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

## I. Genes, Proteins, Structures and DNA Maturation

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The functions of ten known late genes are required for the intracellular assembly of infectious particles of the temperate *Salmonella* phage P22. The defective phenotypes of mutants in these genes have been characterized with respect to DNA metabolism and the appearance of phage-related structures in lysates of infected cells. In addition, proteins specified by eight of the ten late genes were identified by sodium dodecyl sulfate/polyacrylamide gel electrophoresis; all but two are found in the mature phage particle. We do not find cleavage of these proteins during morphogenesis.

The mutants fall into two classes with respect to DNA maturation; cells infected with mutants of genes *5*, *8*, *1*, *2* and *3* accumulate DNA as a rapidly sedimenting complex containing strands longer than mature phage length. $5^-$ and $8^-$ lysates contain few phage-related structures. Gene *5* specifies the major head structural protein; gene *8* specifies the major protein found in infected lysates but not in mature particles. $1^-$, $2^-$ and $3^-$ lysates accumulate a single distinctive class of particle ("proheads"), which are spherical and not full of DNA, but which contain some internal material. Gene *1* protein is in the mature particle, gene *2* protein is not.

Cells infected with mutants of the remaining five genes (*10*, *26*, *16*, *20* and *9*) accumulate mature length DNA. $10^-$ and $26^-$ lysates accumulate empty phage heads, but examination of freshly lysed cells shows that many were initially full heads. These heads can be converted to viable phage by *in vitro* complementation in concentrated extracts. $16^-$ and $20^-$ lysates accumulate phage particles that appear normal but are non-infectious, and which cannot be rescued *in vitro*.

From the mutant phenotypes we conclude that an intact prohead structure is required to mature the virus DNA (i.e. to cut the overlength DNA concatemer to the mature length). Apparently this cutting occurs as part of the encapsulation event.

## 1. Introduction

During the growth of DNA-containing bacteriophage, DNA strands longer than the mature phage DNA are produced. These long DNA molecules (called concatemers) are precursors of the mature phage DNA found in progeny phage particles. (T4: Frankel, 1968; λ: Smith & Skalka, 1966; Skalka *et al.*, 1972; Wake *et al.*, 1972; T7: Thomas *et al.*, 1968; P22: Botstein & Levine, 1968*b*.) In many of these cases, it has been shown that the maturation of the DNA, i.e. the cutting of the overlength concatemer to the mature size, requires the formation of phage heads (T4: Frankel, 1968; λ: Dove, 1966;

Salzman & Weissbach, 1967; MacKinlay & Kaiser, 1969). This connection between DNA maturation and head assembly is expected in the cases of phage that have circularly permuted and terminally repetitious DNA in the phage particles: such a connection was predicted by the "headful" encapsulation model proposed by Streisinger and his collaborators (Streisinger *et al.*, 1964,1967; Sechaud *et al.*, 1965). The model envisages, as a precursor to the mature DNA, a long DNA molecule consisting of several repeated phage genomes laid end to end. If this precursor is cut non-specifically into headfuls, and the headful of DNA is slightly larger than the amount of DNA required to encode the genome, then the resulting phage-size DNA molecules will be circularly permuted and terminally repetitious.

Even though the existence of a connection between DNA maturation and head assembly has been clear for some time, the exact mechanism of the relationship has proved difficult to study, largely because of the complexity of the head assembly pathways of the best-studied large DNA phages (Laemmli, 1970; Luftig *et al.*, 1971; Wang & Kaiser, 1973).

The temperate *Salmonella* phage P22 is an isometric particle that contains a single, double-stranded DNA molecule (mol. wt $27 \times 10^6$), which is circularly permuted and terminally repetitious (Rhoades *et al.*, 1968). The DNA metabolism of phage P22 has been investigated in some detail, and it appears that many of its features are consistent with the Streisinger model, including the formation of intracellular concatemers of phage DNA during growth of the phage (Botstein, 1968; Botstein & Levine, 1968*a*,*b*; Botstein & Matz, 1970; Chan *et al.*, 1972). Mutants exist in many phage genes that have gross phenotypes implicating them in DNA maturation, head assembly, or both (Botstein & Levine, 1968*a*; Botstein *et al.*, 1972). The biology of phage P22 has recently been thoroughly and comprehensively reviewed by Levine (1972).

In the hope that P22 head assembly might be more amenable to analysis than head assembly in the other DNA phages, we examined the late mutants of phage P22 in some detail. The phenotypes of these mutants in terms of DNA maturation and the production of head-related structures visible in the electron microscope, along with analysis of the proteins in particles and infected lysates in terms of the genes specifying those proteins, are presented in this paper.

Most of the mutant phenotypes can be simply explained in terms of accumulation of intermediates on a relatively simple pathway for head assembly and DNA encapsulation. This pathway, and the evidence for it are the subject of the accompanying paper (King *et al.*, 1973).

## 2. Materials and Methods

### (a) *Bacterial strains*

All the bacterial strains used are derivatives of *Salmonella typhimurium* LT2. For most experiments strain DB21 (prototroph *su*$^-$), called strain 18 in Botstein (1968), was used. For experiments where host protein synthesis was inhibited by irradiation with ultraviolet light, strain TA1530 (*his*G46 [*chl*D-*uvr*B-*bio-gal*] deletion; kindly provided by B. Ames) was used. For genetic experiments involving amber alleles, DB74 (*cys*A1348 *his*C527 *su*$^+$19) was used as the permissive host (Botstein & Matz, 1970).

### (b) *Phage strains*

The isolation and characterization of phage P22 mutants is described in Botstein *et al.* (1972). For the experiments in this and the accompanying paper, a representative amber allele was chosen for each of the phage genes. The actual alleles chosen were: 1$^-$*am*N10,

2-*am*H200, 3-*am*N6, 5-*am*N114, 8-*am*H202, 9-*am*N110, 10-*am*N107, 12-*am*N14, 13-*am*H101, 16-*am*N121, 19-*am*N111, 20-*am*N20, 23-*am*H791 and 26-*am*H204. Each allele was combined with a clear-plaque mutation ($c1^7$) to ensure entry into the lytic cycle upon infection (Levine & Curtiss, 1961). In most cases, it was also useful to introduce into the amber *c*1 strains an additional mutation in one of the lysis genes. Mutations in gene *19* (*ts*19·1 was used here) affect the phage lysozyme, which is temperature sensitive *in vivo* and in crude extracts of cells infected with a 19-*ts*19·1 phage (Waddell & Botstein, unpublished results). Mutations in gene *13* (*am*H101 was used) result in the normal production of phage lysozyme, but the cells do not lyse and phage production continues linearly for at least 3 h beyond the normal lysis time at 37°C. Phage strains were constructed by recombination (Botstein *et al.*, 1972) and each allele in a multiply-marked recombinant was checked by complementation against strains carrying the parental alleles. Occasionally deletion mapping methods (Chan & Botstein, 1972) were used to check the recombinants as well.

### (c) *Media*

A Tris-based medium containing casein hydrolysate (LCG20) was used in all the DNA synthesis and maturation experiments; it was modified from the formula given by Botstein (1968) by the addition of a solution of trace metals. M9CAA medium (phosphate-based medium containing casein hydrolysate) is described by Smith & Levine (1964). Experiments in which protein was labeled by the addition of $^{14}$C-labeled amino acids were done in M9 minimal medium, which consists of M9CAA with the casein hydrolysate omitted. LB broth, buffered saline and all the solid media used are described by Chan & Botstein (1972).

### (d) *DNA maturation experiments*

The methods used for these experiments are identical to those previously described (Botstein, 1968). To summarize briefly, infected cells were exposed to high specific activity [$^3$H]thymidine for 2 min (pulse) at 10 min after infection. Labeling was terminated by the addition of excess cold thymidine (chase). At intervals thereafter, samples were lysed gently in a mixture containing chelating agents, buffer, cyanide and lysozyme. Complete lysis was assured by incubation at high temperature (60 to 65°C) in the presence of a detergent (Sarkosyl); this also lyses mature phage particles. As shown previously (Botstein, 1968) this procedure permits the isolation of extremely high molecular weight DNA.

Analysis of the lysates was done in neutral (pH 8) and alkaline (pH 12) sucrose density gradients containing about 0·02 M total salt. The details are the same as those described by Botstein (1968). The conditions are arranged so that mature phage DNA sediments through about 0·2 of the gradient, and the replication complex (intermediate I) sediments to the bottom, where it accumulates on a high-density "shelf" consisting of 80% sodium iothalamate (Angio-Conray) in 20% sucrose. This method of analysis enables us to distinguish not only the partition of DNA between the replication complex and the supernatant, but also any changes in the molecular size of the DNA (especially in alkaline gradients). In all the experiments reported here, $^{32}$P-labeled purified phage were added to the lysis mixture in order both to provide marker in the sucrose gradients and to monitor any degradation of the DNA during lysis or subsequent handling.

Recovery of labeled DNA in these gradients was always monitored; in all cases it exceeded 85% of the trichloroacetic acid-precipitable radioactivity applied. In some cases, we found some degradation of DNA inside the infected cells during the chase; this loss was usually quite modest (less than 25% of the total precipitable radioactivity) and was ignored in all calculations in which the proportion of DNA in various regions of the gradients was determined.

### (e) *Electron microscopy*

Lysates were prepared from DB21 (*su*$^-$) cells infected in LB broth with each of the standard amber mutants carrying *c*1 and 13-*am*H101. After 60 min of infection at 37°C (about twice the length of a normal latent period) the cells were lysed by the addition of chloroform followed by vigorous agitation. Bacterial debris was removed by centrifugation at 9000 *g* for 10 min. The supernatant was then centrifuged at 35,000 *g* for 2 h to pellet

head-related structures. These were resuspended in buffered saline. The crude lysates, low-speed supernatants and high-speed pellets were all examined in the electron microscope.

A drop of each sample was placed on a carbon-coated grid. The grid was washed with 2 drops of distilled water and 1 drop of 2% (w/v) uranyl acetate. Then a drop of the stain was placed on the grid, washed with 1 drop of distilled water and the residue blotted with torn filter paper. The grids were examined in a Jeolco JEM100B at 80 kV accelerating voltage at initial magnifications of 40,000 or 100,000. All the plates were taken from grids prepared from the high-speed pellets.

(f) *Suppression of host protein synthesis after infection by irradiating with ultraviolet light*

Ptashne (1967) introduced, and Hendrik (1971) developed the idea that host-specified protein synthesis after phage infection can be suppressed by irradiation with u.v. light before infection of a u.v.-sensitive host mutant. We have used this technique using the *Salmonella* strain TA1530, which contains a deletion of the *uvr*B gene. Unfortunately, part of the *gal* region is deleted as well, with the result that P22 (whose receptor site includes galactose residues) adsorbs very poorly to this strain. This problem was overcome by providing galactose in M9 minimal medium (0·05% w/v) in the presence of a 4-fold excess of glucose (0·2% w/v). Under these circumstances, adsorption was adequate.

Control experiments were carried out in order to find the optimum u.v. dose for suppressing host-specified protein synthesis without abolishing the phage yield. The optimum condition for these experiments is about 2500 ergs/mm$^2$, which decreases the phage yield to about 10 to 20 phage/infected cell, but totally abolishes the host bands in gels. Thus, our experiments were done under conditions which allow the normal synthesis and maturation of about 10% of the normal phage yield. Most of the critical experiments have been done with and without u.v. light; to the extent that one can determine, there is no change in the pattern of phage protein synthesis after infection caused by the u.v. dose before infection; only the amount is affected. This statement must, of course, be qualified by the fact that many phage-specified proteins are hidden by the host background in the absence of prior u.v. irradiation.

(g) *Analysis of phage proteins in lysates*

(i) *Ultraviolet light irradiated system for gene-protein identification*

Strain TA1530 cells growing exponentially in M9 glucose medium containing 0·05% galactose were concentrated to about $7 \times 10^8$ cells/ml by centrifugation in the cold and resuspension in the same medium. They were irradiated with 2500 ergs/mm$^2$ u.v. light from a GE germicidal lamp. The cells were then diluted to $2 \times 10^8$ cells/ml with warm medium in separate flasks containing the number of phage necessary to give a multiplicity of infection of 7 phage/cell. The phage all carried a *c*1 allele (to ensure entry to the lytic cycle) and a 13$^-$ allele to prevent lysis. Controls included no phage (referred to as cells in Plate IV), 13$^-$*c*1 (referred to as 13$^-$ in Plate IV), and *c*1 (referred to as + in Plate IV). After 30 min of incubation at 37°C with shaking, $^{14}$C-labeled amino acids (approx. 200 mCi/mmol) were added to a final concentration of 5 $\mu$Ci/ml. After 5 min, excess unlabeled amino acids in the form of 0·25% salt-free casein hydrolysate (Nutritional Biochemicals Co.) were added. The cells were immediately concentrated 10-fold by centrifugation in the cold, resuspended in half-strength buffered saline containing 0·01 M-$MgSO_4$ and frozen in a solid $CO_2$/acetone bath. After thawing in ice, an equal volume of a mixture of sodium dodecyl sulfate (4%), glycerol (20%), 2-mercaptoethanol (10%), 0·125 M-Tris·HCl (pH 6·8) and 0·002% Bromphenol blue was added. This mixture was then heated in boiling water for 5 min immediately before application to the gel. Cylindrical discontinuous sodium dodecyl sulfate/polyacrylamide gels were prepared and run as described by Laemmli (1970) and King & Laemmli (1973). Slab gels were run using the methods of Studier (1972) and Maizel (1972).

