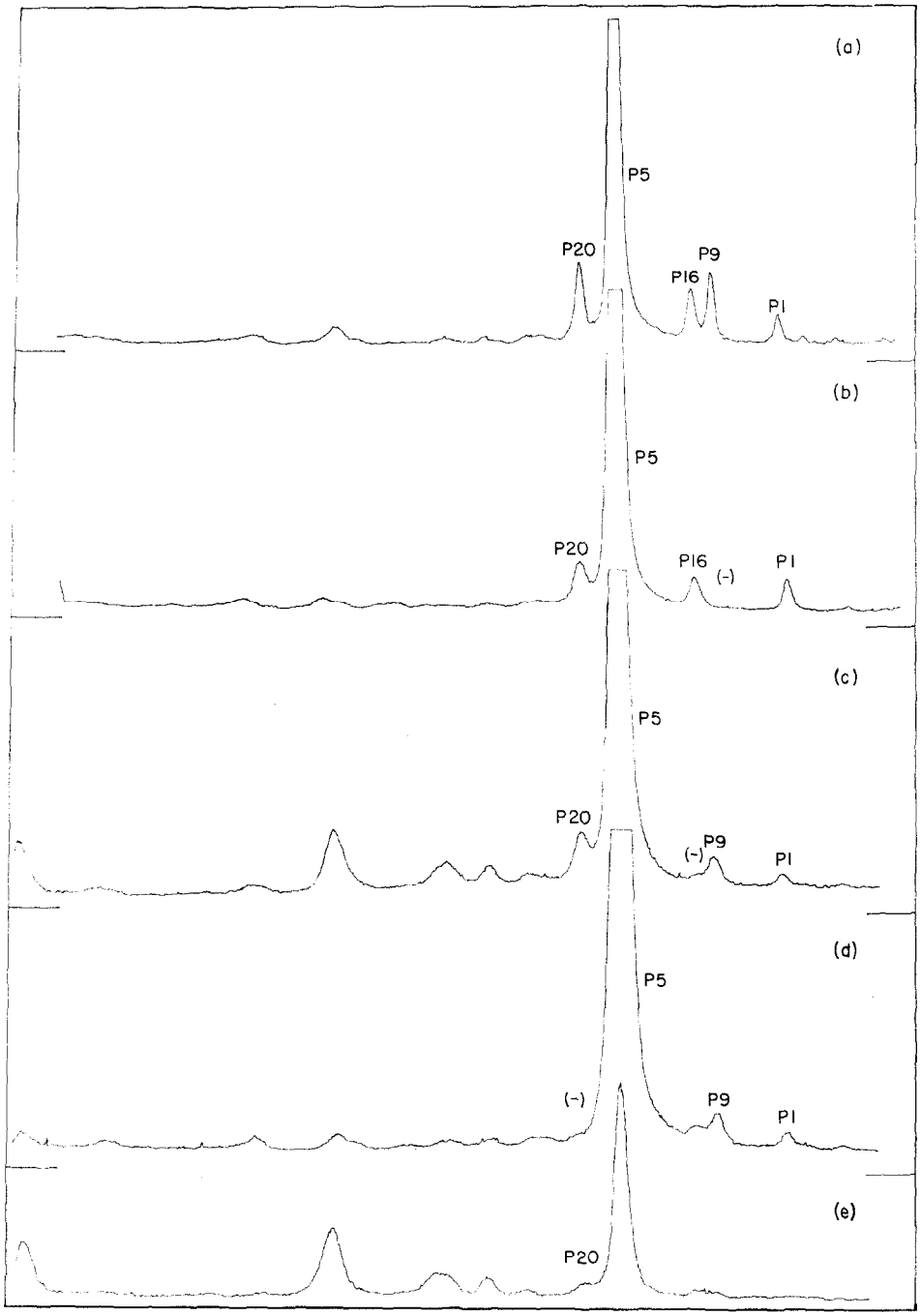

FIG. 3. Sodium dodecyl sulfate gel electrophoresis of the 500 S peak fractions from sucrose gradients of mutant and wild type lysates. Peak fractions from the gradients shown in Fig. 1 were mixed with sample buffer and heated to 100°C to dissociate the proteins. Samples (0·1 ml) were analyzed by electrophoresis through cylindrical 10% polyacrylamide gels. The gels were fixed, sliced and dried, and then applied to X-ray film for autoradiography. Tracings of appropriately exposed films were made with a microdensitometer. The tracings are of a single film to facilitate comparison between gels. Since the 500 S fraction from the 26^- lysate contained much less radioactivity than the other samples, we could not be sure if the minor bands were present. We are also uncertain of the identity of the band present in increased amount in the lower molecular weight region of the gels of the 16^- and 26^- particles.

The positions of missing bands of interest are indicated by (—). (a) Wild type; (b) 9^-; (c) 16^-; (d) 20^-; (e) 26^-.

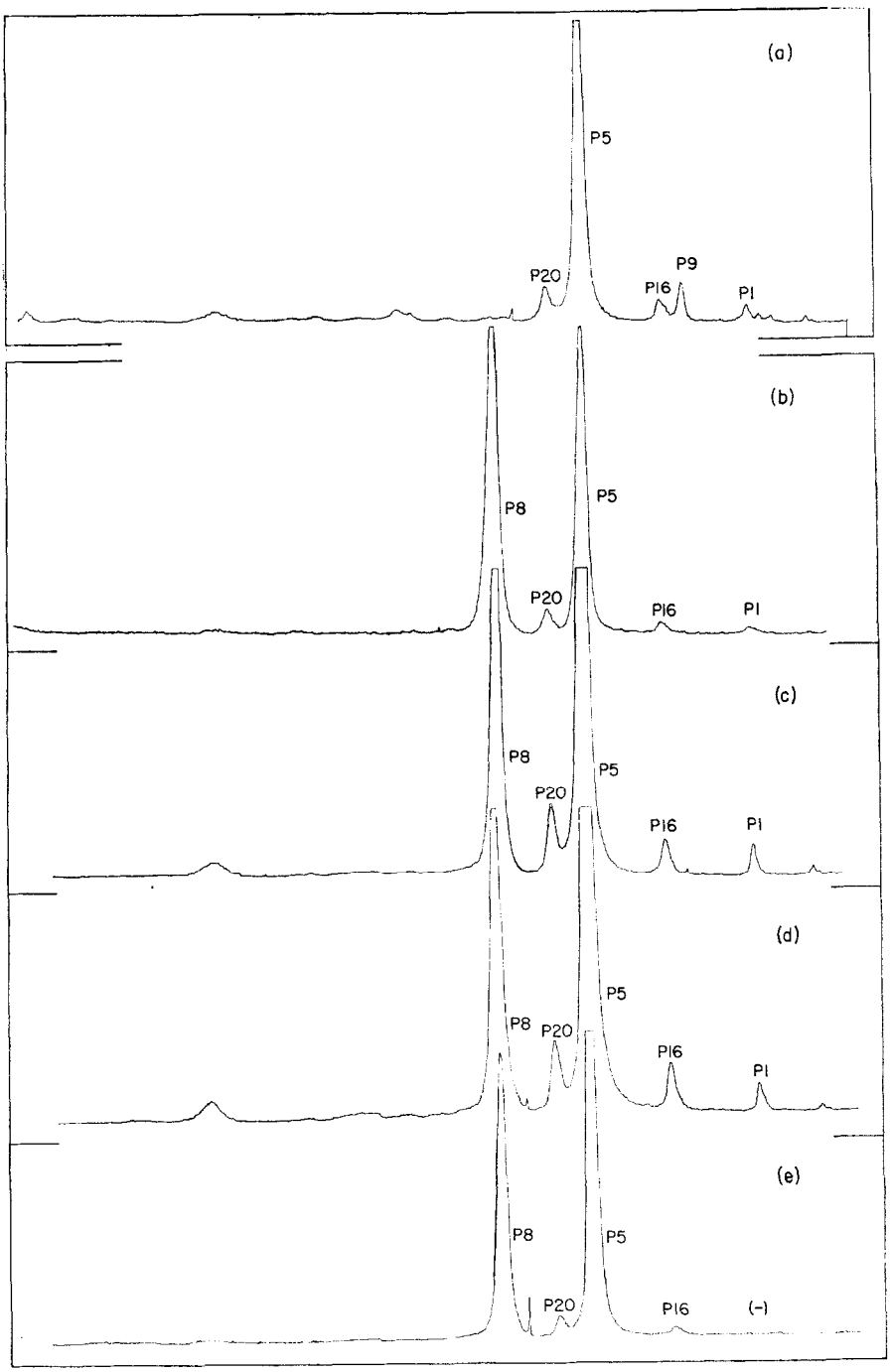

FIG. 4. Sodium dodecyl sulfate gel electrophoresis of 240 S peak fractions from sucrose gradients of mutant and wild type lysates. Experimental details as in the legend to Fig. 3. (a) 500 S wild type phage included in the electrophoresis as a reference sample. (b) Wild type 240 S; (c) 3^- 240 S; (d) 2^- 240 S; (e) 1^- 240 S; note the large P8 band in the prohead fractions. Samples are from gradients shown in Fig. 2. The 240 S peaks from the remaining gradients are shown in the next Figure. Note that (b) shows the 240 S peak from the same wild type lysate from which the top phage sample was derived.

The ratios of the optical density under the P5 peaks to that under the P8 peak gave a molar ratio for P5 : P8 of 1 : 1.

