JOURNAL OF VIROLOGY, Feb. 1976, p. 550-567

Vol. 17, No. 2
Printed in U.S.A.

Bacteriophage T4 Head Morphogenesis

VII. Terminal Stages of Head Maturation

DANIEL L. HAMILTON AND RONALD B. LUFTIG*

Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545

Received for publication 24 June 1975

Several aspects of the terminal stages of T4 head maturation were investigated using *ts* and *am* mutants blocked at single steps of the assembly pathway. We had previously found that cells infected with mutants of gene 13, e.g., *ts*N38 and *am*E609, accumulated both stable (10 to 20%)- and fragile (80%)-filled head precursors (Hamilton and Luftig, 1972). Here we showed the following for such gene 13-defective, mutant-infected cells. (i) Using thin-section analysis the pool of phage precursor structures observed under nonpermissive conditions was one-third of that observed when the cells were cultured under permissive conditions. (ii) In order for complete conversion of the precursors into viable phage to occur, there were apparent requirements of metabolic energy, protein, and DNA synthesis. (iii) The intracellular DNA pool under nonpermissive conditions exhibited a 50% distribution between 63*S* (mature size) and 200 *S* (concatenate size) DNA, with the latter DNA serving as a precursor pool. Further, this DNA pool when spread onto a protein monolayer exhibited a dispersed array of DNA strands around a core, which was less dense than that found for the >1,000*S* DNA concatenate isolated from gene 49-defective infected cells. (iv) When precuations were taken to stabilize the head precursors, such as lysis of the cells into glutaraldehyde, there was a 30% increase in the yield of 1,200*S* filled heads. Correlating these results and previous results concerning gene 49-defective unfilled heads, we propose that there are several forms of gene 13 fragile head precursors which serve as intermediates between gene 49 unfilled heads and gene 13 stable filled heads. We cannot, however, rule out the possibility that all gene 13-defective heads represent a single class of unstable particles, which decay slowly. In either case, we have shown that gene 13-defective particles are unstable to some degree inside the cell and are highly unstable outside the cell; yet all particles can still be efficiently converted to phage in vivo.

T4 head maturation has proven to be an exceptionally complex process, involving assembly of an initial head or prohead structure (2, 24, 25), the packaging of DNA from a >1,000*S* phage concatenate (12, 14) into a modified, intermediate head structure (26, 28, 30, 34), and, finally, the cleavage of a DNA "head full" from the intracellular DNA concatenate (37), followed by subsequent plugging of the head. These steps are controlled by at least 17 gene products, both structural and catalytic in nature (10, 15, 35). Moreover, this entire process is intimately associated with the DNA synthetic machinery of the cell (5, 28, 29). The role of the gene 13 product, namely, P13, acting at the terminal stage of this morphogenetic pathway has been of particular interest to us since: (i) it may act as a plug to hold the DNA inside the filled head, and (ii) it, together with P14, is needed for tail attachment to occur (9) and thus controls the formation of the junction through which DNA passes during infection of the host.

Previous experiments using conditional lethal mutants of gene 13 revealed that, under restrictive conditions, two classes of head precursors accumulated (16). One class, denoted stable-filled heads, was isolated as a filled head structure that contained a full complement of DNA and could be complemented in vitro. The second class, denoted as fragile-filled heads, was isolated as a partially filled head containing 12 to 32% of a phage DNA complement in a DNase I-resistant form. Both head precursors were efficiently converted to mature viable phage particles in vivo.

In this report we wish to further expand on the role of the precursors to gene 13 action in these mutant-infected cells. In particular, we wish to report on: (i) the metabolic requirements necessary for complete conversion of the precursor particles to mature phage; (ii) the nature of the intracellular, nucleic acid pool during this conversion; and (iii) the relationship of gene 13$^-$ precursor structures to gene 49$^-$ precursor structures.

(This work was presented in part at the 75th Annual Meeting of the American Society for Microbiology, May 1975).