The amount of trichloroacetic acid-insoluble radioactivity was measured for each of the samples; from this measurement and the amount of sample applied to each well of the slab gel (10 $\mu$l) we estimate that about 6000 to 15,000 disints/min of $^{14}$C in protein were applied to each gel. At this level, we could see the major bands on the autoradiograms after a few days. Autoradiograms were prepared on Kodak no-screen X-ray film. Films were analyzed

in a Joyce–Loebl microdensitometer; several exposures were made to verify that the density of each band was being measured within the linear range of the film. Integrated density of the peaks was estimated by cutting out the peaks from tracings on heavy, uniform weight paper and weighing the pieces.

(ii) *Lysates labeled without ultraviolet irradiation*

Since incorporation of radioactivity into protein was much greater when cells were not irradiated before infection, it was not necessary to concentrate cells after labeling. Thus infected cells were simply labeled at appropriate times with 2 $\mu$Ci of the $^{14}$C-labeled amino acid mixture/ml, chased with the casein hydrolysate, frozen directly in the medium and analyzed in the electrophoresis system as described above.

(h) *Analysis of proteins in purified phage particles*

A lysozyme-defective, temperature-sensitive mutant of phage P22 (*19⁻ts*19·1c1) was used to infect an exponentially growing culture of strain DB21 in M9 minimal medium at 40°C. At 5-min intervals, $^{14}$C-labeled amino acids were added to portions of the infected culture; after 5 min further incubation, excess cold amino acids were added. After 45 min total incubation, the cells were concentrated 10-fold by centrifugation in the cold and lysed by the sequential addition of 0·1 M-EDTA (pH 8), 100 $\mu$g of lysozyme/ml, and, after 20 min at 25°C, a few drops of chloroform. The lysates were shaken vigorously, debris removed by centrifugation at low speed, and the supernatant layered on a linear 5% to 20% sucrose gradient (see accompanying paper for details) and centrifuged at 20,000 revs/min in a Spinco SW27 rotor (small buckets) for 40 min. The phage band was visible in the middle of the gradient; it was collected and dialyzed against M9 medium without glucose. The samples were then centrifuged through a discontinuous CsCl density gradient, as described by Botstein (1968). The band was collected from these gradients and dialyzed against half-strength buffered saline containing 0·01 M-$MgSO_4$. These samples were then analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis as described above.

The relative amount of radioactivity in each of the bands on the electrophoretograms was estimated from microdensitometer tracings of several exposures of each gel in order to be sure than no band was exposed beyond the linear range of the film. The samples that had been labeled at 20, 25 and 30 min after infection had comparable ratios of radioactivity among the bands; therefore, we assumed that this represented the steady-state. We assumed that the total amount of protein in the phage is $27 \times 10^6$ daltons, since that is the molecular weight of the P22 DNA (Rhoades *et al.*, 1968), and because the phage particle is 50% DNA and 50% protein (Israel *et al.*, 1967).

## 3. Results

(a) *DNA synthesis and maturation in cells infected with phage mutants*

If one pulse-labels DNA in P22-infected cells with radioactive thymidine during the lytic cycle and then examines the distribution of radioactivity in linear neutral sucrose gradients, one finds most of the radioactivity in a rapidly sedimenting replication complex called intermediate I (Botstein, 1968). Little or no host DNA is labeled by this procedure, since P22 shuts off host DNA replication after infection (Smith & Levine, 1964; Botstein, 1968). If one continues incubation of the pulse-labeled infected cells in the presence of excess unlabeled thymidine (chase) until late in the latent period, analysis of the radioactivity shows that the radioactivity disappears from intermediate I and appears in slower-sedimenting forms including, eventually, mature phage DNA. The phage DNA in intermediate I consists of polynucleotide chains greater in length than those of mature form; i.e. intermediate I contains phage DNA concatemers. Removal of DNA from intermediate I is a late phage function (Botstein, 1968) and this function was shown to be blocked in certain phage mutants (Botstein & Levine, 1968*a*).

We have examined DNA maturation during non-permissive infections with amber mutants representative of each of the known late genes of phage P22 (Botstein *et al.*, 1972). Botstein & Levine (1968*a*) had difficulty interpreting some of their data because lysis of the infected cultures occurs soon after the time DNA is removed from the replication complex; the DNA from lysed cells is then degraded in the lysate by nucleases. In order to avoid this difficulty, all of the amber mutations were combined with a temperature-sensitive mutation in gene *19*, which specifies the phage lysozyme (Botstein *et al.*, 1972; Botstein & Waddell, unpublished data). All of the strains also carried a *c*1 allele, to ensure entry into the lytic cycle after infection (Levine, 1957). The resulting amber 19$^-$*ts*c1 strains fail to cause lysis of non-permissive (*su*$^-$) cells at 40°C.

Each amber mutant strain was allowed to infect the non-permissive host (DB21) at 40°C; ten minutes later, [$^3$H]thymidine was added, followed two minutes later by an excess of unlabeled thymidine. Samples were taken at intervals, lysed and analyzed in sucrose density gradients, exactly as described previously (Botstein, 1968). In all cases sucrose gradients had a shelf of high-density material at the bottom in order to ensure quantitative recovery of material that might otherwise sediment to the bottom of the tube.

Typical sedimentation patterns in neutral sucrose gradients are shown in Figure 1. Figure 1(a), (b) and (c) show the results of a control (19$^-$*ts*c1) infection. Shortly after the chase (Fig. 1(a)), essentially all of the $^3$H radioactivity sediments to the bottom of the tube (on the shelf). This is the replicating complex (intermediate I). After 48 minutes chase (close to what would ordinarily be the lysis time) all the radioactivity is in mature DNA, as judged by its co-sedimentation with added $^{32}$P-labeled mature phage DNA (Fig. 1 (b)). After a further 40 minutes (Fig. 1(c)) the profile is still the same, which is expected since this infection produces stable, normal, viable phage. The cells had not lysed before the samples were taken.

Figure 1(d), (e) and (f) show a parallel infection with an amber mutant in gene *8*. In this case again, all the pulse label is in the replicating complex shortly after the pulse (Fig. 1(d)). However, unlike the control, at 48 minutes after the chase (Fig. 1(e)), most of the radioactivity still remains in this form. From Figure 1(f) (88 min after chase) it is clear that although most of the radioactivity has disappeared from intermediate I, it has not appeared in the mature form but in small fragments at the top of the gradient. It should be emphasized that the normal lysis time is 60 to 65 minutes, and at this time DNA maturation in the 8$^-$ infection has not progressed beyond intermediate I.

Figure 1(g), (h) and (i) show the results obtained after infection with a 2$^-$ amber mutant. The results are identical with those found with the 8$^-$ mutant. After the pulse, the label remains in the replication complex until the normal lysis time (Fig. 1(g) and (h)). Very late after infection (100 min) the DNA seems to have been degraded to small fragments (Fig. 1(i)). At no time during infection is there any peak of mature size DNA. Very similar results were obtained with mutants in genes *5*, *1* and *3* (gradients not shown). Failure to remove newly synthesized DNA from intermediate I was also observed by Botstein & Levine (1968*a*) for a temperature-sensitive mutation in gene *5*.

Figure 1(j), (k) and (l) show the results with a mutant in gene *10*. Here the pattern is identical with the control until 48 minutes chase (Fig. 1(k)), at which time most of the DNA is in the mature form. However, this DNA is unstable (even though the cells

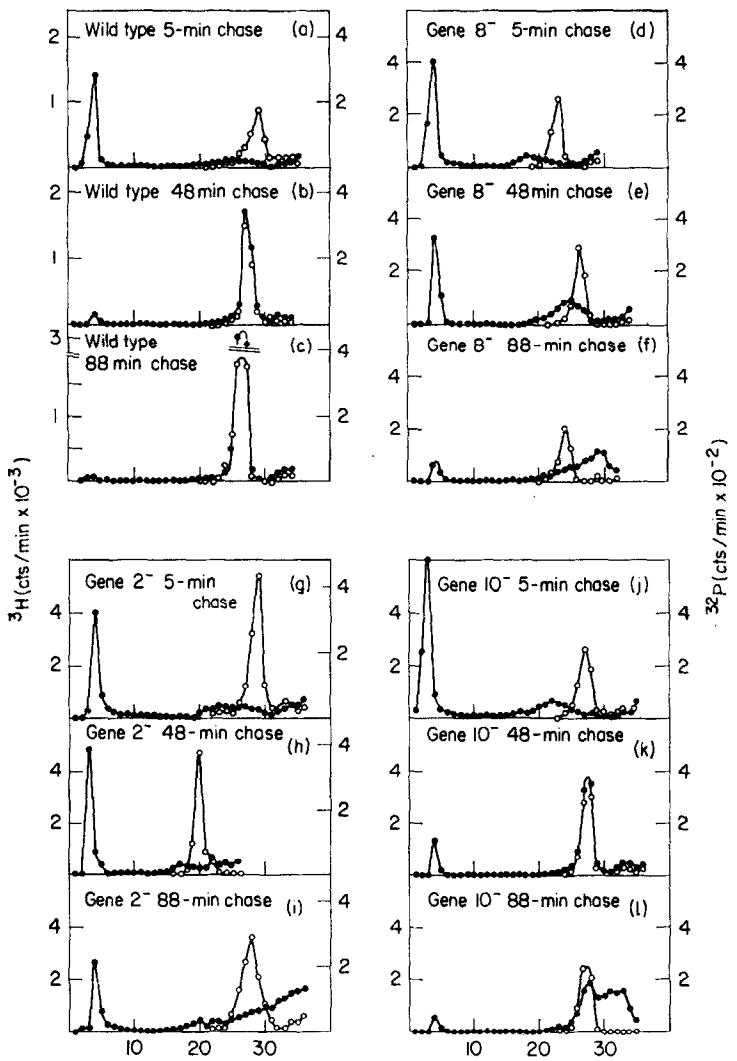


FIG. 1. Maturation of intracellular phage DNA: neutral sucrose gradients. Cells infected with wild type or mutant phage were pulse-labeled with [$^3$H]thymidine at 10 min after infection. Excess unlabeled thymidine was added 2 min later. At the indicated times thereafter, samples were lysed and analyzed as described by Botstein (1968). In addition to the amber mutation, each phage strain carried a temperature-sensitive mutation in phage lysozyme; the infections were carried out at 40°C to prevent spontaneous lysis. The lysis mixture contained $^{32}$P-labeled purified phage: the DNA from these phage is released by the lysis procedure and serves as a sedimentation marker in the gradients. Each gradient was prepared with a shelf of high-density material at the bottom to ensure complete recovery of rapidly sedimenting materials. Sedimentation is from right to left; recovery of both $^3$H (—●—●—) and $^{32}$P (—○—○—) radioactivity exceeded 85%.

did not lyse) and ultimately is degraded to small fragments (Fig. 1(l)). These results are identical with those found for mutants in gene *26*. It should be pointed out that our results for gene *10* differ from our previous results (Botstein & Levine, 1968*a*); this discrepancy is due to the ambiguities caused by the previous failure to inhibit lysis of the cells.

Figure 2 summarizes the results of the analysis of DNA maturation in all the P22 late mutants. The first panel shows the kinetics of disappearance of pulse-labeled phage DNA from the replicating complex (intermediate I). Each point represents the proportion of total radioactivity in the replication complex. It is clear that mutations in genes *10* and *26* resemble wild type with respect to the kinetics of removal of pulse-labeled DNA from intermediate I. Mutations in genes *1*, *2*, *5*, *8* and *3* cause defects in the removal of DNA from intermediate I. At what would ordinarily be the lysis time (60 min after infection) most of the labeled DNA is still in the replication complex. This defect is greatest in $3^-$ mutants. Very late in infection, the DNA appears to leave intermediate I even in the mutants; as shown above, this is the consequence of a degradative process rather than delayed maturation of the DNA.