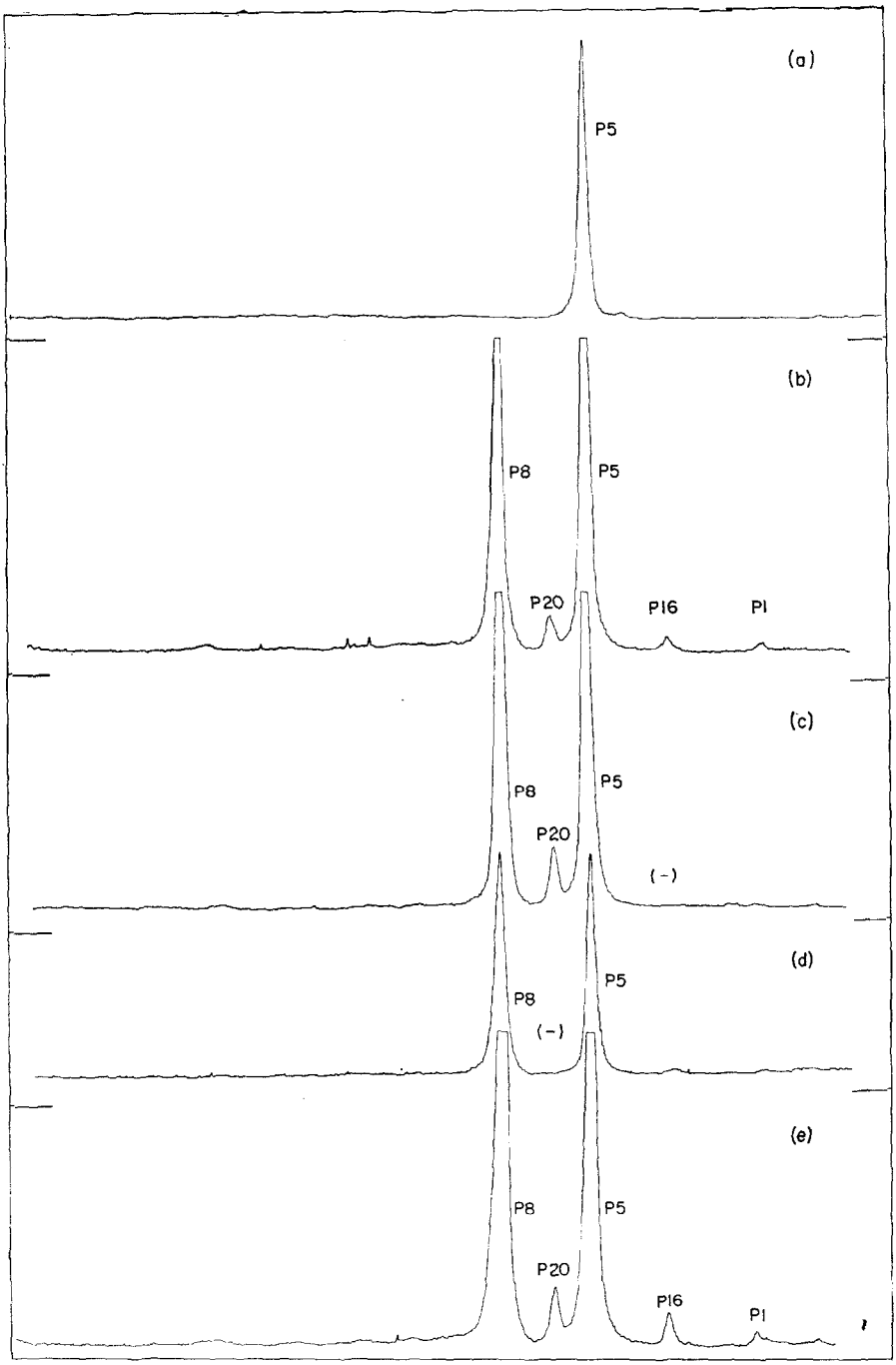

FIG. 5. Sodium dodecyl sulfate gel electrophoresis of 240 S peak fractions from gradients of mutant lysates, and of the 195 S peak from the 8$^-$ lysate. (a) 8$^-$ peak; (b) 10$^-$ peak; (c) 16$^-$ peak; (d) 20$^-$ peak; (e) 26$^-$ peak.

The proteins are from the 240 S peak fractions of the remaining gradients of Fig. 2. The strong P8 band is present in the 240 S peaks derived from the wild type lysate (Fig. 4) and all the mutant lysates, except for the 8$^-$ lysate, as can be seen in (a).

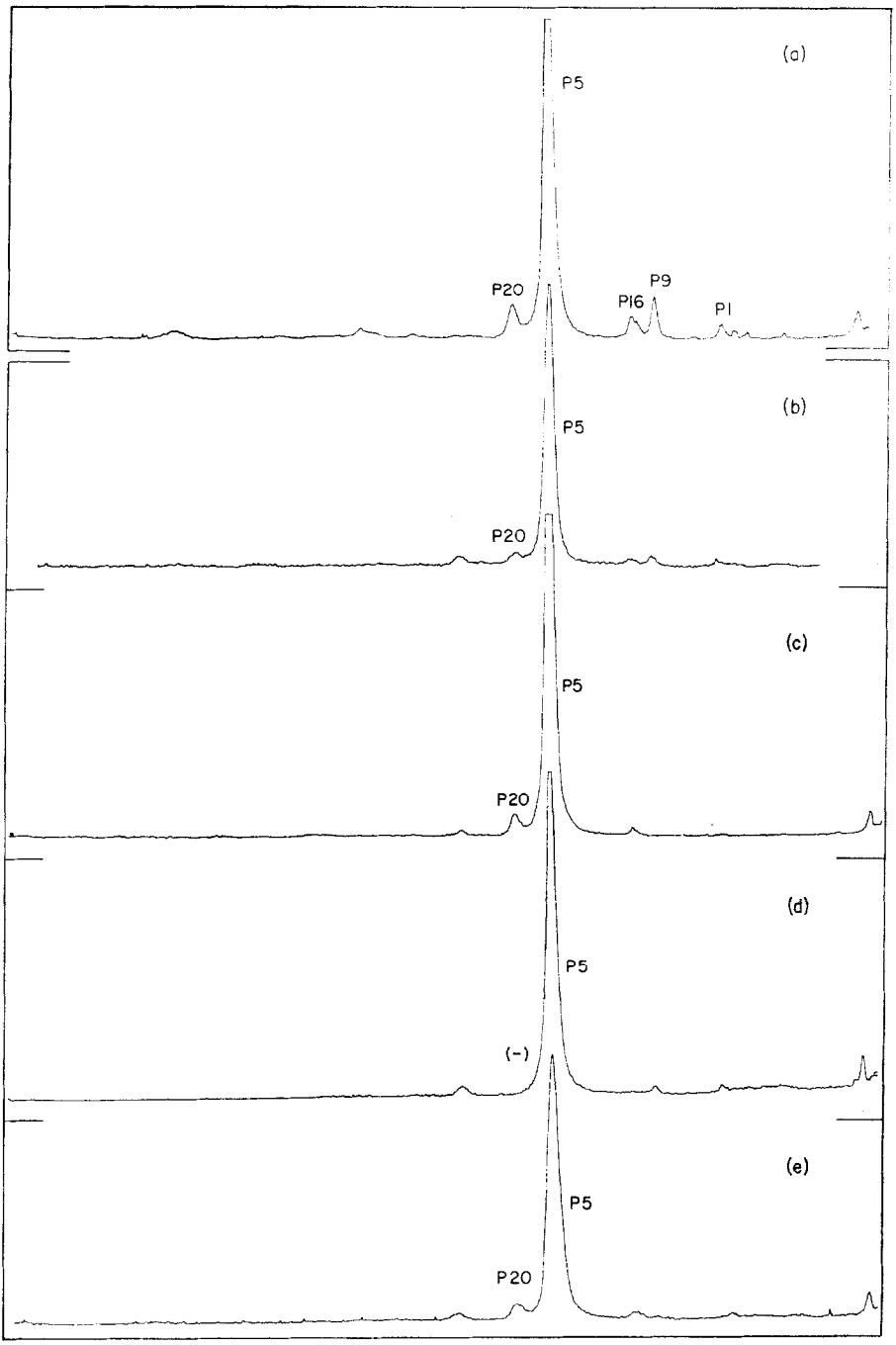

FIG. 6. Sodium dodecyl sulfate gel electrophoresis of the 170 S peak fractions from sucrose gradients of mutant lysates. (a) The labeled proteins of complete wild type phage particles for reference. (b) Wild type 170 S peak; (c) 10^- peak; (d) 20^- peak; (e) 26^- peak. The 9^- and 16^- 170 S peaks were similar to those shown, except that the 16^- peak lacked P16, as expected. However, none of the 170 S peak fractions displayed clearcut P16 bands. The peak fractions from the 8^- lysate contained only P5.

P1, P9 and P16 in these empty particles. We suspect that these empty particles are derived from filled, unstable heads. Some of these proteins may be incorporated into the particles, but may be lost with the DNA.

None of the particles we were able to identify in mutant or wild-type lysates contained P2. Since P2 is easily identified in whole lysates, this indicates that P2 is not directly assembled into particles, though it clearly is essential for the formation of the mature DNA-containing particle (preceding paper).

Since we were unable to identify P3 in lysates, we do not know if it has a structural role in the particle assembly process. However, the fact that it accumulates 240 S prohead particles and uncut DNA suggests that it too is involved in the packaging step.

TABLE 2

Protein composition of structures from phage P22-infected cells

Lysate	Structure	P9	P26	P1	P16	P20	P5	P8
Wild type	Phage	+	+	+	+	+	+	−
	Empty heads	+	−	+	+	+	+	−
	Proheads	−	−	+	+	+	+	+
9⁻	Heads	−	+	+	+	+	+	−
	Empty heads	−	−	+	+	+	+	−
	Proheads	−	−	+	+	+	+	+
16⁻	Defective phage	+	+	+	−	+	+	−
	Empty heads	−	−	+	−	+	+	−
	Proheads	−	−	+	−	+	+	+
20⁻	Defective phage	+	+	+	+	−	+	−
	Empty heads	−	−	+	+	−	+	−
	Proheads	−	−	+	+	−	+	+
10⁻	Empty heads	−	−	+	+	+	+	−
	Proheads	−	−	+	+	+	+	+
26⁻	Empty heads	+	−	+	+	+	+	−
	Proheads	−	−	+	+	+	+	+
1⁻	Proheads	−	−	−	+	+	+	+
2⁻	Proheads	−	−	+	+	+	+	+
3⁻	Proheads	−	−	+	+	+	+	+
8⁻	aberrant	−	−	−	−	−	+	−

P9 is the base-plate protein, P5 is the major coat protein and P8 is the scaffolding protein.