MATERIALS AND METHODS

Media and buffers. Depending on the experimental design, growth of *Escherichia coli* cultures occurred in either minimal medium, to which various radioactive labels could be added, or in an enriched medium, which permitted rapid bacterial growth. The minimal medium (M9) contained 0.026 M Na_2HPO_4, 0.022 M KH_2PO_4, 0.019 M NH_4Cl, 0.1 M NaCl, 0.022 M glucose, 0.01 M $MgSO_4$, 10^{-4} M $CaCl_2$, and 6×10^{-9} M $FeCl_3$. The enriched medium of Hershey broth (H-broth) contained nutrient broth and peptone (Difco) in addition to 0.085 M NaCl and 0.006 M glucose. A detailed description of the preparation of top and bottom agars, used in plaque assays, as well as H-broth, is described by Steinberg and Edgar (36). Phosphate (PO_4) buffer (0.05 M, pH 7.2) and dilution buffer (PO_4 buffer containing gelatin) for phage serial dilutions were prepared as described by King (21). Tenfold-diluted PO_4 buffer containing 0.02 M $MgSO_4$ is termed 0.1× PO_4-Mg buffer and was used in preparing sucrose gradients.

T4 phage strains. The phage mutants used in this study are listed in Table 1. All are derivatives of T4D from the collection of R. S. Edgar and W. B. Wood and have been described previously (10, 11). Amber (*am*) mutants form plaques on the permissive host *E. coli* CR63, but not on the restrictive hosts *E. coli* Bb or B3. Temperature-sensitive (*ts*) mutants form plaques at 25 C but not at 42 C. Phage stocks were prepared as described by Epstein et al. (11) by using either H-broth or M9 as the growth medium.

For some experiments, double mutants of the (*am*,*ts*) classification were required. They were constructed by mixedly infecting a permissive culture (*E. coli* CR63 at 30 C) with four phage of each mutant per cell. After aerating for 10 min, 0.1 ml of the infected culture was transferred to a fresh bubbler containing 10 ml of medium and aerated for an additional 60 min. At the end of 60 min, 4 to 5 drops of $CHCl_3$ was added and the culture was agitated to cause lysis. After lysis, an aliquot was removed and diluted to yield 50 to 100 plaques on a double-agar plate. Individual plaques were picked by inserting the tip of a sterile Pasteur pipette over the plaque. After agitating the picked plaque in 1.0 ml of H-broth plus $CHCl_3$, a drop was spotted on several plates designed by culture strain and incubation temperature to determine if it was (*am*:+), (+:*ts*), (*am*:*ts*), or (+:+). After replicating to confirm its identity, high-titer stocks of the double recombinant (*am*:*ts*) were made as previously described.

E. coli host strains. *E. coli* Bb grows in M9 medium with generation times of 47 and 90 min at 41.5 and 25 C, respectively, and, like other *E. coli* B strains, it is restrictive for *am* mutants. Although a prototroph, it takes up ^{14}C-labeled amino acids efficiently and was used for isotope incorporation experiments. *E. coli* B3 (kindly provided by D. Hall, Duke University) was grown in M9 medium plus thymidine (5 μg/ml of culture medium) with roughly the same generation times as above. This *E. coli* B strain was used in experiments where [^{3}H]thymidine incorporation into phage DNA was required, since it increases the amount of label taken up by roughly 10-fold over *E. coli* Bb. *E. coli* B/5 and S/6/5 were also used as restrictive hosts for *am* mutants and for making stocks of *ts* mutants. *E. coli* CR63 was used for plaque assays and making stocks of *am* mutants.

Reagents. Chloroform for lysing infected cultures was Baker and Adams reagent grade. Sucrose for gradients was from Baker and Adams. Crystalline DNase I, used in preparing extracts, was obtained from Sigma Chemical Co. Lysozyme (salt free) for DNA extraction was from Worthington Biochemical Corp. Chloramphenicol was obtained from Calbiochem, as were fluorodeoxyuridine (FUdR) and rifampin. L-[U-^{14}C]lysine monohydrochloride and L-[U-^{14}C]leucine monohydrochloride at about 300 mCi/mol and [*methyl*-^{3}H]thymidine, [8-^{3}H]deoxyguanosine, [5-^{3}H]uridine, [^{3}H]arginine at 6 Ci/mmol were obtained from Schwarz/Mann. Glutaraldehyde was from Ladd.