Figure 2(b) shows the appearance of pulse-labeled DNA at the position of mature DNA. Here it is obvious that the mutants defective in DNA removal from intermediate I do not mature the DNA. Mutants in genes *5*, *8*, *1*, *2* and *3* are thus

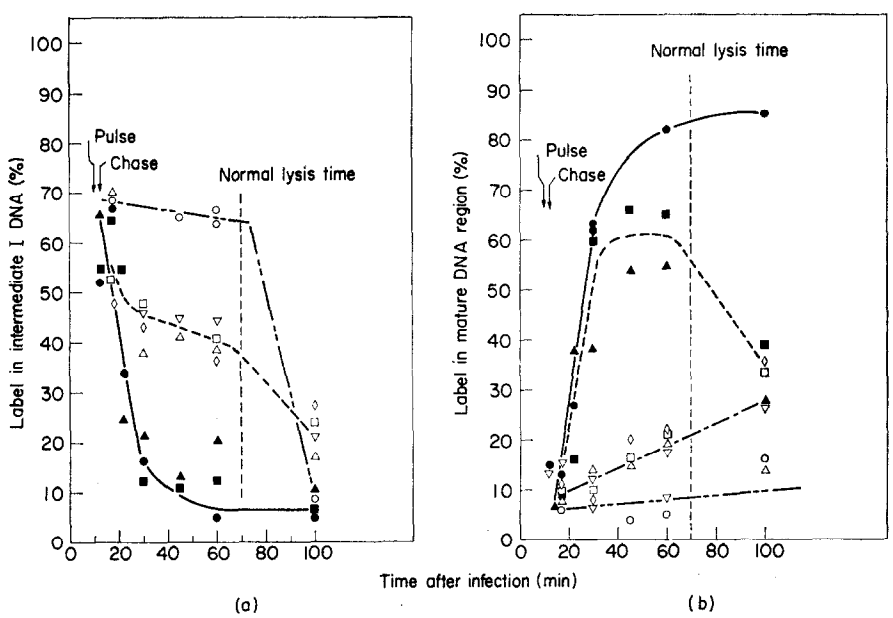


FIG. 2. Analysis of DNA maturation in cells infected with phage mutants. Pulse–chase experiments, identical to (and including) those shown in Fig. 1 were done with cells infected with amber mutants in genes *1*, *2*, *3*, *5*, *8*, *10* and *26*. Each strain also carried a temperature-sensitive mutation in gene *19* (lysozyme) and the experiments were done at 40°C to prevent spontaneous lysis. [$^3$H]thymidine was added at 10 min after infection; excess unlabeled thymidine was added 2 min later. At the times indicated on the abscissa of each plot, samples were lysed and analyzed in neutral sucrose gradients as in Fig. 1. (a) The proportion of radioactivity that sedimented with the replication complex (intermediate I) as a function of time after infection. (b) The proportion of radioactivity that sedimented with mature phage DNA marker as a function of time after infection. (◇) Gene $1^-$; (▽) gene $2^-$; (○) gene $3^-$; (□) gene $5^-$; (△) gene $8^-$; (■) gene $10^-$; (▲) gene $26^-$; (●) wild type.

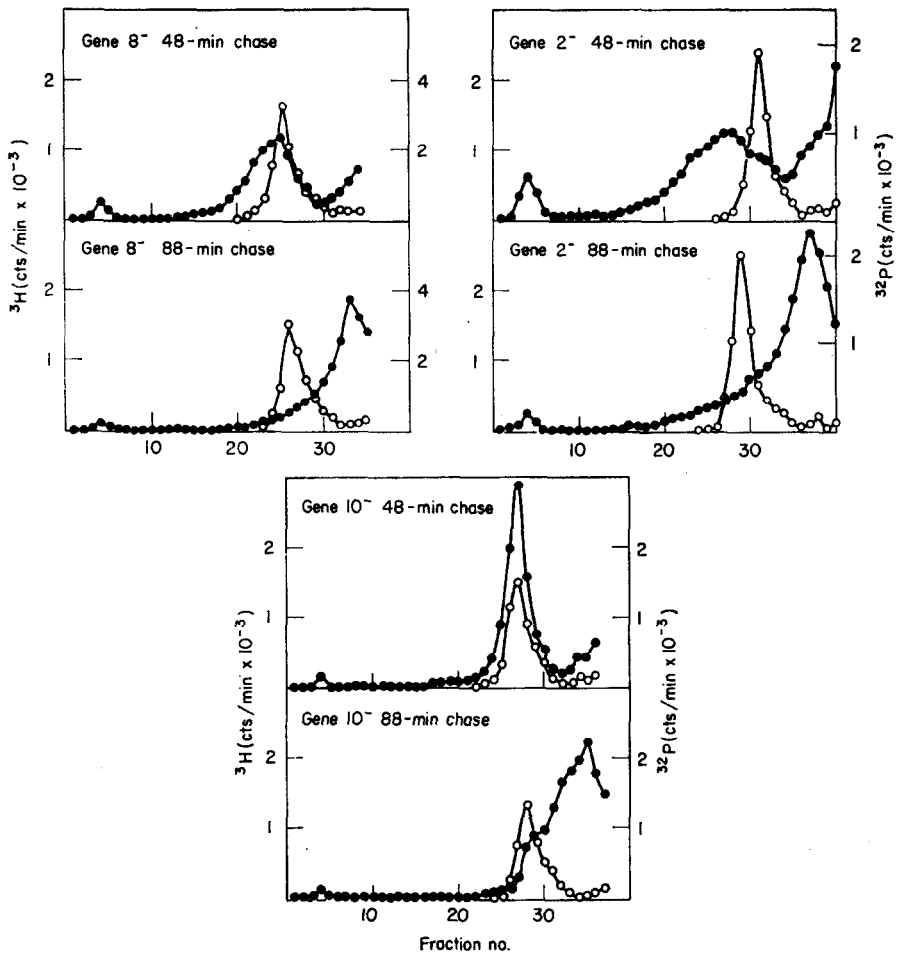


FIG. 3. Maturation of intracellular phage DNA: alkaline sucrose gradients. Samples from the experiment shown in Fig. 1 were analyzed in linear 5% to 20% sucrose gradients at pH 12·1. —○—○—, $^{32}P$ radioactivity (mature phage DNA marker); —●—●—, $^{3}H$ radioactivity.

defective at some point in the pathway of DNA maturation between intermediate I and mature DNA.

Mutants in genes *10* and *26* appear to mature their DNA normally at first, just as the removal of the DNA from intermediate I is normal. However, unlike wild type, the DNA that appears at the mature DNA position in the gradient ultimately breaks down.

Figure 3 shows a set of alkaline sucrose gradients of mutant lysates. Samples were taken at times chosen to demonstrate that concatemers are indeed formed in these mutants, as they are in all late mutants. The alkaline gradients show in each case that some of the DNA that is in intermediate I (as seen in neutral sucrose) is in fact of length greater than mature length. Thus the defects in genes *1*, *2*, *3*, *5* and *8* are apparently defects in DNA maturation rather than DNA synthesis.

(b) *Examination of phage mutant lysates by electron microscopy*

In order to characterize further mutants with defects in DNA maturation, lysates of mutant phage in a non-permissive host (DB21) were prepared and examined by electron microscopy. Although we examined the crude lysates as well as high-speed pellets from these infections, all the Plates shown are of resuspended high-speed pellets. All preparations are negatively stained with 2% uranyl acetate.

(i) *Mutants that produce "full" heads (wild type, 9⁻, 16⁻ and 20⁻)*

Plate I(a) shows a wild type P22 phage lysate. The characteristic P22 morphology is visible (Anderson, 1960; Yamamoto & Anderson, 1961; Israel *et al.*, 1967); a polyhedral head about 58 nm in diameter with a short base-plate some 20 nm across. Occasionally a long spike about 20 nm long emanating from the center of the base-plate can be seen. This spike was also observed by Yamamoto & Anderson (1961). Most of the heads in Plate I(a) appear to be full; in the upper left corner, an empty (presumably ghosted) head is visible, and is typically present in wild type lysates.

The rest of Plate I shows lysates of mutants that also produce full heads: non-permissive infections with mutants in genes *16* and *20* (Plate I(c) and (d)) produce particles morphologically indistinguishable from wild type. Lysates of mutants in gene *9* (Plate I(b)) contain full heads that are missing the base-plate, but which still retain a "neck" and the spike. As shown by Israel *et al.* (1967), such particles can be reconstituted *in vitro* by mixing with purified base-plate parts. Higher magnification photographs of a wild type and of a 9⁻ head are shown in Plate III(f) and (e), respectively.

All of these mutants which produced full heads (16⁻, 20⁻ and 9⁻) were completely normal with respect to DNA synthesis and maturation (data not shown; the curves are the same as wild type in Fig. 2).

(ii) *Mutants that produce no visible head structures (5⁻ and 8⁻)*

Lysates of mutants defective in genes *5* and *8* contain few, if any, structures recognizable as being related to phage heads. Occasionally, we saw particles in an 8⁻ lysate which might be aberrant head membranes. We have also seen spiral head-related structures in 8⁻ lysates; these are discussed in greater detail in the accompanying paper. As shown above, mutants in genes *5* and *8* synthesize, but do not mature intracellular concatemers. As will be shown below, gene *5* specifies the major capsid protein.

(iii) *Mutants that produce "prohead" structures (1⁻, 2⁻ and 3⁻)*

Plate II shows preparations of lysates of mutants in (a) gene *1*, (b) gene *2* and (c) gene *3*. These all produce particles that are similar in size to full phage heads, but not as impenetrable to stain. They appear to be less "full" than wild-type, but are also not as "empty" as the ghosted particles one sees in wild type lysates. There is a distinctive pattern within the perimeter of these particles, which suggests the presence of some regular internal structure; furthermore, these particles are rarely collapsed as ghosts usually are. These 1⁻, 2⁻ and 3⁻ heads are more round than phage particles; clear vertices are rarely seen.

In the higher magnification photograph of a 3⁻ head (Plate III(c)) one can clearly discern a small protrusion at the edge of the particle; similar structures can also be discerned near some of the particles in Plate II. The significance of this is not certain; not all of the particles have this ancillary structure.

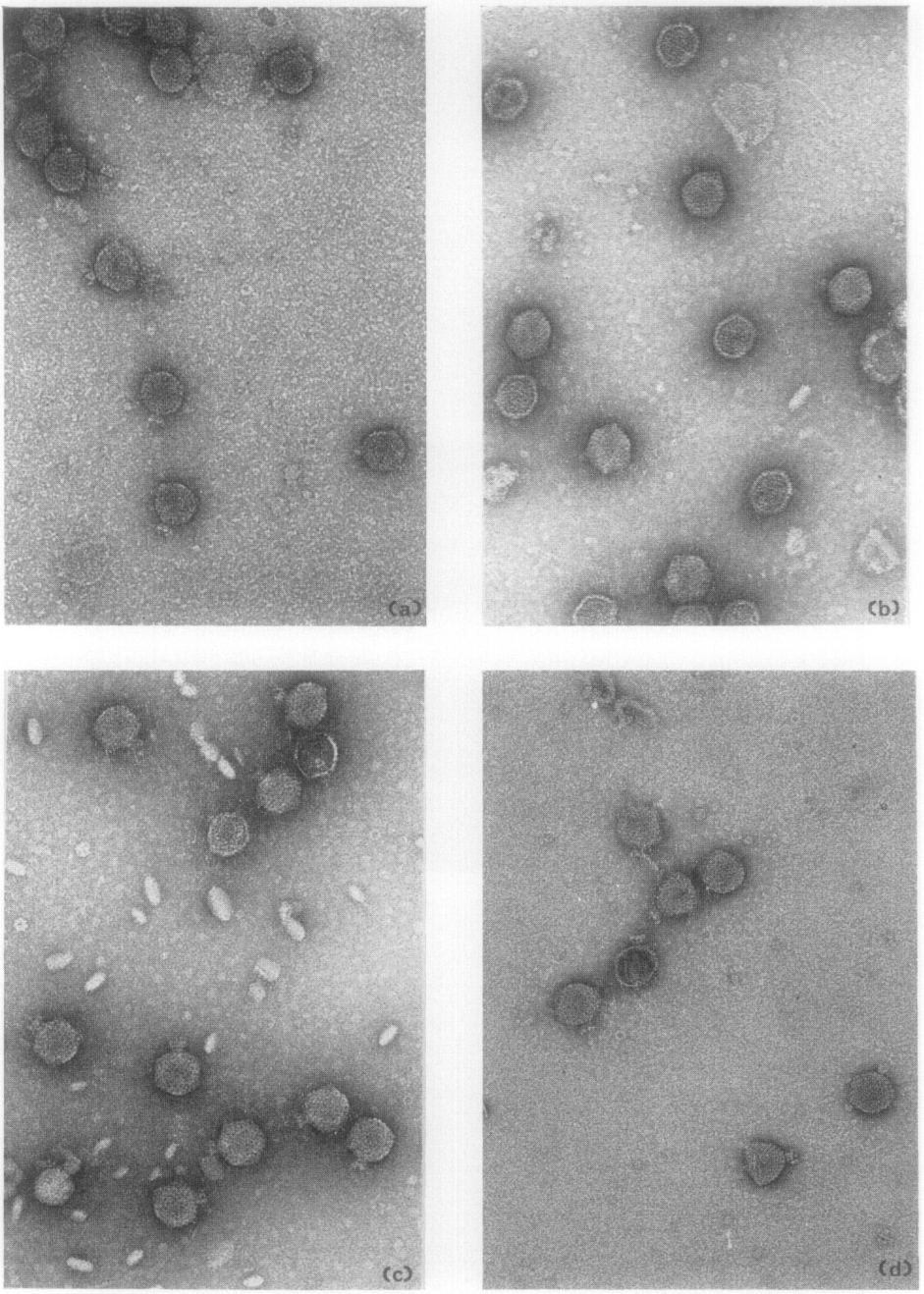


PLATE I. Electron micrograph of phage mutant lysates. Details are given in Materials and Methods; high-speed pellets of lysates were used; crude lysates do not differ significantly except for concentration of particles and the presence of larger amounts of total protein. All the phage carried, in addition to the amber mutation of interest, a *c*1 and a 13$^-$*am*H101 (lysis-defective) allele. (a) Wild type; (b) 9$^-$ (base-plate defective); (c) 16$^-$; (d) 20$^-$.