We have not yet characterized these structures in terms of residual DNA content. They clearly do not contain a substantial amount of DNA, as evidenced both by their sedimentation rate and their appearance in the electron microscope. However, this does not exclude the possibility that a small piece of DNA, such as is found in T4 49⁻ heads (Luftig *et al.*, 1971), is still retained. We have done experiments with and without DNase in the lysis mixture; this treatment had no effect on the sedimentation of the mutant structures.

The results of the characterization of the particles accumulating in P22 lysates are summarized in Table 2.

(b) *Pulse–chase experiments with wild type infected cells*

(i) *Precursor particles*

The presence of the head-related 240 S and 170 S structures in wild type lysates suggested that these particles might represent, or might be derived from, true intermediates in phage head assembly. The fact that 1^-, 2^- and 3^- lysates accumulated the P8-containing prohead structures, but not empty heads, also suggested that the proheads might be precursors to empty heads.

To find out the relation of the P8-containing structures and the empty structures to the normal encapsulation process, we carried out pulse–chase experiments with phage-infected cells.

For this purpose we used phage carrying an amber mutant in gene *13*, which results in continued phage synthesis past the normal lysis time. This avoids problems of premature cell lysis, and allowed us to label during a time when phage formation was

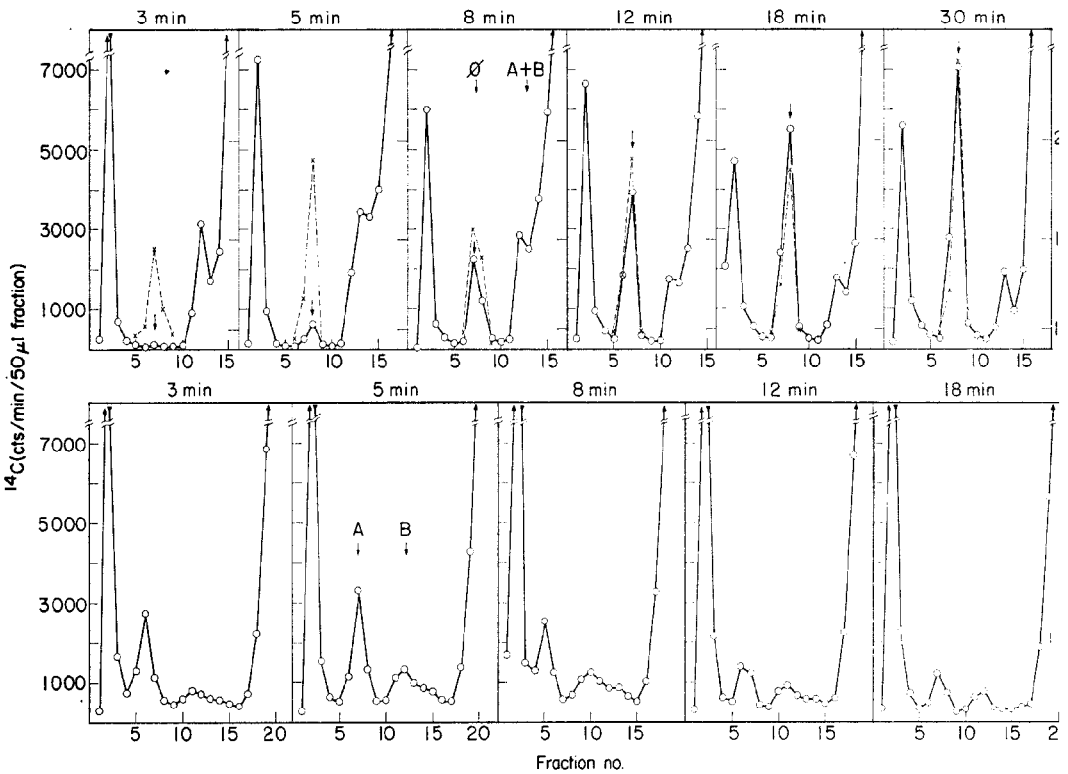

FIG. 7. Appearance of labeled proteins in phage, proheads (peak A) and empty heads (peak B) after a short pulse of radioactivity. The top series of sucrose gradients displays the appearance of radioactivity in phage particles isolated from samples lysed at various times after a pulse of radioactivity. The times shown are minutes after the addition of radioactivity. The bottom series of gradients are the same samples centrifuged at high speed to resolve the A and B peaks.

The P22-infected cells incubating at 37°C were exposed to a pulse of ^{14}C-labeled amino acids from 60 to 63 min after infection, as described in Materials and Methods. At various times afterward samples were taken, chloroform added and shaken vigorously to lyse the cells. These samples were left at room temperature until the last sample was taken at 90 min, at which time 0·2-ml samples were layered on the 2 sets of sucrose gradients. Samples were taken for scintillation counting, and fractions were also assayed for viable phage (– – × – – × – –).

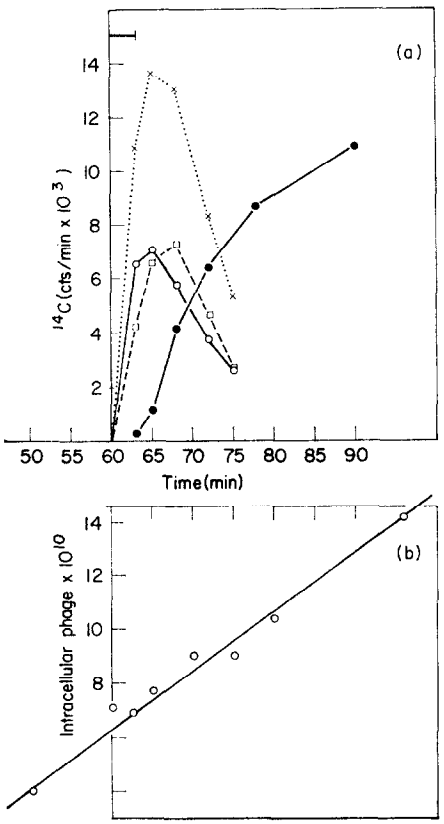

FIG. 8. Kinetics of the incorporation of label into proheads (—○—○—), empty heads (--□--□--) and phage at 37°C (—●—●—); sum of proheads plus empty heads (. . . × . . . × . . .). (a) Data from the gradients of the pulse–chase experiment shown in the previous Figure are summarized here. The radioactivity in the fractions under the various peaks was summed to give the total radioactivity under each peak for each time point. (b) The formation of total viable phage, determined by plating, during the course of the experiment.

proceeding linearly. As before, the phage was also *c*1, to ensure entry into the lytic growth cycle upon infection. The infected cells were exposed to a pulse of ^{14}C-labeled amino acids and at various times afterwards samples were withdrawn and lysed immediately by shaking vigorously with chloroform. The samples were then analyzed by centrifugation through sucrose gradients. After chloroform lysis, samples were kept at room temperature until the last time-points were taken, and then centrifuged through sucrose gradients to separate the structures present. Two gradients were run for each time-point, as in the previous experiments. These are shown in Figure 7. The upper panels show the appearance of radioactivity in complete particles. The broken line shows the viable phage, as determined by plating, and the solid line shows the appearance of radioactivity in the particles.

The lower series of graphs shows the results of centrifugation in which the two slowly sedimenting peaks are resolved. Incubating the sample with DNase did not change the distribution of radioactivity, nor did storage for a few days in the cold. The kinetics of the labeling of the various peaks is summarized in Figure 8. The lower panel shows the increase in viable phage during the course of the experiment. Radioactivity

appears almost immediately in both peaks A and B. The radioactivity in these structures then decreases, and subsequently appears in phage, indicating that proheads and empty heads are either precursors of phage or are derived from such precursors. The delay in the labeling of empty heads suggests that these structures might be derived from a later intermediate in the assembly process than proheads.

In the hope of separating more clearly the labeling of the various structures we carried out a similar pulse–chase experiment at 25°C. The kinetics of labeling of peaks A, B and phage are shown in Figure 9. The lower panel shows the incorporation of ^{14}C-labeled amino acid into trichloroacetic acid-insoluble material, and the open circles show the formation of viable phage during the same time period. These data are presented on the same time scale as Figure 8 to facilitate comparison. The labeling of all three structures of interest is slowed by about a factor of two. The initial labeling of proheads is better demarcated in this experiment, but the difference between the pattern of labeling of proheads and empty heads is even less than at 37°C.

The experiments strongly suggest that a 240 S prohead is a precursor in phage assembly. The status of the 170 S empty head is less certain.

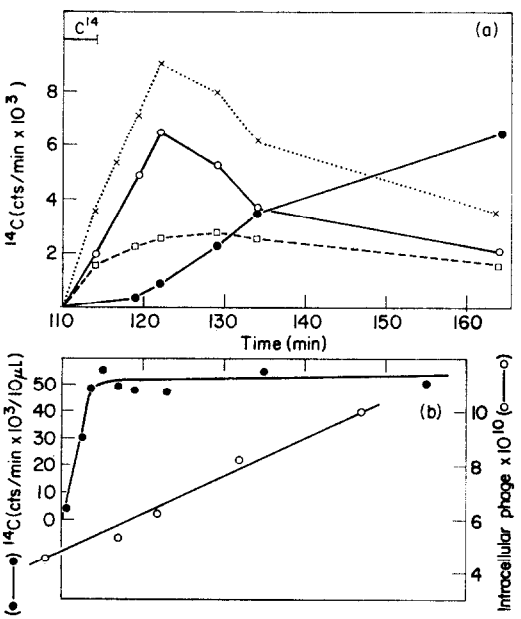

FIG. 9. Kinetics of phage head assembly at 25°C. A pulse–chase experiment was carried out as described in the legend to Fig. 7, except that the infection was at 25°C and the labeling and sampling times were altered accordingly. The graph shows the amounts of radioactivity under the various peaks from the gradients of the pulse–chase samples. —○—○—, Proheads; --□--□--, empty heads; —●—●—, phage; . . . × . . . × . . ., summed data. (b) The formation of viable phage, and also the total incorporation of label. The data have been been plotted on the same horizontal scale of Fig. 8, for ease of comparison.

(ii) *Kinetic analysis of the morphogenetic proteins during head assembly*

To identify directly the proteins involved in precursor structures, we analyzed the radioactive proteins in the peak, supernatant and pellet (shelf) sucrose gradient fractions from the pulse–chase experiments of Figures 8 and 9. Figure 10 shows tracings of the autoradiograms from sodium dodecyl sulfate gel electrophoresis of

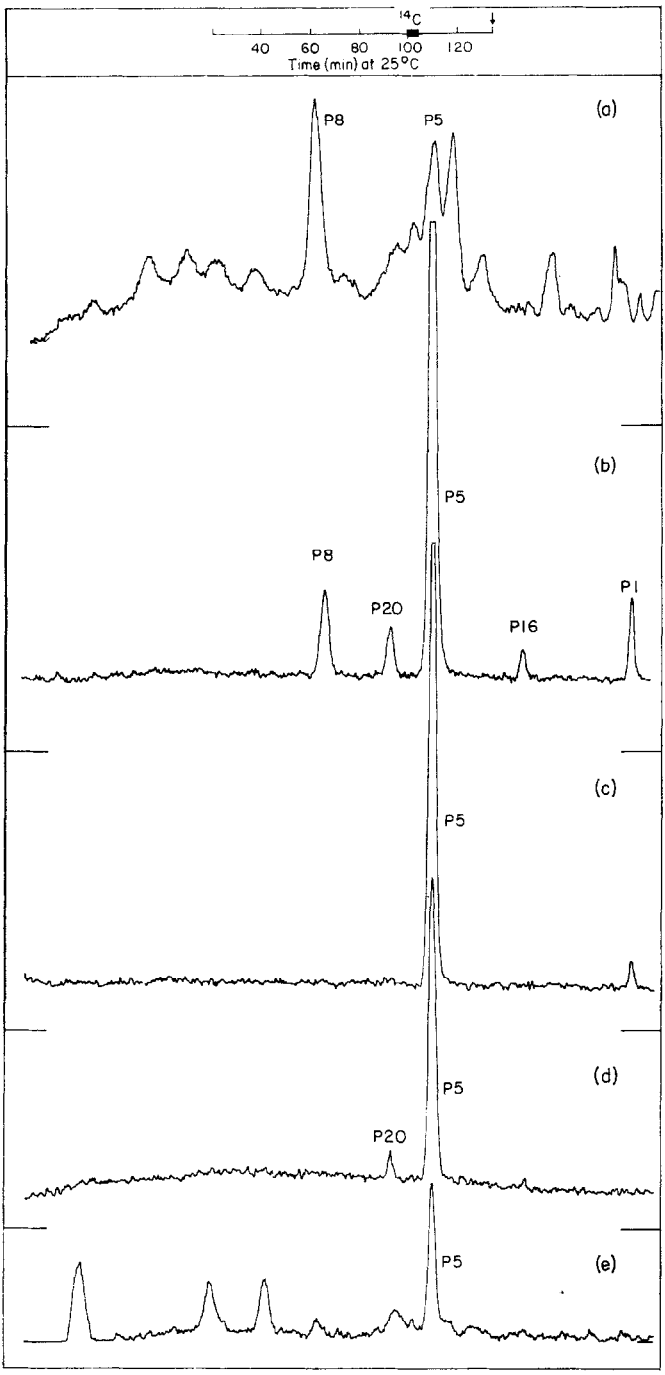

FIG. 10. Identification of pulse-labeled proteins in proheads (peak A), empty heads (peak B) and phage. The tracings are sodium dodecyl sulfate/acrylamide gel autoradiograms of sucrose gradient fractions. The fractions are from the pulse–chase experiment summarized in Fig. 9. Samples of the pellet (shelf), phage, A, B and supernatant fractions were heated with sample buffer and analyzed by electrophoresis through a 10% polyacrylamide slab gel. The pellet fraction was from the low-speed centrifugation and the supernatant fraction was from the high-speed centrifugation. This tracing is of fractions derived from the sample taken 19 min after the addition of radioactivity to the infected cells (Fig. 9). (a) Supernatant; (b) proheads (peak A); (c) empty heads (peak B); (d) phage; (e) pellet.

one such set of fractions, from a sample taken 15 minutes after the chase of the label.

The upper tracing shows the labeled proteins present at the top of the gradient (faster centrifugation). The second tracing shows the proteins present in peak A; P8 is clearly present in addition to P5, the coat protein. The proheads in this fraction also contain P1, P16 and P20. The pattern in fact is very similar in protein composition to the 240 S proheads that accumulate in 2^- and 3^- lysates. The only significant difference is the decrease in the amount of labeled P8 with respect to P5; this difference will be considered in the Discussion. Peak B, the empty heads, lacked P8, thus resembling the empty heads accumulating in 10^- and 26^- mutant lysates.

The phage sample analyzed in Figure 10 did not yet contain enough radioactivity for the minor bands to be clearly visible. Phage fractions from samples taken later after the pulse showed the expected additional bands, P1, P9 and P16.

Very little P5 and P8 was found in the pellet fraction of the gradient (bottom tracing). The majority of the P5 is incorporated into A and B head structures and phage: in contrast, P8 is divided between the prohead A structure and the supernatant fractions from the top of the gradient.

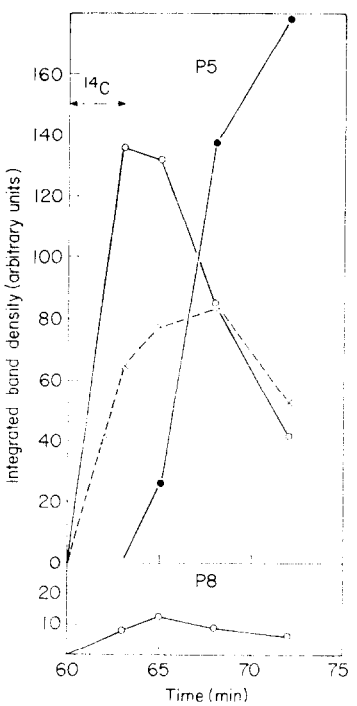

FIG. 11. Kinetics of P5 and P8 assembly at 37°C. Samples of the peak fractions from the sucrose gradients of the 37°C pulse–chase experiment (Figs 7 and 8) were analyzed by gel electrophoresis as described in the legend to Fig. 10. The P5 and P8 peaks were cut from the tracings of the gel autoradiograms and weighed.

The passage of P5 through intermediates in head assembly is shown in the upper panel; the passage of P8 into proheads is in the lower panel. Though plotted separately for clarity, P5 and P8 share the same ordinate scale. No labeled P8 was detected in empty heads or phage. A small amount of P5 appears in the pellet fractions, but this did not change significantly in samples taken at different times after the chase.

—○—○—, P5 or P8 radioactivity in prohead fractions (peak A; 240 S); – – × – – × – –, P5 radioactivity in empty head fractions (peak B; 170 S); —●—●—, P5 radioactivity in phage fractions (500 S).

Gel analyses, such as those shown in Figure 10, were done on the various fractions derived from lysate samples taken at various times after the chase of radioactivity. Peaks from the tracings were cut out and weighed to quantitate the amount of radioactive P5 and P8 in the relevant fractions. These data from the 37°C pulse–chase experiment are summarized in Figure 11. The top panel shows the time-course of appearance of pulse-labeled P5 in proheads, empty heads and phage. The bottom panel shows the appearance of P8 in proheads. P5 clearly passes through a 240 S stage before entering phage. About four minutes is required for this process at 37°C. We are less certain about the status of the 170 S B structure. The data are consistent with the idea that proheads fill with DNA rapidly after their assembly, forming unstable filled heads. Before maturation into phage, some fraction of these lose their DNA either intracellularly or on lysis, yielding the 170 S empty heads (preceding paper).

Similar data on the transit of pulse-labeled P5 and P8 through the various intermediate structures is summarized in Figure 12, for the experiment (Fig. 9) carried out at 25°C. The results are similar to the 37°C experiment. The lag between the synthesis of P5 and its appearance in mature phage is about 12 minutes.

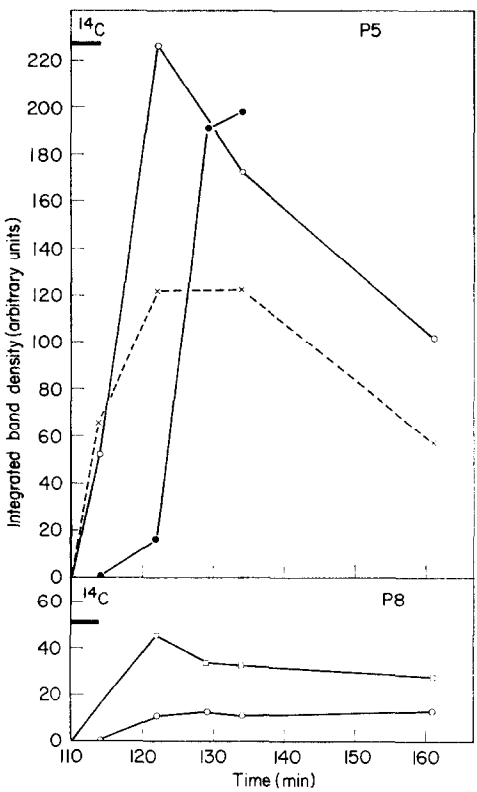

FIG. 12. Kinetics of P5 and P8 assembly at 25°C. Summary of the gel analysis of the pulse-labeled fractions from the 25°C experiment (Figs 9 and 10). The passage of P5 into phage is shown in the top panel, and of P8 into proheads in the bottom panel.