[*facing p. 678*

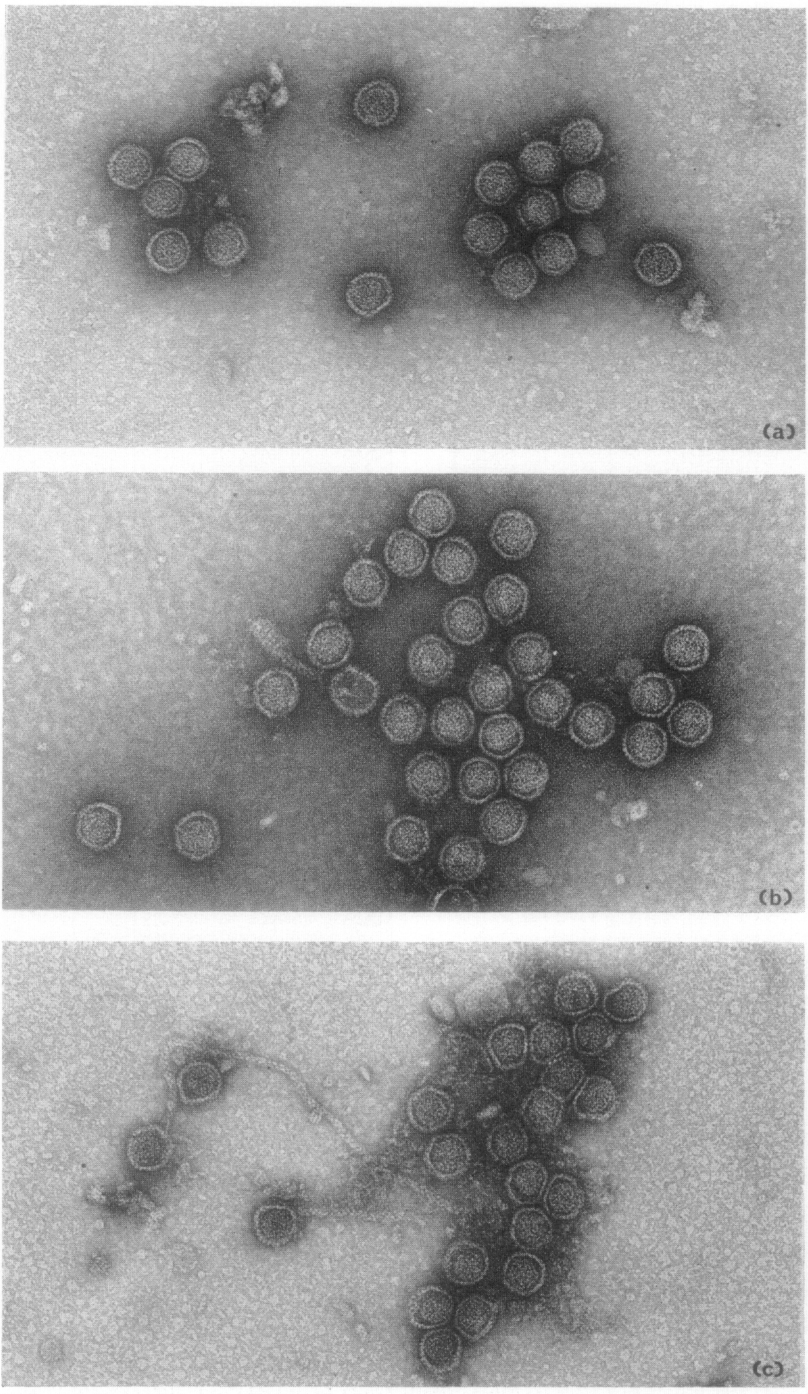


PLATE II. Electron micrographs of phage mutant lysates. See the legend to Plate I and Materials and Methods for details. (a) $1^-$; (b) $2^-$; (c) $3^-$.

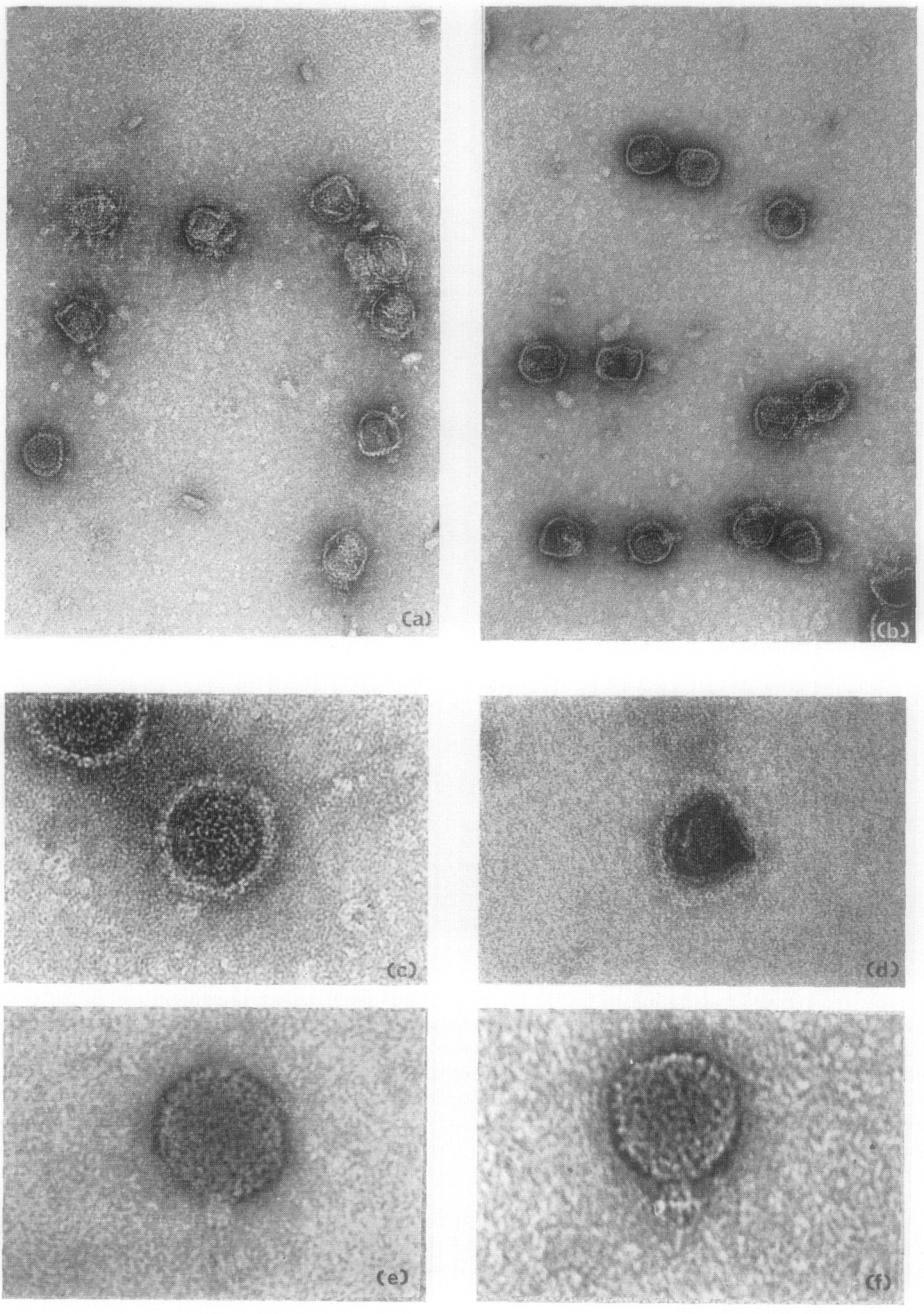


PLATE III. Electron micrograph of phage mutant lysates. See the legend to Plate I and Materials and Methods for details. (a) $26^-$; (b) $10^-$; (c) higher magnification view of a typical particle from a $3^-$ lysate showing the partially empty morphology; (d) higher magnification view of a typical empty particle from a $10^-$ lysate; (e) higher magnification view of a full but base-plateless particle from a $9^-$ lysate. The spike at the bottom is clearly visible; (f) higher magnification view of a wild type P22 particle.

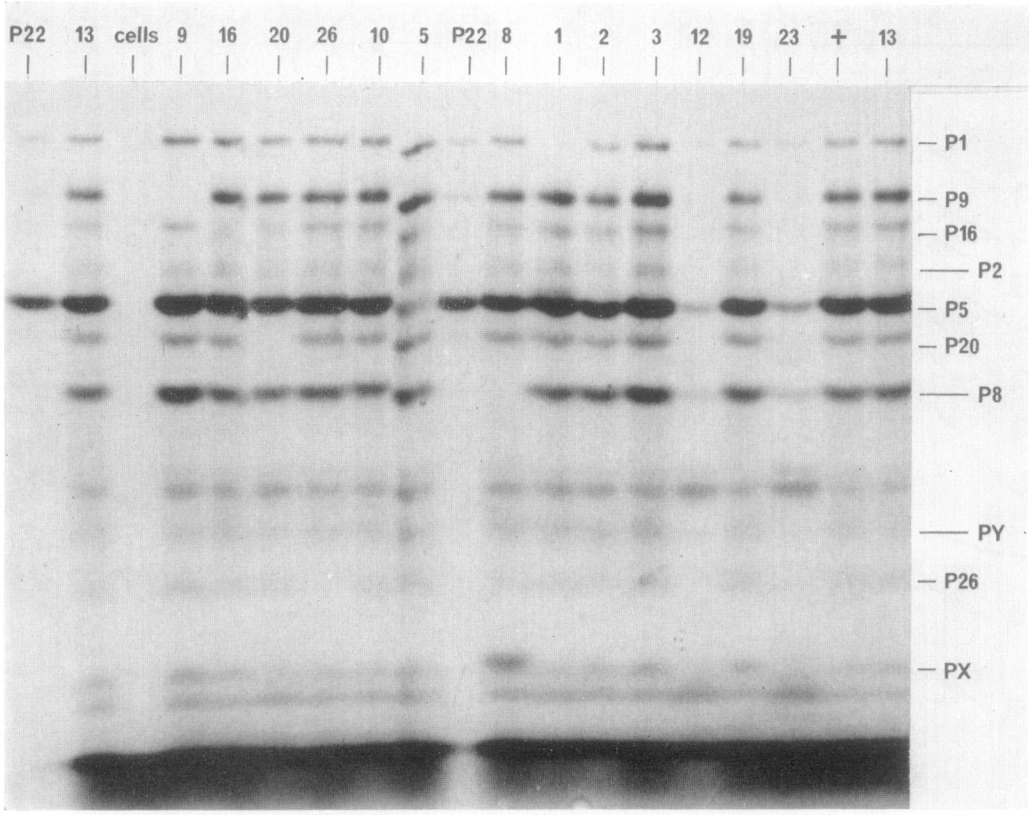


PLATE IV. Identification of phage gene products on sodium dodecyl sulfate/polyacrylamide gels. Proteins synthesized in cells infected with phage mutants were analyzed as described in Materials and Methods. The cells were irradiated before infection to reduce the background of host-specified protein synthesis. Each sample is marked at the top: P22, purified phage particles; this sample contained less total radioactivity, resulting in only the major bands being readily visible; cells, a control in which no phage were added, the negligible incorporation of radioactivity shows that the irradiation efficiently suppressed host protein synthesis; +, infection with c1 phage; 13, infection with a $13^-$ mutant; the other numbers refer to the mutant gene in the infecting phage; besides carrying the standard amber mutation in that gene, they all carry a $13^-$ mutation to inhibit lysis, and a c1 allele.

At the right of the Plate are the identified proteins labeled according to the gene which specifies them. Proteins listed at the extreme right are proteins found in lysates but not in phage particles; the others are found both in phage particles and lysates.