—○—○—, P5 or P8 radioactivity in prohead fractions (peak A; 240 S); --×--×--, P5 radioactivity in empty head fractions (peak B; 170 S); —●—●—, P5 radioactivity in phage fractions (500 S); —□—□—, P8 radioactivity in supernatant.

(iii) *Evidence that P8 may be re-used*

The kinetic behavior of P8 (Figs 11 and 12, bottom panels) is quite different from that of P5; pulse-labeled P8 slowly enters the prohead structures, and chases slowly or not at all. The latter is not surprising, since P8 is not present at all in mature phage. Still, after losing its association with the pulse-labeled P5, which has become phage, we would expect the pulse-labeled P8 to appear either in the supernatant or to sediment differently from proheads. The 25°C experiments (Fig. 12), in which we quantitated the appearance of P8 in both prohead and supernatant (top) fractions, shows clearly that P8 does not chase out of proheads into the supernatant. Two possibilities that could account for these results are: (1) after dissociation from P5, P8 gets trapped in non-functional 240 S aggregates, or (2) P8 recycles during morphogenesis and takes part in many rounds of head assembly.

(c) *Temperature shift experiments*

The prohead intermediates in the pulse–chase experiments were indistinguishable from the proheads accumulating in 2^- and 3^- mutant lysates. This suggested that the structures from the mutant-infected cells were true precursors in phage assembly. To test this idea we carried out a temperature shift experiment with a temperature-sensitive mutant in gene *3* (*ts*3.1). This mutant is thermoreversible; upon shifting infected cells from restrictive to permissive temperature, phage formation commences immediately, even in the presence of inhibitors of protein synthesis (Jarvik & Botstein, 1973). By exposing the cells to a pulse of radioactivity during growth at restrictive temperature we labeled the 3^- proheads, and then determined whether after shift to permissive temperature the radioactivity transferred to mature phage (Luftig *et al.*, 1971).

The protocol of this experiment was similar to the pulse–chase experiment (Fig. 8). (The temperature-sensitive mutant also carried a *c*1 mutation to ensure lytic infection, and an amber mutant in gene *13*, to extend phage synthesis.) Cells were infected at high temperature and incubated with ^{14}C-labeled amino acids from 25 to 35 minutes, at which time the label was chased with excess unlabeled amino acids. The cultures were incubated further for 20 minutes at high temperature, to allow the newly synthesized labeled proteins to accumulate in proheads. At 55 minutes the cultures were shifted to the permissive temperature. Samples were taken before and after the temperature shift, lysed with chloroform, and centrifuged through sucrose gradients to determine the fate of the label.

The results of the experiment are shown in Figure 13; the high-speed centrifugations (to resolve proheads) are shown at the right, the low-speed centrifugations, for phage, are to the left. The upper group of panels displays the distribution of label in the wild type and temperature-sensitive infections before the temperature shift, while the lower group displays the distribution of label in the lysates after the temperature shift. The wild type pattern is unchanged after the temperature shift, since the assembly of the labeled protein into phage is completed before the shift. In contrast, at high temperature the 3^- temperature-sensitive lysate accumulates only 240 S proheads, and after shifting to low temperature, the radioactivity from these proheads is converted to 170 S empty heads and to phage particles.

To follow the course of the individual proteins in this experiment we analyzed the peak fractions from the gradients by sodium dodecyl sulfate gel electrophoresis.

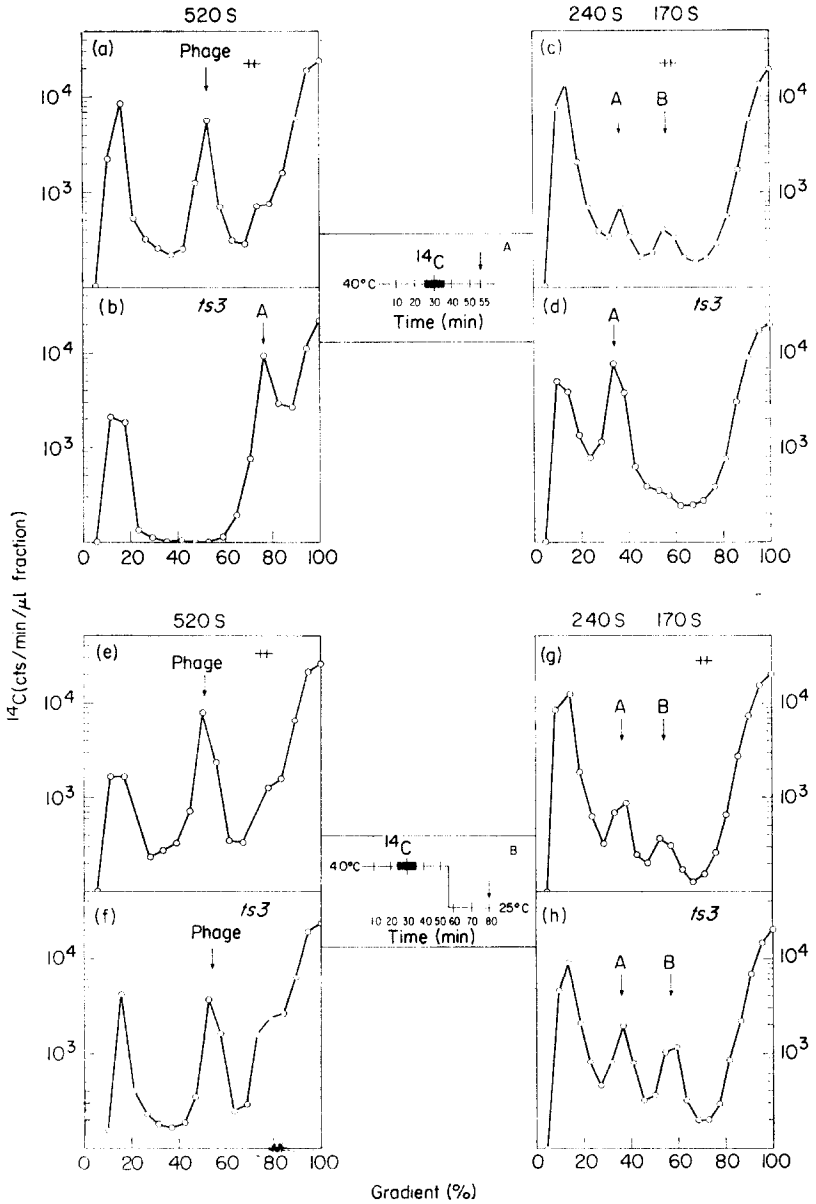

FIG. 13. Pulse–chase temperature shift experiment with a temperature-sensitive mutant in gene *3*: sucrose gradient analysis. A culture infected with 3^- (temperature-sensitive) 13^-, and the other with 13^- control phage (++), were labeled at high temperature and then shifted to low temperature as described in Materials and Methods. Samples were taken before the temperature shift (a) to (d) and after the shift (e) to (h), and lysed with chloroform before centrifugation through sucrose gradients. The left-hand gradients are the low-speed centrifugations for the separation of particles, and the right gradients are the high-speed centrifugation for separating structures A and B.

The viable phage titers of the lysates were as follows:

	Before shift	After shift
Control	$2{\cdot}3 \times 10^{10}$	$5{\cdot}4 \times 10^{10}$
*ts*3.1	$1{\cdot}0 \times 10^{6}$	$2{\cdot}3 \times 10^{10}$

The portion of the temperature-sensitive culture left at high temperature for the duration of the experiment had $1{\cdot}6 \times 10^{6}$ phage/ml.

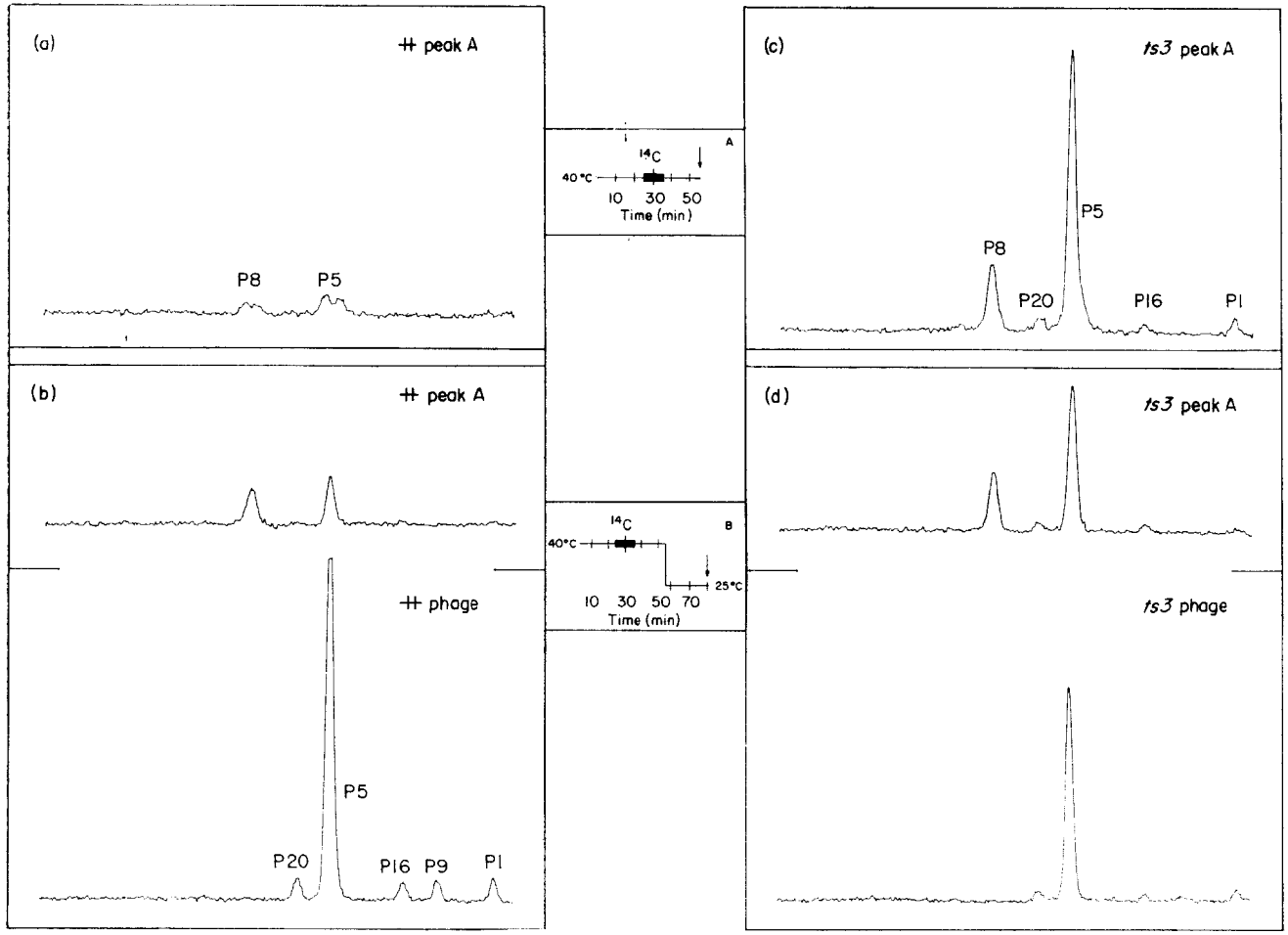

FIG. 14. (*See facing page for legend.*)

Tracings of some of the autoradiograms of these gels are shown in Figure 14; the right-hand gels are of 3⁻ temperature-sensitive samples, the left-hand gels are of samples derived from the wild type lysate. As before, the upper panels represent samples taken before the temperature shift, and the lower panels represent samples taken after the temperature shift. Note the similarity in protein composition between the 3⁻ temperature-sensitive proheads, and the proheads from the wild type pulse–chase experiment shown in Figure 10. The prohead fraction from the wild type infection in this experiment shows very little label, since there has been a 20-minute chase at 40°C.

The main feature of the experiment, however, is that a substantial fraction of the P5 that has accumulated in proheads at high temperature, appears as phage after shift to low temperature. The pulse-labeled P8, on the other hand, remains in the prohead fraction. Thus, in this 3⁻ lysate, the 240 S proheads represent an intermediate state in the assembly of the major coat protein into phage particles. P8 appears to take part in this process, but is neither assembled into phage nor degraded in the course of it.

4. Discussion

The experiments described above delineate the pathway for the assembly of the phage P22 capsid and the encapsulation of the virus DNA into a mature infectious particle. For the purpose of discussion the process can be divided into three major stages; (1) the formation of a prohead, (2) the packing of the DNA into the prohead, including the cutting of the chromosome from the concatemer, and (3) the stabilization and maturation of the newly filled head. The most striking aspect of these experiments is the identification of a scaffolding protein species, P8, which polymerizes with the major coat protein (P5) into a prohead, but departs from the prohead in conjunction with the entry of DNA. This is reminiscent of the situation in phage T4, in which the product of T4 gene *22* forms part of an "assembly core" complex (Showe & Black, 1973), and is cleaved during head assembly and DNA packaging (Laemmli, 1970; Dickson *et al.*, 1970). However, with P22 we find no evidence for proteolytic cleavage of P8 or any of the other major phage-specified proteins (preceding paper). Nonetheless, these experiments and those of Showe & Black (1973) and Laemmli (1973) indicate that the assembly of both T4 and P22 might proceed through a 'disposable morphopoietic core' (Kellenberger & Edgar, 1971).

We first discuss our findings on P22 assembly, in roughly the order mentioned above, and subsequently consider the relation with other viruses.

(a) *Prohead formation*

Central to our interpretation of phage head assembly is the conclusion that a large number of molecules of P8, a protein species not found in phage, associates with the

FIG. 14. Sodium dodecyl sulfate gel electrophoresis of structures from the temperature shift experiment. Samples of the peak fractions from the gradients shown in Fig. 13 were heated with sample buffer and analyzed by electrophoresis through a 10% slab gel. Tracings of some of the autoradiograms are shown. (a) and (c) Peak A fractions from lysate samples taken before the temperature shift; (b) and (d) Peak A and phage fractions from samples taken after the temperature shift. Gels of the temperature sensitive lysate samples are to the right, gels of the control (++) samples, to the left.

The gel pattern of the phage peak from the control lysate sample taken before the temperature shift (not shown) did not differ in composition or intensity from the pattern shown, taken after the shift, showing that no further incorporation took place after the chase.

major coat protein, P5, to form a 240 S particle. P8 is clearly required for phage assembly, since cells infected with amber or temperature-sensitive mutants in gene *8* fail to synthesize phage or phage particles (preceding paper). In wild type and mutant lysates P8 is found associated with P5 in a rapidly sedimenting (240 S) form. In 5^- lysates, however, no rapidly sedimenting P8 is found; we deduce from this that the two species of proteins must associate in order to produce the 240 S particles.

Examination with the electron microscope of the 240 S fractions from lysates show that they represent the morphologically distinctive class of particles we have termed proheads. The proheads partially exclude the negative stain, giving the impression of an internal core, though this could be a superposition artefact of the staining. From their morphology and sedimentation coefficient it is clear that they do not contain a great deal of DNA.

Proheads are the major class of particles accumulating in 1^-, 2^- and 3^- lysates, but are present in all lysates that accumulate phage or phage-like structures. They contain, in addition to P5 and P8, P1, P16 and P20. These proteins are not essential to prohead formation, since proheads lacking each of these species are found in 1^-, 16^- and 20^- lysates. The functions of these proteins are discussed later.

(b) *Amount of gene* 8 *protein in proheads*

In the experiments reported here we used radioactive labeling to detect the phage proteins. Examination of the sodium dodecyl sulfate gel autoradiograms of proheads from mutant lysates (Figs 4 and 5) shows that the molar ratio of radioactivity in P5 to that in P8 was about 1 : 1. However, these lysates were labeled relatively early during infection. In lysates, and in proheads isolated from them, in which the radioactivity was present later during infection, the ratio of radioactivity in P8 to that in P5 was much lower. Furthermore, from the pulse–chase experiments it is clear that the ratio of P5 : P8 radioactivity in proheads changes during the experiment, since P5 chases whereas P8 does not. Clearly, we cannot reliably estimate the ratio of P5 : P8 in proheads from the radioactive labeling experiments. We have measured the synthesis of P5 and P8 at various times during infection and find that the rate of synthesis of P8 decreases, while that of P5 increases after the onset of late protein synthesis (unpublished experiments). In order to determine more reliably the protein composition of proheads, we have purified proheads from 3^- lysate in high concentration, and determined the P5 : P8 ratio in acrylamide gels by staining the proteins directly (Casjens, unpublished results). Staining with Coomassie blue gave a molar ratio for P5 : P8 of about 2 : 1. In any event, it is clear that P8 is a major component of the prohead structure, and is stably associated with it.

(c) *Prohead is a normal intermediate in head assembly*

Given that P5 and P8 are associated into some kind of shell structure, is that structure really an intermediate in head assembly and DNA packaging? The pulse–chase experiments show clearly that the P5, and P1, P16 and P20, all proteins of the mature particle, pass through a 240 S state in their assembly into phage. Newly synthesized P8 is also associated with such structures.

Further evidence for the precursor nature of the prohead particles comes from the temperature shift experiment using a temperature-sensitive mutant in gene *3* (Fig. 13). Proheads accumulate at the restrictive temperature only. On shift to permissive

temperature, a substantial fraction of the coat protein that had accumulated in proheads is converted into phage. The P8 that was synthesized at high temperature remains associated with the 240 S fraction. We can account for this result if on release from one structure P8 can again complex with more P5 from the pool to form further proheads. The evidence for this view is reviewed below. We have not done density shift experiments (Luftig *et al.*, 1971) to see if the P5 from proheads mixes with the pool of free P5 during conversion to phage. Thus we can only establish that a prohead aggregate is an intermediate state in the assembly process. More detailed aspects of prohead function are discussed below.

(d) *DNA encapsulation and cutting*

The preceding paper shows that phage DNA accumulates as a rapidly sedimenting replicating structure in cells infected with mutants defective in genes *1*, *2* and *3*. The DNA strands in these structures are longer than the mature phage length. Since the temperature shift experiment showed that the proheads accumulating in 3^- *ts* lysates are precursors to phage, they must also be precursors to the cutting of the chromosome from the concatemer. Since proheads indistinguishable from the 3^- proheads are an intermediate in the normal assembly process, we believe the prohead is the normal precursor in the cutting process.