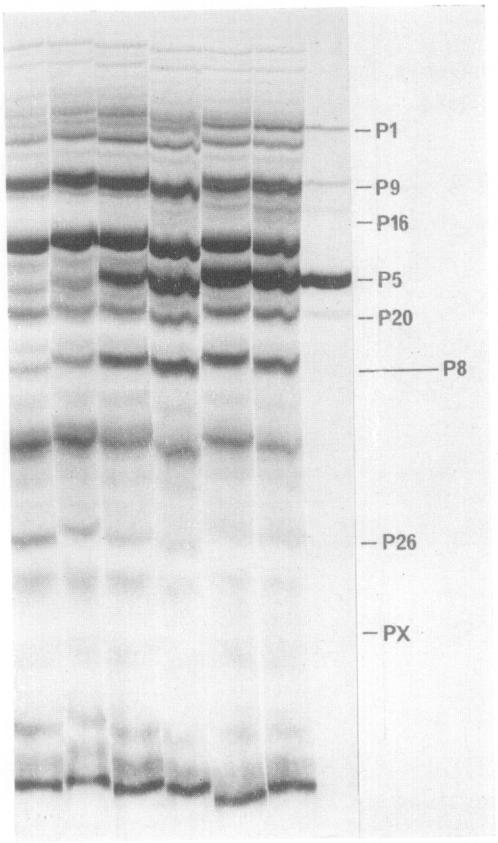


PLATE V. Electrophoresis of proteins in purified phage (extreme right) and in lysates of unirradiated cells infected with wild-type phage. The details of this experiment are described in Materials and Methods. Shown in the Plate are cylindrical gel electrophoretograms of whole lysates of infected cells labeled at (from left to right) 5, 10, 15, 20, 25 and 30 min after infection. Radioactive amino acids were present for 5 min, then excess cold amino acids were added, and incubation was continued until 45 min after infection, at which time the cells were lysed and samples taken for electrophoresis.

Phage from the sample labeled at 30 min after infection were purified as described in Materials and Methods and the electrophoretogram is shown at the extreme right. The proteins listed at the extreme right are found in lysates but not in phage particles. A microdensitometer tracing of 2 of these autoradiograms is shown in Fig. 4.

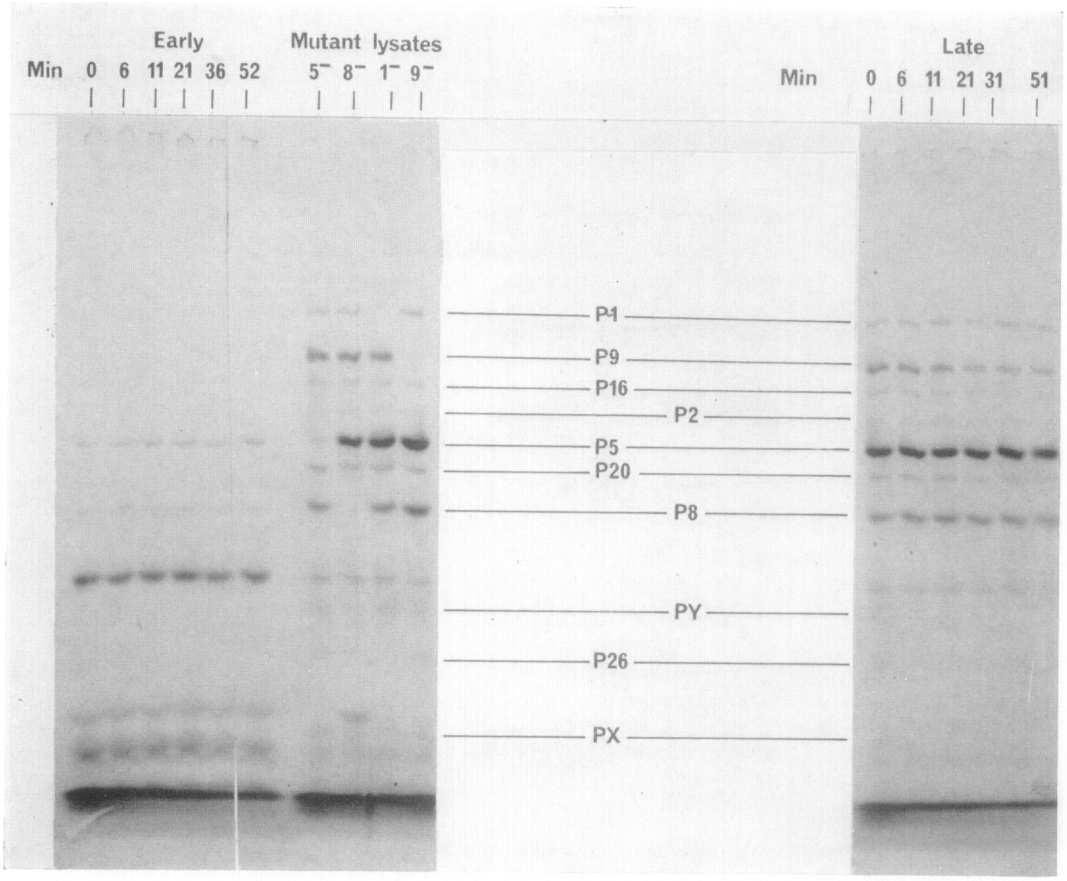


PLATE VI. Pulse–chase experiment with wild type phage. Strain TA1530 was irradiated and infected as described in Materials and Methods with 13⁻c1 (lysis-defective) phage. At 10 min after infection (marked early) and at 30 min after infection (marked late) $^{14}C$-labeled amino acids were added to a final concentration of 5 $\mu$Ci/ml. After a further 3 min, excess unlabeled amino acids were added. At various times thereafter (as shown at the top), samples were taken by freezing in sample buffer at −70°C and prepared for electrophoresis by directly placing in boiling water. All samples were run on the same slab gel. Samples of mutant lysates from the experiment shown in Plate IV were run at the same time as reference markers; these are shown in the center of the Plate.

These proheads have, however, a very characteristic appearance; substantial numbers (10 to 30%) of heads of this type are visible in all lysates that produce any head-related structure. Such partially full heads are visible in Plates I(a) wild type (near the top of the Plate) I(b) $9^-$; I(c) $20^-$; III(a) $26^-$ and III(b) $10^-$.

The mutants ($1^-$, $2^-$ and $3^-$) which produced primarily proheads were all defective in cutting of the DNA concatemers. They share this phenotype with mutants in genes *5* and *8*, which produce few visible head-like structures (Fig. 2).

(iv) *Mutants that produce "empty" heads ($10^-$ and $26^-$)*

Plates III(a) and (b) show the structures we found in $26^-$ and $10^-$ mutant lysates. These seem to be empty, like phage ghosts, and are often mis-shapen as if they were collapsed. In the case of the $26^-$ lysate, the particles often have base-plates (Plate III(a)). However, $10^-$ particles rarely displayed base-plates, although occasionally a "neck" was visible. Otherwise, they closely resembled the $26^-$ particles.

These "empty" particles derive from lysates in which concatemers are cut to the mature phage length, but which apparently are ultimately unstable, even in the cell, since the DNA eventually breaks down (Fig. 2). Therefore, one might suspect that the empty heads observed in the lysate might be breakdown products of full, but unstable, heads. In order to test this, grids of crude lysates were prepared immediately after lysis of the cells. On these grids we found a substantial number of full heads. When lysis was immediately followed by the addition of gluteraldehyde as fixative, full heads were also seen in $10^-$ lysates. These measures had no such effect on the appearance of lysates of $1^-$, $2^-$ or $3^-$ mutants, which contained only the head structures. Therefore, we believe that the empty heads visible in $10^-$ and $26^-$ lysates are probably the result of the breakdown of an unstable full head. We (Lenk, Casjens, King & Botstein, unpublished results) have found substantial numbers of full heads in sectioned cells infected with $10^-$ and $26^-$ but not $1^-$, $2^-$ or $3^-$ mutants.

(v) *Examination of double mutant lysates*

The observation that all lysates which contain any head structures also contain partially full heads suggests that the latter might represent some sort of precursor form or some abortive product. Therefore, we constructed some double amber mutants: $10^-$ $2^-$ and $10^-$ $3^-$. Examination of the double mutant lysates (Table 1)

TABLE 1

*Analysis of double-mutant phenotypes by electron microscopy*†

| Phage‡ | Heads (number) Partially empty | Empty | Full | Partially empty (%) |
|---|---|---|---|---|
| $10^-$ | 45 | 134 | 2 | 25 |
| $2^-$ | 103 | 4 | 0 | 97 |
| $3^-$ | 100 | 5 | 0 | 95 |
| $10^-$ $2^-$ | 248 | 6 | 0 | 98 |
| $10^-$ $3^-$ | 253 | 6 | 0 | 98 |
| $9^-$ | 35 | 10 | 45 | 38 |
| Wild type | 26 | 11 | 72 | 24 |

† See Plates I, II and III for the appearance of the 3 types of head. Grids prepared from high-speed pellets were photographed and printed to a final magnification of 50,000. All particles in 3 different prints were counted.

‡ All strains carried $c1^7$. Double mutants were tested by complementation.

showed that the structures typical of $2^-$ or $3^-$ mutants predominated in lysates of both double mutants. This is consistent with the idea that the $2^-$ and $3^-$ forms represent an earlier intermediate in head morphogenesis than the "empty" form found in $10^-$ (and $26^-$) lysates. It is for this reason, and because of more conclusive evidence in the accompanying paper, we call these partially full structures proheads (see Laemmli, 1973).

TABLE 2

*Assembly of phage P22* in vitro

| | $9^-$ | $10^-$ | $26^-$ | $16^-$ | $5^-$ |
|---|---|---|---|---|---|
| $5^-$ | $2\times10^{13}$ | $1{\cdot}5\times10^{12}$ | $3{\cdot}7\times10^{10}$ | $5{\cdot}1\times10^{9}$ | $5{\cdot}8\times10^{8}$ |
| $16^-$ | $6{\cdot}2\times10^{10}$ | $4{\cdot}6\times10^{11}$ | $4{\cdot}2\times10^{10}$ | $3{\cdot}4\times10^{9}$ | |
| $26^-$ | $1{\cdot}5\times10^{13}$ | $9{\cdot}5\times10^{11}$ | $1{\cdot}6\times10^{9}$ | | |
| $10^-$ | $1{\cdot}4\times10^{13}$ | $1{\cdot}8\times10^{9}$ | | | |
| $9^-$ | $3{\cdot}6\times10^{8}$ | | | | |

Strain DB21 (*su*$^-$) cells growing in M9CAA medium at 37°C were infected with amber mutants in the indicated genes; each phage also carried c1$^7$ and 13$^-$*am*H101 (lysis-defective). After 90 min, the cells were concentrated 150-fold and resuspended in 2×M9A, $10^{-2}$ M-$MgCl_2$. Equal volumes were mixed and vigorously shaken with chloroform, incubated for 2·5 h at 37°C, and titered for viable phage.

### (c) *Rescue of 10$^-$ and 26$^-$ heads* in vitro

The hypothesis that $10^-$ and $26^-$ infections result in the accumulation of full, but unstable, phage heads encouraged us to attempt to rescue these heads *in vitro*. Table 2 shows the results of experiments in which concentrated lysates of $10^-$ and $26^-$ infections were mixed with $5^-$ (major capsid subunit defective) lysates. The $10^-$ and $26^-$ heads were reconstituted *in vitro* into active viable phage. However, under these conditions none of the other heads could be rescued. This result suggests that the $10^-$ and $26^-$ mutant-infected cells initially accumulated full, though unstable heads, which are the true intermediates in head assembly. It should be noted that the failure to reconstitute $16^-$ and $20^-$ heads *in vitro* suggests that these heads are not later intermediates, but are irreversibly dead structures.

### (d) *Identification of phage gene products*

The polypeptide chain specified by a particular phage gene carrying an amber nonsense mutation is prematurely terminated when this gene is expressed in a restricting (*su*$^-$) host (Sarabhai *et al.*, 1964). If one fractionates proteins synthesized after infection with phage using a method that separates polypeptides on the basis of molecular weight, one can correlate genes with the proteins they specify by observing which of the proteins synthesized after infection with an amber mutant phage is reduced in molecular weight (or is missing).

In order to fractionate the proteins in phage particles and in lysates of infected cells, we used the discontinuous high-resolution gel system described by Laemmli (1970). This method of separation of polypeptide chains separates them according to molecular weight (Weber & Osborn, 1969; Neville, 1971). In a typical experiment, cells infected with phage were labeled with $^{14}$C-labeled amino acids. At a suitable time

after infection, the cells were lysed and were placed in boiling water in the presence of sodium dodecyl sulfate and a reducing agent and were applied to the gels. After electrophoresis, the gels were dried and autoradiographed (Fairbanks *et al.*, 1965; Maizel, 1972).

In our initial experiments, we found that infection with phage P22 did not shut off host protein synthesis as it does host DNA synthesis. As a result, only the major phage proteins could be identified in infected cells. To identify the remaining proteins, we selectively inhibited the synthesis of host proteins by using a mutant host (*uvr*B) unusually sensitive to u.v. light and irradiating with u.v. before infection (Ptashne, 1967; Hendrix, 1971). Under our conditions, a dose of 2500 ergs/mm$^2$ reduces host protein synthesis, so that in the absence of infection we see no bands on the gels. After infection, total protein synthesis is stimulated tenfold, and a burst of phage is produced that is about 10 to 20% of the normal burst, indicating no gross aberrations in phage DNA, RNA or protein synthesis.

In order to avoid double nomenclature, we present the identification of genes with the proteins they specify before showing the results of our analysis of the protein composition of purified phage particles. Plate IV shows the autoradiogram of a slab gel in which the proteins synthesized after infection of u.v.-irradiated cells with P22 late mutants are displayed. Parallel infections with wild type phage, a few early mutants, and uninfected irradiated cells are also included in Plate IV. In all cases, $^{14}$C-labeled amino acids were added 30 minutes after infection; after five minutes further incubation, excess unlabeled amino acids were added and the samples concentrated, lysed and analyzed as described in the methods.

Thirteen bands are clearly visible in the autoradiograms of wild type, 13$^-$ and 19$^-$ (lysis defective) infections. This represents the wild type pattern. In two cases (12$^-$ and 23$^-$), all but two or three of the bands are greatly diminished in intensity or totally absent. Gene *12* is an "early" gene implicated in DNA replication (Smith & Levine, 1964; Botstein & Levine, 1968*a*; Botstein *et al.*, 1972; Levine & Schott, 1971). Gene *23* apparently is involved in control of late gene expression (Margolskee & Botstein, unpublished results). Thus, most of the bands seen in the wild type autoradiogram seem to represent "late" phage gene products.

Beginning with the top of the gel pattern, one can identify most of these late protein bands with particular genes by virtue of their absence from lysates from infections with mutants in that gene. The band nearest the top of the gel is missing only in the 1$^-$ infection: we infer that this band is the gene *1* product. The second band is missing only from the 9$^-$ lysate: indicating that it is the gene *9* product. The third band is diminished in intensity or missing in two of the lysates, 20$^-$ and 16$^-$. However, the sixth band is missing only from the 20$^-$ lysate. Since genes *16* and *20* are adjacent on the genetic map (Botstein *et al.*, 1972) we suspect that gene *20* specifies the sixth band and the mutation we used exerts a polar effect on gene *16*, whose product would be the third band. We have isolated the particles from 20$^-$ lysates (see accompanying paper): these are missing the sixth band. Similarly, particles from 16$^-$ lysates are missing the third band only, indicating that we are correctly identifying these gene products.

The fourth band from the top of the gel is missing only in the 2$^-$ lysate: we identify it as the gene *2* product. The fifth band, which is one of the two most intense bands, is the product of gene *5*. As indicated above, the sixth band is probably the gene *20* product. The seventh band is the product of gene *8*; the amber fragment is clearly

visible near the bottom of the gel. The eighth and ninth bands are apparently early proteins, since they are not diminished in the 23$^-$ and 12$^-$ infections. The tenth band is missing in none of the lysates examined; apparently it is the product of a late phage gene for which we have no amber mutation as yet.

The 11th band is, by virtue of its absence from the 26$^-$ lysate, the product of gene *26*, and the 12th is the product of another unidentified late gene. Finally, the 13th band is undiminished in the 12$^-$ and 23$^-$ infections; we suspect it to be the product of an early gene.

We have detected the amber fragment in only one case (gene *8*). The others may be too small to detect, or they might be degraded (Goldschmidt, 1970; Platt *et al.*, 1970). We have detected fragments in preliminary experiments with different amber mutants in genes *9* and *16*.

Thus from Plate IV we can assign most of the bands in phage-infected lysates, labeled late after infection, to late genes: we will call these henceforth by their gene numbers: P1 being used to designate the product of gene *1*. The two unidentified bands we will call PY (the tenth from the top) and PX (the twelfth).

### (e) *Detection of phage proteins after infection of an unirradiated host*

An experiment was carried out to show that the pattern of phage protein synthesis is not grossly aberrant in a u.v.-irradiated host. Wild type (DB21) *Salmonella* were infected with a lysozyme-defective (*ts*19·1c1) but otherwise wild type P22 phage. At five-minute intervals, $^{14}$C-labeled amino acids were added for five minutes, excess unlabeled amino acids were then added and incubation was continued until 45 minutes after infection. Concentrated lysates of these cells were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. The autoradiograms (Plate V) clearly show bands corresponding in position to P1, P16, P20, P5 and P8, which increase markedly in intensity during the latter part of the latent period. P9 and the other major bands found in lysates are apparently obscured by host proteins of similar mobility in the gels.

### (f) *Protein composition of phage particles*

To determine which proteins are physically present in P22 phage, particles were purified from the lysates described in the previous section. Each sample was purified by sedimentation through a linear sucrose gradient. The peak of phage (determined by titer) was then purified further in a discontinuous CsCl density gradient. An electrophoretogram of the phage purified from the sample labeled at 30 to 35 minutes after infection is shown at the right of Plate V. Figure 4 shows a densitometer tracing of this gel of purified phage together with the gel of the whole lysate labeled at the same time.

From a comparison of these electrophoretograms it is possible to derive the protein composition of the phage in terms of the gene products as identified above. The phage particle contains seven different proteins: namely, in order from the top of the gel, P1, P9, P16, P5 (the major protein species), P20, P26 and PX. It may be noticed that in Plate IV, as well as in Plate V and Figure 4, the purified phage samples show P1, P9, P5 and P20 quite clearly.

Three of the ten late proteins are not found in the phage particle. One of these, P8, is present in the lysate in amounts comparable to P5, which is the major structural protein of phage P22. This is as true in the case of infections of unirradiated cells

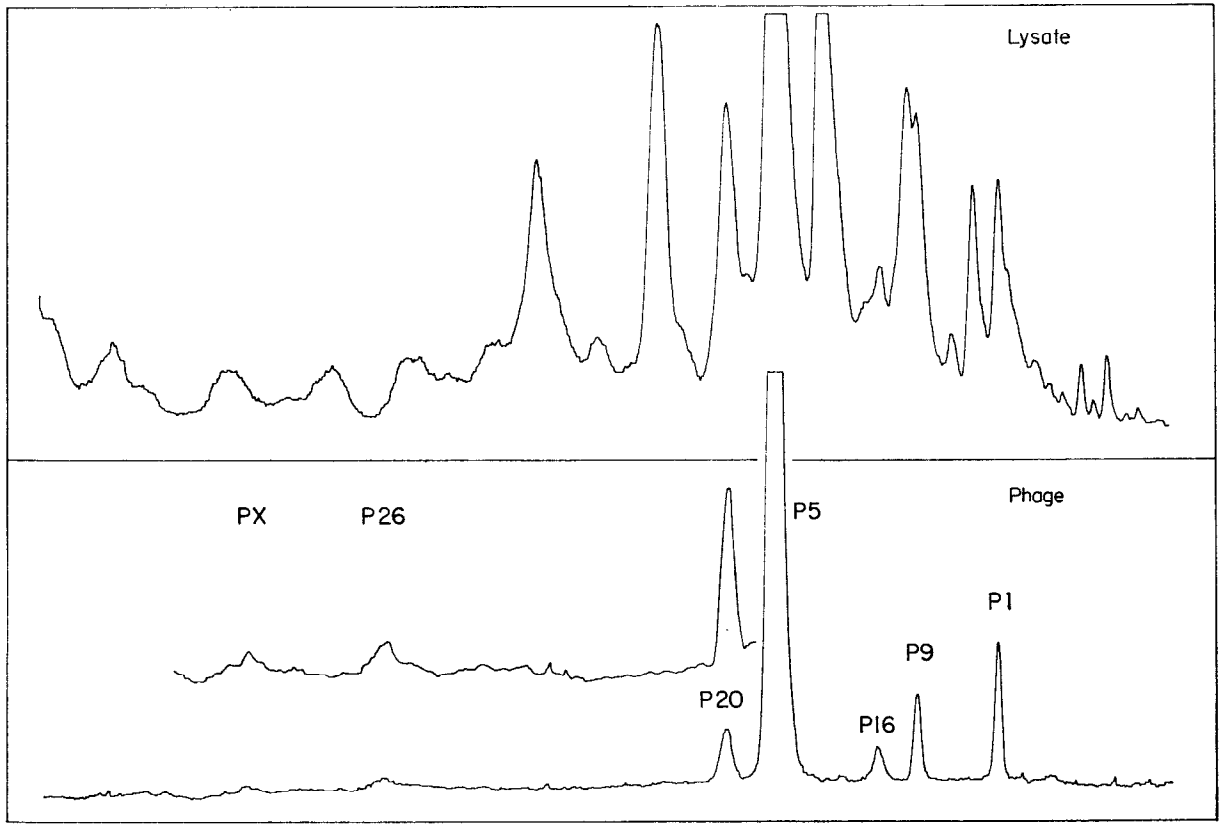


FIG. 4. Electrophoretic analysis of phage protein. Autoradiograms of sodium dodecyl sulfate/polyacrylamide gels (shown in Plate V) were traced with a Joyce–Loebl microdensitometer. Electrophoresis is from from right to left. (a) Lysate of wild type infected cells labeled with $^{14}C$-labeled amino acids at 30 min after infection. (b) Highly purified phage particles from the same infection. Cells were labeled and excess unlabeled amino acids were added 5 min later, and incubation continued until 60 min, at which time the cells were lysed and the phage purified. The inset shows the low molecular weight region of the gel at an expanded scale.

(Plate V and Fig. 4) as it is in infections of irradiated cells (Plate IV). P2 and PY, although not as abundant in the lysate as P8, are nevertheless clearly also not present in phage particles. From the phenotypes of mutants in the various late genes we can see that the functions of some of the genes are clarified by the analysis of the phage proteins. Gene *5* clearly is the structural gene for the major capsid protein; the fact that DNA is not cut from the concatemer by mutants in gene *5* implies that the head structure is vital to this cutting of the DNA. A similar conclusion can be drawn for the function of gene *1* in the cutting of DNA.

### (g) *Determination of molecular weights of phage polypeptide chains*

In sodium dodecyl sulfate/polyacrylamide gel electrophoresis, the denatured polypeptides migrate in proportion to the logarithm of their molecular weight (Shapiro *et al.*, 1967; Weber & Osborn, 1969). This is also true for the high-resolution discontinuous system (Neville, 1971). We used four commercially available marker proteins to calibrate our electrophoretic systems in order to estimate the molecular weights of the polypeptides that we have identified as the products of the late genes of phage P22. Figure 5(a) shows the mobility of the four proteins that we used as standards ($\beta$-galactosidase: 130,000; phosphorylase A: 94,000; pyruvate kinase: 57,000; and alcohol dehydrogenase: 37,000) and which were run together with a preparation of purified phage labeled with $^{14}$C-labeled amino acids in the cylindrical gel system. From the known molecular weights of the standards, we estimated the molecular weights of P1 (94,000), P9 (76,000), P16 (69,000), P5 (55,000) and P20 (50,000). In order to detect possible anomalies in the migration of phage polypeptides, two different gel concentrations were used: the two gels agreed in all molecular weight assignments to within 10%.

Figure 5(b) shows the relation of mobility to the logarithm of molecular weight for another gel (10%) of purified phage (this is the gel shown in Plate V and Fig. 4(b)). Since we know the molecular weights of the first five proteins from Figure 5(a), the fact that they fall on a straight line indicates that these proteins are migrating normally. By extrapolation we could tentatively assign molecular weights to P26 (23,000) and PX (18,000). This completes the set of polypeptides that we can detect in purified phage particles.

In order to find the molecular weights of the late phage proteins present in the lysate but not found in phage particles, we plotted the mobility (Fig. 5(c)) of the bands in the slab gel shown in Plate IV. Using the straight line produced by the proteins we estimated the molecular weights of P2 (63,000), P8 (42,000) and PY (27,000). We could also check the assignments for P26 and PX; the result was identical with that found from Figure 5(b).

### (h) *Are phage polypeptides cleaved during head assembly?*

Since the discovery that the cleavage of large precursor polypeptides is an integral part of the morphogenesis of poliovirus (Jacobson & Baltimore, 1968) and coliphage T4 (Laemmli, 1970; Hosoda & Cone, 1970; Dickson *et al.*, 1970; Kellenberger & Kellenberger-van der Kamp, 1970) it has become clear that this mechanism is common, if not universal, in the processes of head assembly and nucleic acid encapsulation in a variety of virus systems. Plate VI shows the results of an experiment designed to detect such cleavage, if it occurs, during the growth of phage P22. Cells previously irradiated with u.v. light were infected with phage P22 and pulse-labeled

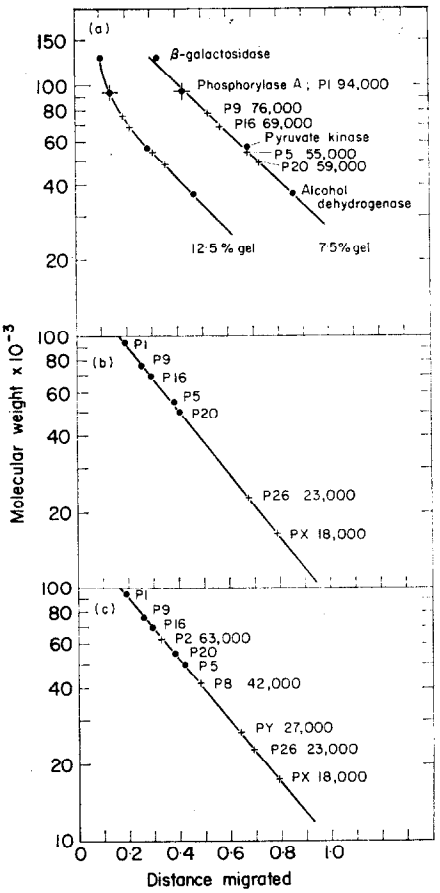


FIG. 5. Determination of the molecular weights of phage polypeptide chains. (a) P1, P9, P16, P5 and P20 from purified phage labeled with $^{14}C$-labeled amino acids, relative to standards of known molecular weight. The standards were stained with Coomassie blue before autoradiography. The method is described in detail by King & Laemmli (1973); in this case the discontinuous cylindrical gel system was used. (b) P26 and PX from purified phage relative to the other 5 phage proteins. The gel shown in Plate V and Fig. 4 was used. (c) P2, P8 and PY relative to the 7 phage particle proteins. The slab gel shown in Plate IV was used for these determinations. (●) Mol. wt known; (+) mol. wt calculated.

with $^{14}C$-labeled amino acids for a short time either early (5 min after infection) or late (25 min after infection). Excess unlabeled amino acids were added, and samples taken at intervals, concentrated, and the total lysates examined by sodium dodecyl sulfate/acrylamide gel electrophoresis. The results show no indication of the preferential disappearance of any of the phage bands from the lysate during the chase, nor is there any indication of the preferential appearance of any new bands. Both might be expected if there was cleavage of any of the major proteins that we can detect on these electrophoretograms. There is some general loss of radioactivity during the chase; however, this affects all bands, equally, as shown by densitometer tracings of each of the autoradiograms (data not shown). The reason for this general degradation of proteins is not understood, although it was regularly observed in our experiments.