We also believe that the prohead is a precursor to the encapsulation process. We have recently examined thin sections of phage P22-infected cells using the procedures of Simon (1972), and we find that the proheads in thin sections look just like proheads in negative stain (Lenk & Casjens, unpublished results). They are easily distinguishable from full heads, and also from the ghosts absorbed to the cells, as they are in lysates (preceding paper). Admittedly, we cannot prove that these structures did not contain the DNA in a hyperunstable state, which was lost during fixation. Nonetheless, the experiments are consistent with the idea that the proheads are not formed with the DNA condensed within them, but are filled after their assembly.

In sum, although we have not rigorously excluded alternative hypotheses, interpretation of the results is much simplified if P22 proheads are first synthesized and afterward filled with the bulk of the DNA. We do not know whether or not a small piece of DNA, as has been reported by Hosoda & Mathews (1969) for phage T4, might already be associated with the prohead.

(e) *Role of genes* 1, 2 *and* 3

Given that proheads are precursors to encapsulation and cutting, the product of genes *1*, *2* and *3* must mediate this process, since defective lysates accumulate proheads and uncut DNA. The product of gene *1* is a protein found in both the prohead and the mature phage. P2, however, is not associated with any of the particles found in phage-infected cells. Since we did not identify the product of gene *3* in these experiments, we could not determine if it was a particle protein. Since P2, and probably P3 (Casjens, unpublished results), are not proteins of the phage, but are necessary for the formation of filled heads, we presume that they function in the encapsulation and cutting of the chromosome from the concatemer. In the preceding paper we noted that non-specific DNA degradation *in vivo* is reduced in 3^- infections. This suggests that P3 is directly involved in the chromosome cutting process. Perhaps P2 is responsible for threading in the phage DNA (Richards *et al.*, 1973).

(f) *170 S empty heads: head stabilization by P26 and gene 10 product*

Cells infected with wild type phage and with mutants that do not block the conversion of the concatemeric DNA to phage-sized molecules yield a 170 S structure. Electron microscopy of the 170 S fractions reveals that these structures are empty phage heads. The protein composition of these structures resembles mature phage rather than proheads, in that they lack P8. Such structures are the major particle class in 10^- and 26^- lysates. Observations with the electron microscope and *in vitro* studies indicated that these empty heads were in fact derived from the breakdown, both intracellularly and extracellularly, of full but unstable heads (preceding paper). Although it seems unlikely, we cannot rule out the possibility that these unstable heads contain P8 as well as DNA before they break down.

The pulse–chase experiments with wild type phage were ambiguous with respect to the origin of the 170 S heads during the course of normal phage assembly. On the other hand, the temperature shift experiments showed clearly that the 170 S empty heads in the experiment were derived from a stage in phage assembly after prohead formation, and depended on DNA cutting for its existence. All these results are consistent with the idea that 170 S empty heads are derived in all cases from the breakdown of a full but unstable head, which is a true intermediate, later than the prohead in phage assembly. According to this interpretion the phage chromosome is encapsulated into a prohead under the control of genes *1*, *2* and *3*, and coupled with the exit of P8. This newly filled head, containing DNA and lacking P8, is unstable until it has been acted on by the products of genes *10* and *26*.

(g) *Properties and functions of P8*

(i) *P8 and form determination*

In our initial characterization of phage P22 mutant lysates in the electron microscope, we found that related structures were rare in 8^- lysates, though a unique class of spiral structures were seen occasionally. Sucrose gradient analysis subsequently revealed two classes of structures, sedimenting at about 195 S and 125 S, but these structures were present in only low concentration, making it difficult reliably to identify them morphologically. Electron microscopy of sucrose gradient fractions from more concentrated preparations revealed that the 125 S structures were small spherical particles, analogous to petit λ (Karamata *et al.*, 1962), and that the 195 S particles resemble proheads. A further class of structures sedimented as a broad distribution around 300 to 400 S and turned out to be the spiral structures seen in whole lysates. Such spiral structures, and the petit structures, were very rarely seen in other lysates. This result is in marked contrast to the situation in phage λ, where most lysates contain petit λ (Mount *et al.*, 1968). We are uncertain of the frequency of occurrence of the 195 S particles, since they resemble proheads and would not have been resolved as a separate peak in our gradients.

Sodium dodecyl sulfate/polyacrylamide gel analysis of the structures from 8^- lysates showed that they contained only P5 and none of the other proteins usually associated with phage or proheads. They are apparently aberrant aggregates like the polyheads of T4 (Kellenberger & Boy de la Tour, 1965; Yanagida *et al.*, 1970) or monsters and tubular forms of phage λ (Mount *et al.*, 1968). It is of particular interest that we have never observed tubular forms of phage P22. Thus P5 itself, the major coat protein, has considerable form-determining specificity. However, in the absence of P8 poly-

merization appears to be inefficient and structures of varying dimensions form; particles that are too small, the petits, and particles that look as if one edge has missed the other edge and the shell has kept on growing in some three-dimensional nested shell fashion. Thus P8 is in part form-determining, and is literally a scaffolding protein; it is required for the efficient polymerization of P5 into shells with the proper dimensions, but is not a protein of the completed head.

The appearance of the proheads in the electron microscope originally suggested to us that P8 was forming a core around which P5 assembled. The presence of cores within phage heads was suggested by Kaiser (1966) for phage λ, and by Kellenberger *et al.* (1968) for the tau particles of T4. Kellenberger explicitly suggested that the structures they saw were morphopoietic cores, which played a critical role in morphogenesis. Though, as described above, we believe P8 is playing a morphopoietic role in phage P22 assembly, we think it likely that P8 is not a separate core, but is complexed with P5 into the prohead shell. The appearance of inner material would then be due to the superposition of both top and bottom surfaces in the negative stain. This still leaves the problem of why the proheads are not penetrated by the negative stain. Perhaps the prohead is formed as a closed shell and an aperture for DNA entry is made subsequently.

The above discussion raises the question of the initiation of prohead formation. Neither P1, P16 nor P20 can be prohead initiation proteins, since proheads form in their absence. We think it unlikely that P8 and P5 simply polymerize *de novo*, since this makes it particularly difficult to understand how a unique site is generated for the site of the neck and base-plate. The gel analysis of the proheads from the wild type pulse–chase experiment did reveal an additional protein, which we had not resolved in the analysis of the mutant particles. This protein, PX, of 18,000 molecular weight, is found in mature phage. It is one of the two proteins for which we do not have a gene (preceding paper). If there is a specific initiation protein for prohead formation it must be PX. Alternatively, proheads could initiate on some region of the DNA molecules in the concatemer, or even on the cell membrane (Georgopoulos *et al.*, 1972). We have not as yet determined whether proheads contain a fragment of DNA, as was found with the 49^- heads of T4 (Luftig *et al.*, 1971).

We are also lacking a good candidate for corner proteins. PX is probably not present in sufficient numbers to represent fivefold vertices. The proheads may thus be true quasi-equivalent structures (Caspar & Klug, 1962). This leaves open the question of the origin of the corners of the mature phage as opposed to the prohead.

(ii) *Gene* 8 *protein and DNA encapsulation*

Since all phage-associated structures that are full of DNA have no detectable P8, P8 must either be removed or remove itself from the prohead on the entry of the DNA. The pulse–chase experiment on the proteins of the whole lysate (preceding paper) and on the precursor structures (Figs 8 and 9) show that P8 is not removed by proteolytic cleavage. In describing P8 thus far, we have mentioned its scaffolding or form-determining function. In addition, we could describe P8 as a DNA packaging protein, since DNA entry and P8 removal seem closely coupled. How P8 gets out of the prohead is quite mysterious. The P8 molecules might leave the prohead through the same aperture that the DNA enters. In fact, this departure might serve to thread the DNA in. Alternatively, if P8 were near the surface of the prohead shell, it might be released directly to the outside by some shell rearrangement on displacement by the DNA.

One can imagine that P8 is a DNA-binding protein, which, because of its position in the prohead, binds DNA into the shell. Alternatively, neither P8 nor P5 alone might have any affinity for DNA, but in their combination in the prohead, DNA binding sites are generated. These sites would then be lost when P8 departs (or is ejected) from the prohead during the encapsulation reaction. On the other hand, the departure of P8 from the shell might create the environment that collapses DNA (Laemmli, 1973).

(iii) *Does gene 8 protein recycle during morphogenesis?*

Since few if any molecules of P8 are incorporated into mature phage, and since the molecules are not cleaved to smaller fragments, P8 molecules could participate in multiple cycles of prohead assembly. This would explain the results of the pulse–chase experiments showing that the molecules of P8 synthesized are always found in the 240 S fraction and do not chase. That is, after release from a prohead that has just encapsulated a DNA molecule, the released P8 molecules complex with new P5 molecules to form a new prohead, thus forming a repeating cycle. This interpretation is consistent with our observations that the synthesis of P8 decreases at late times, while the synthesis of P5 continues to increase. If P8 is recycled it need not be synthesized in as large a quantity as the coat protein.

(h) *Kinetics of assembly*

The results from the pulse–chase experiments showed that radioactive label appeared in 170 S particles very quickly after the appearance of label in proheads. There was considerable lag, however, before the appearance of label in stable full heads and phage (500 S). This suggests that proheads are filled with DNA very soon after they form, but that the stabilization of the newly filled head is a slow process.

We do not know why these steps are so slow. An alternative explanation for these results is that the proheads do in fact contain considerable DNA within the cell. Thus the step from prohead to filled but unstable head would require not the encapsulation of the whole chromosome, but just the last steps in the process. These steps would have to include the exit of P8.