In order to exclude the possibility that prior irradiation might interfere with cleavage of a polypeptide which might otherwise occur, a similar experiment was carried out without u.v.-irradiation. As shown above, only P1, P20, P16, P5 and P8 can be seen above the background of host protein synthesis in this circumstance; however the experiment clearly showed that these four proteins are neither subject to cleavage nor the result of cleavage of a larger precursor (data not shown).

Thus phage P22 seems to constitute an exception to the rule that cleavage of polypeptides accompanies virus head assembly and nucleic acid encapsulation.

## 4. Discussion

In the Results section we have described the genes and proteins involved in head assembly and DNA encapsulation of phage P22. We examined three different aspects of the defective phenotype of mutants in each of the known late genes: aberration in DNA maturation; the accumulation of head-related structures in the mutant lysates; and the identification of the proteins specified by each gene. The phenotypes that we found are summarized below (Table 3 and Fig. 6).

TABLE 3

*Gene products and mutant phenotypes*

| Gene | Phenotype | | Protein products | | |
|---|---|---|---|---|---|
| | DNA-cutting | Heads (EM) | In mature phage | Mol. wt | Number per phage |
| *1* | No | Prohead | Yes | 94,000 | 20 |
| *2* | No | Prohead | No | 63,000 | — |
| *3* | No | Prohead | | not identified | |
| *5* | No | None | Yes | 55,000 | 400 |
| *8* | No | Few aberrant | No | 42,000 | — |
| *10* | Yes | Full (unstable) | | not identified | |
| *26* | Yes | Full (unstable) | Yes | 23,000 | 30 |
| *9* | Yes | Full (tailless) | Yes | 76,000 | 20 |
| *16* | Yes | Full | Yes | 67,000 | 10 |
| *20* | Yes | Full | Yes | 50,000 | 20–30 |
| *X* | not identified | | Yes | 18,000 | 10 |
| *Y* | not identified | | No | 27,000 | — |

(1) DNA maturation. With respect to DNA maturation we found three kinds of mutant phenotypes. During infections with mutants in genes *1*, *2*, *3*, *5* and *8*, DNA accumulates in the DNA replication complex (intermediate I). In all of these cases the replication of the DNA is apparently normal; concatemers are formed, but essentially none of the DNA is cut to the mature phage length. Another DNA maturation phenotype is that represented by mutants in genes *10* and *26*, in which DNA is cut to the mature length after synthesis, but ultimately is degraded into small fragments. The third class of mutants (in genes *16* and *20*) have normal patterns of DNA synthesis and maturation.

(2) Head-related structures. Three distinguishable kinds of head-related structures accumulated in mutant lysates: one of these (found with mutants in genes *1*, *2* and *3*) is a rounded structure that seems to be neither empty (in the sense that a phage ghost is empty) nor full. Instead, these head structures seem to be full of something

other than DNA; we believe these structures to be precursors of final heads (see accompanying paper). For convenience, we call them proheads (Laemmli, 1973).

Two direct pieces of evidence can be adduced from the present results to support the precursor hypothesis: first, any lysate that contains head-related structures in large

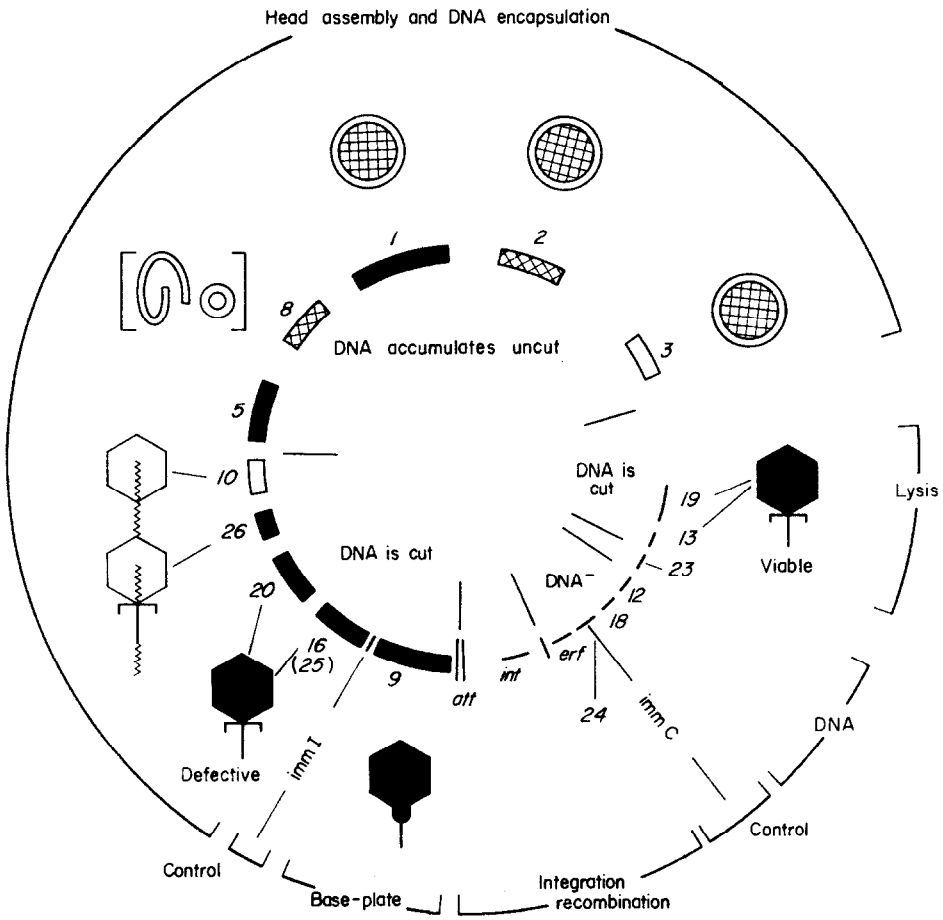


FIG. 6. Genetic/functional map of phage P22. Each of the late phage genes is located on the genetic map according to Botstein *et al.* (1972). Where the proteins have been identified, the length of the bar is in proportion to the protein mol. wt; the map is normalized to the coding capacity of the phage P22 genome. Solid bars represent proteins found in phage particles; crosshatched bars represent proteins found in lysates but not in mature particles; open bars show genes not yet identified with a protein product. Inside the circular map the DNA maturation phenotype is indicated; outside are diagrams showing the morphology of particles (if any) that accumulate in amber mutant lysates.

numbers contains some of these partially empty forms; second, double mutants where one of the mutants alone would normally accumulate the partially empty form, and the other some other (by hypothesis, more advanced) form always accumulate the partially empty form. A second kind of head-related structure that we found in mutant lysates is an empty (ghost-like) head. This kind of particle accumulates in

lysates of $10^-$ and $26^-$ mutants; it is a collapsed structure, which may have a base-plate ($26^-$) or not ($10^-$). By the reasoning presented above (and in the accompanying paper), we believe that this form represents a later stage in head morphogenesis than the $1^-$, $2^-$ or $3^-$ prohead, since double mutants such as $10^- 3^-$ accumulate the prohead and not the empty head form. We also have presented evidence that the empty head form is probably not the form that actually accumulates inside the mutant-infected cell. Instead, we believe that the empty heads represent the breakdown product of a full head that is unstable. Possibly the most convincing evidence that this is the case is our ability to rescue whole active phage from $10^-$ and $26^-$ mutant lysates by *in vitro* complementation. The third kind of head structure that we have observed is an apparently normal but inviable phage particle that accumulates in $20^-$ and $16^-$ mutant infections. Of course, we also observed, in $9^-$ lysates, the base-plateless viable head reported by Israel *et al.* (1967); this observation suggests that gene *9* is the structural gene for the base-plate protein which they had purified.

(3) Gene-protein identification. Using sodium dodecyl sulfate/polyacrylamide gel electrophoresis of labeled lysates of cells infected with chain-terminating (amber) mutants, we have identified proteins specified by eight of the ten late phage genes and we have identified the genes that specify six of the seven polypeptide chains found in purified phage particles (Table 3). We also did experiments designed to detect specific cleavage of polypeptide chains during the times that head morphogenesis occurs; these experiments show no cleavage of any of the proteins that we can detect in our system, leading us to the conclusion that cleavage of a major protein species may not be a normal part of morphogenesis of phage P22. This conclusion must be tempered by the following: cleavage of very small pieces or large pieces in small amount would not have been detected; also, extraordinarily rapid cleavage (restricted to the pulse period) might possibly have gone undetected. In any event, the gross sort of cleavage found in other phages appears not to occur in P22.

In Figure 6, our results are summarized in graphic form; each gene is marked by a bar proportional in length to protein molecular weight; the bar also indicates whether or not its product is found in the mature virion (heavy solid bar), found only in the lysate (hatched bar), or not identified (open bar). The kind of structure found in lysates is also shown, as is the phenotype of mutants with respect to DNA maturation.

### (a) *Relation of DNA maturation to head encapsulation*

We found a close relation between the cutting of the intracellular DNA concatemers and the encapsulation of the DNA into phage particles. Mutants in genes *1*, *2*, *3*, *5* and *8* fail to mature the phage DNA (as measured by removal of the DNA from the replication complex and the appearance of phage-sized DNA); these also produce either no normal head-related structure at all (genes *5* and *8*) or an early precursor form (the prohead; genes *1*, *2* and *3*). Some head structures accumulate in $8^-$ infections (see accompanying paper), but these are clearly aberrant. Since gene *5* specifies the major capsid subunit, this result implies that efficient DNA maturation cannot proceed in the absence of the morphological head. On the other hand, mutants in genes *10* and *26* mature the DNA; they also encapsulate it, although the resulting capsid is unstable unless acted on, either *in vivo* or *in vitro* by the gene *10* or gene *26* protein. The stabilization of these unstable heads *in vitro* is quite efficient, which leads us to conclude that the unstable head is probably quite a late intermediate in head assembly. More definitive evidence for this view is presented in the accompanying

paper. Mutants in genes *20*, *16* and *9* mature the phage DNA stably, and they also encapsulate it into a stable head. In the case of gene *9* mutants, the heads produced are simply lacking the base-plate; it can be attached *in vitro* (Israel *et al.*, 1967) to produce normal infectious phage particles.

Thus the results indicate that the removal of phage DNA from the replication complex (intermediate I) and the cutting of the DNA concatemers to mature phage length is associated intimately with the encapsulation of this DNA. The cutting event apparently requires that head assembly proceed to the point of encapsulation; although there is some spontaneous cutting late in infection in all mutants, this does not result in mature DNA lengths.

There is one feature of the DNA maturation experiments which suggests that the function of gene *3* is more directly related to the cutting of concatemer DNA than the functions of genes *1*, *2*, *5* and *8*, which have a similar gross phenotype. The DNA in the replication complex in gene 3$^-$ infections is noticeably more stable for the first hour than is the case with mutants in the other four genes. Schmieger (personal communication) has evidence that mutations in gene *3* can result in greatly increased formation of generalized transducing particles; these mutants are much more active in degrading host DNA after infection than wild type (Schmieger, 1972).

### (b) *Protein composition and structure of P22 phage particles*

From our electrophoretic analysis of purified phage particles isolated after labeling of infected cells with radioactive amino acids, we were able to see seven bands, each representing one of the polypeptides that make up the protein of the phage particle. For each of these polypeptides we were able to make an estimate of molecular weight, which is probably accurate to 10%. By integrating the density on the autoradiogram contributed by each band, we could estimate the relative contribution of each polypeptide to the total radioactivity incorporated in the particle protein. This requires that the polypeptides be equally labeled; since we did not carry out steady-state labeling for this experiment, but instead labeled and chased at various times after infection, we must assume that labeling of the various polypeptides was uniform. This assumption is made more reasonable by the observation that the relative contribution to the major bands did not vary significantly if labeling was done between 20 and 40 minutes after infection. In addition, a preliminary experiment, in which purified phage were analyzed and the polypeptides stained with Coomassie blue (Casjens, unpublished experiments), gives a pattern similar to our autoradiograms.

Using the relative contributions of each polypeptide to the total radioactivity in the phage particles, we can estimate the number of polypeptide chains in each phage particle, since we know the approximate molecular weight of all the protein in a phage. The molecular weight of the phage DNA is 26 to $27 \times 10^6$ (Rhoades *et al.*, 1968), and the phage is about 50% protein and 50% DNA (Israel *et al.*, 1967), making the total protein molecular weight about 26 to $27 \times 10^6$. The values for number of chains per particle are shown in Table 3. These values, given all of the assumptions, cannot be more accurate than about 20%, especially for the smaller proteins. Nevertheless, the value of 400 P5 molecules per phage suggests that the isometric P22 head might follow the rule of Caspar & Klug (1962) for icosahedral deltahedra with triangulation number equal to 7, which requires 420 subunits.

The base-plate of phage P22 is made up of P9 and has sixfold symmetry (Anderson, 1960) and its subunits assemble onto the phage head *in vitro* with sixfold stoichio-

metry (Israel *et al.*, 1967). Thus, our value of 20 subunits per phage means either that each base-plate part is a trimer (making 18 subunits per phage) or a tetramer (making 24 subunits per phage). Israel *et al.* (1967) estimated the molecular weight of each base-plate part as between 160,000 and 260,000. Our value for the molecular weight of the polypeptide subunit (P9) is 76,000; thus, we slightly prefer the hypothesis that the base-plate part is a trimer with a molecular weight of 228,000, thus making a total of 18 polypeptide subunits per phage particle.

(c) *Role of late phage gene products found in lysates but not in mature phage particles*

Among the proteins that we have detected in lysates are three (P8, P2 and PY) which are not present in detectable amounts in mature phage, although they are clearly the products of phage genes and are not present in lysates of early mutants that do not turn on late phage gene expression. Little can be said about PY, since we have no mutants in the gene that specifies its synthesis. The phenotypes of mutants in genes *2* and *8*, however, are known. To review, $8^-$ infections lead to the production of few normal head-like structures, whereas $2^-$ infections lead to the production of proheads. In neither case is DNA matured. Thus we conclude that P8 must, in some way, be involved in the proper assembly of P5 subunits into the head structure. In fact, the role of P8 includes this function; its role is much more exactly defined in the following paper. P2, on the other hand, is not necessary for the production of the prohead, although it apparently must act before encapsulation and the concomitant maturation of the DNA. As shown in the accompanying paper, P8 is associated with prohead forms but P2 is not. Thus, P2 might act in a catalytic way; for example, it might produce a change in the prohead and/or the concatemer DNA so as to prepare them as substrates for the encapsulation reaction. If this is the function of P2, it is of some interest that P2 is made in quite large amounts: in the gels the amount is similar to P1 or P20, each of which is made in the ratio of about 20 protein molecules per phage.

(d) *Function of genes* 16 *and* 20

Mutants in genes *16* and *20* have similar phenotypes: phage DNA maturation and encapsulation is apparently normal, producing particles that appear to be identical in appearance and stability to wild type phage particles. However, these particles are not infectious, and each type appears to be missing a particular protein species normally found in phage: $20^-$ particles lack P20 and $16^-$ particles lack P16. The *in vitro* complementation studies indicate that the addition of P20 and P16 to the head structure is probably not a late step in head assembly, because although $10^-$ and $26^-$ particles (which produce unstable full heads) are rescued *in vitro*, the $20^-$ and $16^-$ particles cannot be rescued under the same conditions. Therefore, we believe that P20 and P16 must assemble on the nascent head structure early in its assembly, before P10 and P26 act, and very possibly before encapsulation of the DNA and cutting of the concatemer DNA to mature size. In the absence of P20 and P16, assembly goes forward, but produces an aberrant defective form, which cannot then be rescued by simple addition of P20 or P16. This view is substantiated in the following paper, because P20 and P16 are found in the prohead structures, which are early intermediates in the head morphogenesis pathway.

In this way we are led to the conclusion that both P20 and P16 are assembled in the head structure early, but their function is actually needed only much later, i.e. when the particle infects a cell in the next cycle of infection. Preliminary experiments

(Osburne, Waddell & Botstein, unpublished results) indicate that the function of P20 and P16 is in the injection of DNA into the host; Hoffman & Levine (personal communication) have evidence that P16 may be directly involved in injection of phage DNA; a temperature-sensitive mutation affecting this protein exhibits a temperature-sensitive injection phenotype.

### (e) *Control of late phage protein synthesis*

In the course of identifying the proteins with the genes that specify them, we also obtained evidence about the amount and rate of synthesis of the various late proteins. The most obvious result is that the late proteins are synthesized in very different amounts: P5 and P8 are present in much greater amounts than the others; among the remainder, P9 is present in larger amounts than the remaining identified gene products. This situation is comparable to that found with other types of phage (Hosoda & Levinthal, 1968; Murialdo & Siminovitch, 1972). There might be some correlation in the amount produced from adjacent genes; however, in the absence of specific information about transcription of the late region, it is too early to make any judgements about the mechanism of differential gene expression within this region of the phage genetic map.

The only instance of polarity among the amber mutants that we tested is the reduction of the amount of P16 synthesized by a mutant of P20. If this observation truly represents a case of polarity, the direction of reading in this region would be from gene *20* to gene *16*, which corresponds with the direction found for the late genes of phage λ. In view of the great similarity in functional organization of the P22 and λ genetic maps (Botstein *et al.*, 1972) this result is not unexpected.

We presented evidence that phage P22 early mutants fail to express the late genes. Among the mutants we examined were mutants in genes *12* and *23*. Gene *12* is probably directly involved in DNA replication (Levine & Schott, 1971; Botstein *et al.*, 1972), whereas gene *23* is probably directly involved in control of the late genes, since $23^-$ mutants synthesize phage DNA normally (Margolskee, Hilliker & Botstein, unpublished results). The fact that neither $23^-$ or $12^-$ mutants express any of the late genes to any significant degree means that there is a tight control on late gene expression; the mechanism of this control system is not understood. Especially significant, however, is the fact that gene *9* is not expressed in either of the early mutants, because this means that the postulated late gene control mechanism exerts its effect on both sides of the secondary control region (*imm*I; Chan & Botstein, 1972), which is jointly responsible (with the clear region, or *imm*C) for immunity and repression in lysogens (see Fig. 6). The mechanism that controls late functions must act, therefore, at at least two points: one point to control gene *9* and at least one other point for the rest of the late genes.

### (f) In vitro *head completion*

In our attempts to assemble phage P22 heads *in vitro* we were able to rescue $10^-$ particles efficiently, $26^-$ particles less efficiently, and $16^-$ and $20^-$ particles not at all. These results are in a minor sense paradoxical: the $16^-$ and $20^-$ heads appear, by their morphology, to be more advanced forms in that they contain DNA stably encapsulated, whereas the $10^-$ and $26^-$ particles are unstable. The problem can be resolved if one postulates that P20 and P16, which are components of the mature phage, are in fact assembled into the structure early in the head-assembly pathway, but are not

essential to further progress of the encapsulation process itself. Thus in $10^-$ and $26^-$ particles, P20 and P16 are already part of the structure. This interpretation is supported by the experiments in the accompanying paper; early intermediates in head assembly (before encapsulation) contain both P20 and P16 (accompanying paper). Thus P20 and P16 are, in a sense, exceptions to the rule of Edgar and Wood that morphogenetic processes proceed sequentially (Edgar & Lielausis, 1968; King & Wood, 1969).

With respect to the roles of genes *10* and *26*, it is important to recall that $26^-$ particles appear to have base-plates, whereas $10^-$ particles do not. It is possible that the low efficiency of the $26^-$ particle rescue *in vitro* might in fact represent rescue of a minority of the particles in the lysate, i.e. those to which the base-plate assembly had not yet been attached. This interpretation would place the action of gene *10* before the addition of P26; in the absence of the gene *10* product, P26 does not assemble and all particles are ready for the action of both gene products, whereas in the absence of P26 most of the particles go beyond the gene *10* step, and cannot be rescued; only the minority of heads that have not been acted on by gene *10* product are rescued.

Nevertheless, the fact that $10^-$ particles are efficiently converted to a stable phage *in vitro* must mean that the unstable $10^-$ particle is a normal intermediate in prohead assembly. A similar argument applies to the ability to convert $9^-$ (baseplate-less) particles (Israel *et al.*, 1967) to viable phage.

### (g) *Comparison with other bacteriophages*

Head assembly and DNA encapsulation have been the objects of intensive investigation using bacteriophages T4, λ and P2 (Kellenberger, 1966; Laemmli *et al.*, 1970; Murialdo & Siminovitch, 1971; Kellenberger & Edgar, 1971; Lengyel *et al.*, 1973). Furthermore, work with phage T7 (Studier & Maizel, 1969; Studier, 1972) gives many hints about the probable course of the head assembly of this phage.

All the phages studied save P2 have been shown to synthesize DNA concatemers during the lytic cycle of growth (Frankel, 1968; Smith & Skalka, 1966; Thomas *et al.*, 1968), as is the case with phage P22 (Botstein & Levine, 1968*b*). In the case of phages T4 and λ, head assembly is essential to the cutting of these concatemers to phage length (Frankel, 1968; Dove, 1966; Salzman & Weissbach, 1967). We have found the same to be true of phage P22. However, the DNA structures of the phages under study differ; P22 and T4 have circularly permuted and terminally repetitious DNA, whereas P2 and λ have linear DNA with cohesive ends, and T7 has a terminally repetitious but not circularly permuted form. It has been pointed out that this difference might be less profound than at first seems to be the case (Thomas *et al.*, 1968).

With respect to genetic organization, the most closely related phage to P22 is phage λ (Botstein *et al.*, 1972; Dove, 1971). However, with respect to assembly, these phage are rather unalike. Phage λ has two major proteins in the head (Casjens *et al.*, 1970; Murialdo & Siminovitch, 1971); P22 has but one. Aberrant forms are found in many λ mutants, whereas P22 shows them in only one case (Kemp *et al.*, 1968; accompanying paper). On the other hand, as is the case with genes *10* and *26* of phage P22, lysates of mutants defective in genes *F* and *W* of λ produce unstable heads, which can readily be rescued *in vitro* (Casjens *et al.*, 1972). Recently, Wang & Kaiser (1973) have shown that the product of gene *A* of λ, which is found in lysates

but not in mature phage particles, is responsible for the cutting of λ DNA concatemers; Kaiser & Masuda (1973) have succeeded, although inefficiently, in encapsulating DNA *in vitro*.

Phage T7 is morphologically very similar to P22. A similarity in genetic organization can also be discerned, especially in the region concerned with head assembly. For example, Studier & Maizel (1969) report that there are two major head-related proteins in lysates, but only one in phage particles; the genes specifying these two proteins (*9* and *10*) are adjacent on the genetic map. There is another strong similarity to P22 in the T7 case: two genes of T7 produce morphologically normal, but defective, phage particles (Studier, 1972). As is the case with genes *16* and *20* of phage P22, there is some evidence that the defect in these particles might be in injection of DNA into the next host (Studier, personal communication).

Phage T4 is the most distantly related phage, both in genetic organization and morphology. However, the P22 proheads that we have observed as rounded partially full particles in certain phage mutant lysates bear a distinct morphological resemblance to the tau particles of T4 described by Kellenberger *et al.* (1968). These authors proposed that the material inside the tau particles constituted a "morphopoietic core". This question is discussed with respect to P22 proheads in the accompanying paper. On the other hand, T4, like λ, exhibits tubular and other aberrant structures in many different mutant lysates (Kellenberger, 1966).

P22 also differs from P2, λ and T4, in that there is no cleavage of major late proteins during head assembly.

To summarize, P22 and T4 have similar DNA structures but apparently different modes of head assembly, P22 and T7 have different DNA structures but possibly very similar modes of head assembly, and P22 differs from λ both in DNA structure and head assembly. Obviously, although head assembly and DNA maturation are in all cases intimately related, one has failed to give much of a clue to the other.

### (h) *Phage P22 head assembly as a morphogenetic system*

It appears that the head of phage P22 is a relatively simple structure, containing less than ten proteins, most of which have been identified as products of particular genes. Likewise, the base-plate assembly is extremely simple. The last three steps in the pathway proceed *in vitro*, making the prospect of rescuing earlier intermediates *in vitro* quite favorable. There are several steps in the pathway that produce stable intermediate structures, which (as shown in the accompanying paper) are likely to be true precursors and not aberrant dead-end structures. Finally, the DNA metabolism of this phage is quite simple and relatively well-defined: we have a good idea of the nature of the DNA substrate for the encapsulation reaction. We hope to use this system to elucidate the mechanism through which DNA is folded and encapsulated into an infectious virion.

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