(i) *Model for the over-all pathway of phage assembly and DNA encapsulation on lytic infection with phage P22*

Figure 15 shows a model of the *in vivo* assembly of P22 that is consistent with all our experimental observations. Two major protein species, the P8 scaffolding protein

FIG. 15. Model for the pathway of phage P22 morphogenesis. The pathway shown is consistent with all our experimental results: the major coat protein P5 complexes with scaffolding protein P8, and 3 other proteins (PX, P16 and P20) to form a prohead. (Though P16 and P20 are incorporated at this stage in normal phage development, these steps can be bypassed, producing normal appearing but non-infectious particles lacking P16 or P20.) On incorporation of P1, and with the function of P2 and the gene *3* product, a headful of DNA is encapsulated and cut from the concatemeric DNA. We do not know which stage in head assembly makes the initial complex with DNA. The P8 molecules exit coincident with the entry of the DNA, and may recycle to form further proheads. The newly filled head is stabilized, and the site for base-plate attachment formed by gene *10* product and P26. Finally, the base-plate assembles, yielding a complete phage (Israel *et al.*, 1967).

A P before a gene number refers to the polypeptide chain of that gene. The polypeptide chains found in each structure are listed below it, with their molecular weights. A P after a gene number refers to the active protein, which may be a multimer. Genes whose protein products have not been identified are listed below the arrows. Brackets around a structure indicate that it is unstable and loses DNA during isolation. The open arrows indicate steps that proceed *in vitro*.

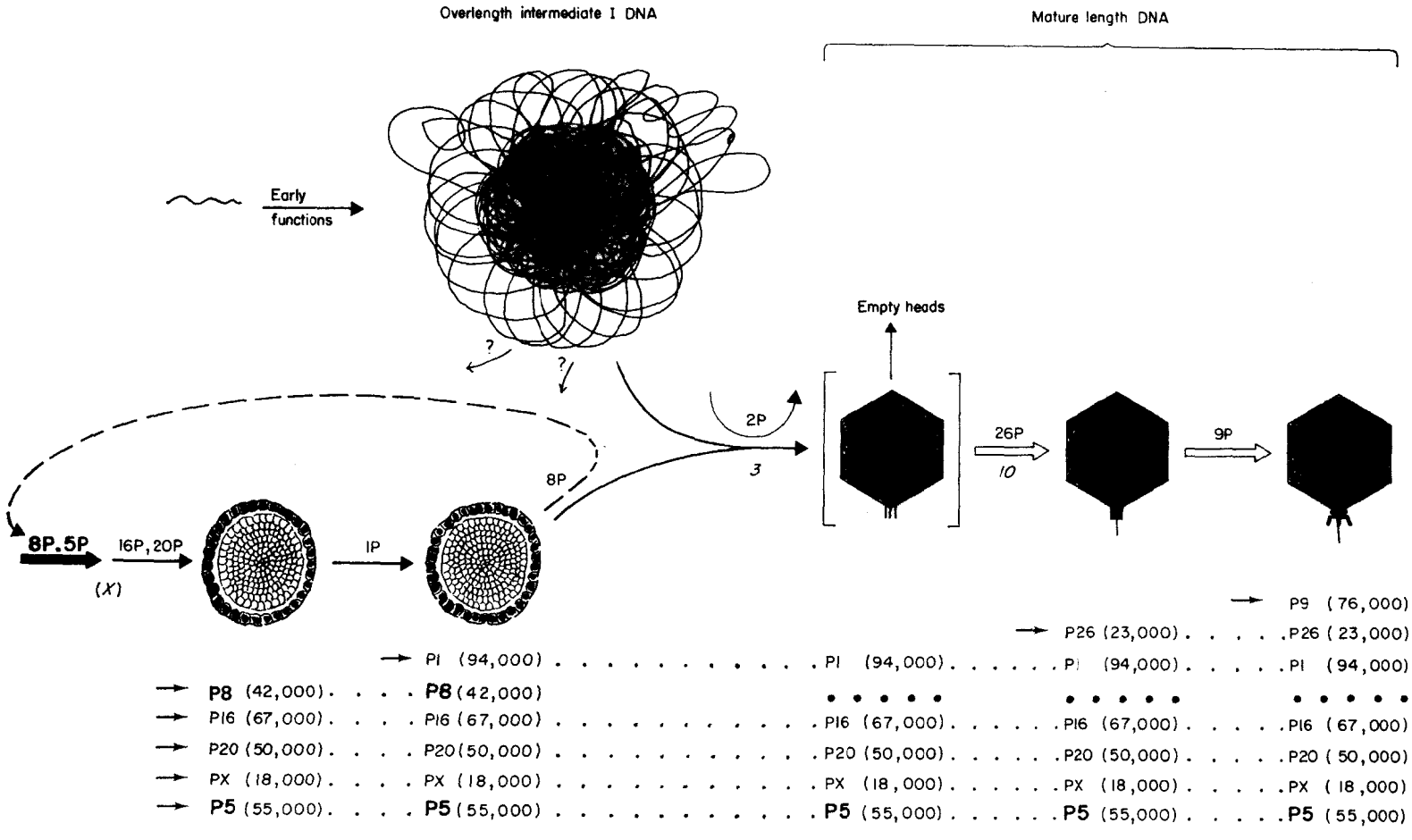

FIG. 15. (*See facing page for legend.*)

and P5 coat protein, complex to form a prohead. Two protein species not required for the formation of the prohead are also incorporated at this early stage. However, these proteins function at the end of the phage life cycle, in the injection of the chromosome into the next host. The prohead also contains a protein of unknown function, PX. P1 is incorporated into the prohead and this structure, in the presence of P2 and P3, encapsulates and cuts a headful of DNA from the concatemer. P2 and probably P3 are not particle proteins; they are needed for the encapsulation of the DNA but are not themselves incorporated into proheads or phage. The scaffolding protein P8 exits from the prohead coincident with the entry of the DNA. It is likely that P8 molecules can then complex with free coat protein to make further proheads.

The newly-filled head is stabilized by the actions of gene *10* product and P26, which presumably form part of the DNA injection apparatus and the site for base-plate attachment. The base-plate, formed of P9, then forms as the final step (Israel *et al.*, 1967).

P22 is a generalized transducing phage (Levine, 1972). We think that particles, which end up encapsulating some host chromosome, pass through essentially the same pathway as described above, since transducing particles do not differ significantly from phage particles (Ebel-Tsipis *et al.*, 1972). We also assume that P22 phage particles forming on induction from the lysogenic state follow the same pathway as those formed on lytic infection.

(j) *Relation to the assembly of other viruses*

(i) *Bacterial viruses*

Kellenberger *et al.* (1968) described a class of particles that accumulated in cells infected with mutants in genes *21* and *24* of phage T4, called tau particles. These structures were not full of DNA but did contain an internal core. Kellenberger suggested that these particles might be related to normal precursors of the phage and that the inner material might be a morphopoietic core. Simon (1972) has examined thin sections of T4 wild type infected cells and shown that particles similar to tau particles form in association with the *Escherichia coli* membrane, then move to the cytoplasm where they are converted to phage. These structures may well be analogous to the proheads of P22.

Luftig *et al.* (1971) showed that mutant particles that were only partially full when isolated (49^- temperature-sensitive heads) were precursors to mature phage. That result, and the electron microscopic observations of Simon (1972) support the conclusion that the head forms first, and the DNA enters later. Recent experiments of Laemmli (1973) suggest that the cleavage of the major head protein of T4, P23, takes place before the packaging of the DNA, and that two other proteins, P22 and IP III are subsequently cleaved in intimate association with the packaging of the DNA. Showe & Black (1973) have demonstrated that the (T4) P22-internal protein complex is an "assembly core" that functions both in initiation of a properly dimensioned head and in the packaging of the DNA. P8 of phage P22 and the T4 assembly core complex are thus very similar; both are responsible for proper head assembly, and are removed coincident with DNA packaging. In T4 the proteins are cleaved, in P22 they are removed intact and perhaps re-used.

Studier & Maizel (1969) have described the genes and proteins of phage T7, and comparison with P22 indicates that the two phages are probably assembled by very

similar mechanisms. T7 lysates contain a top component that has an extra protein not found in phage. This protein is coded for by the gene adjacent to the major head protein gene, suggesting that it is analogous to the P8 scaffolding protein. Many of the other proteins described for T7 (Studier, 1972) can be assigned appropriate roles in morphogenesis by correlating them with P22 gene products.

One clear conclusion emerges from these comparisons; all the well-studied phages containing double-stranded DNA undergo a major change in the protein composition of their head shell in the course of morphogenesis.

(ii) *Animal viruses*

The cleavage of virus structural proteins during assembly was first discovered for polioviruses (Jacobsen & Baltimore, 1968*a*; Maizel *et al.*, 1970). A number of the uncleaved proteins are found associated with a top component in poliovirus and the experiments of Jacobsen & Baltimore (1968*b*) indicated that the poliovirus top component might represent a precursor to the virion. Precursor–product experiments are somewhat more difficult to do in the animal virus systems but recently Ozer (1972) and Ozer & Tegtmeyer (1972) have presented good evidence that empty shells produced on infection with simian virus 40 (SV40) are precursors of mature virus. The proheads of P22 correspond to the "top components" or "empty shells" of animal virus terminology. The top component of adenovirus, though missing proteins present in the mature virus (Maizel *et al.*, 1968), also contains a species of protein not present in the mature virion (Prage *et al.*, 1972). These data are very similar to the P22 result, that a major component of the prohead, or top component, is lost during the formation of the complete virion.

5. Conclusions

The distinctive features of the pathway of phage P22 head morphogenesis as summarized by Figure 15 are as follows.

(1) The identification of a protein (P8) that acts as a temporary (and possibly re-usable) scaffold for the proper assembly of head subunits into the shell of the phage head.

(2) The strong implication that a preformed shell (the prohead) is a substrate for the DNA encapsulation reaction.

(3) The exit, apparently without hydrolysis or cleavage, of the scaffold protein (P8) during the DNA encapsulation reaction.

(4) The participation of two gene products (P2 and P3) in the DNA encapsulation reaction, either of which could be the enzyme that cuts out the "headful" of DNA, but only when the structure is in readiness and not before.

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