General procedure for temperature shift experiments. The procedure for temperature shift experiments has been previously described (30). *E. coli* Bb or B3 was grown in a bubbler tube with vigorous aeration at 41.5 C to 3×10^8 to 4×10^8 cells/ml in M9 from an original inoculum of 1/40 volume of a freshly grown saturated culture. The cells were infected at a multiplicity of four phage per cell and incubated with vigorous aeration at 41.5 C. At 5 min after infection,

TABLE 1. *Characteristics of Mutants used*

Mutant	Gene	Defect	Components accumulated
*ts*N38 or *ts*N49	13	Head	13$^-$-defective heads, tails, tail fibers
(*ts*N38:*am*tA3)	(13; *t*)	Head, lysis inhibited	13$^-$-defective heads, tails, tail fibers
*am*E609 or *am*NG329	13	Head	13$^-$-defective heads, tails, tail fibers
*am*B17	23	Head	tails, tail fibers
*am*E727 or *ts*C9	49	Head	49$^-$-defective heads, tails, tail fibers

samples were removed and plated to determine both unadsorbed phage and bacterial survivors (~20 and ~2%, respectively, in all experiments reported). At 8 min after infection the cells were superinfected at the same multiplicity. Temperature shift-down to 25 C was accomplished by placing the still aerating bubbler in an ice bath for 0.5 to 1.0 min. Samples were taken at times indicated for phage assays, preparation of defective extracts, and electron microscopy, as described in Results.

Samples for assay of intracellular phage were diluted 100-fold into $CHCl_3$-saturated dilution buffer and shaken vigorously before further dilution and plating.

For preparation of defective extracts, a 5-ml sample of the culture was chilled in ice and centrifuged at 3,000 × *g* for 5 min. The cell pellet was then suspended in 0.4 ml of 0.1× PO_4-Mg buffer, shaken vigorously with 2 to 3 drops of $CHCl_3$, and then incubated for 30 min at room temperature after the addition of DNase I to 80 μg/ml. The resulting extract was centrifuged at 8,000 × *g* for 10 min and the pellet was discarded. Experiments were performed with extracts made the same day and left at 4 C before use.

The labeling regime for pulse-chase temperature-shift experiments is described in Fig. 1.

Zone sedimentation through sucrose gradients. Linear sucrose gradients (9.8 to 10 ml [20 to 40%, wt/vol] or 4.8 to 5.0 ml [10 to 30%, wt/vol]) were made in 0.1× PO_4-Mg buffer. Samples of 0.2 to 0.4 ml were layered onto gradients and centrifuged either in an SW41 rotor of a Spinco model L3-50 ultracentrifuge for 40 min at 22,000 rpm at 8 to 12 C (20 to 40% sucrose gradient) or centrifuged in an SW50 rotor for 25 min at 23,000 rpm at 8 to 12 C (10 to 30% sucrose gradient). Fractions were collected through the bottom of the tubes by using a tube-piercing and -collecting device equipped with a 20-gauge needle.

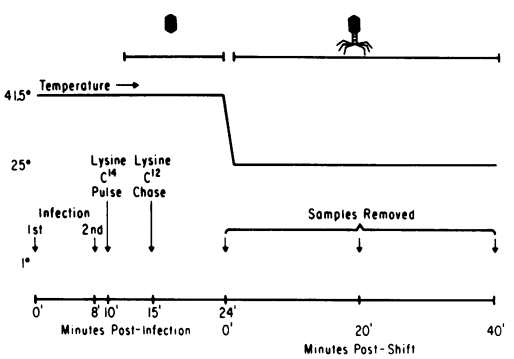

FIG. 1. *Generalized labeling and incubation regime for pulse-chase, temperature shift experiments with tsN38. E. coli Bb or B3 cells were grown, infected, and shifted in temperature as described in the text. [^{14}C]lysine was added at 10 min postinfection to a level of 0.2 to 0.4 μCi/ml, followed by a chase of a 1,000-fold excess of [^{12}C]lysine 5 min later. When present, [^{3}H]thymidine was added at 5 min postinfection to a level of 20 μCi/ml and chased 5 min later with a 1,000-fold excess of cold thymidine. After the temperature shift, samples were removed and placed on ice. Subsequent treatment of the samples is described in the text.*

Absorbancies at 260 nm were determined on 20-drop fractions by using a Gilford 2400 spectrophotometer equipped with microcuvettes. For measuring radioactivity, 20-drop fractions were precipitated in 5% trichloroacetic acid and the precipitates were collected on a membrane filter. The filters were then dried and placed in glass vials containing 6 ml of scintillation fluid (20 ml of Liquifluor [New England Nuclear Corp.] to 1 pint [0.47 liter] of toluene) and counted in a Beckman liquid scintillation counter at approximate efficiences of 80% for ^{14}C and 30% for ^{3}H. Background was substracted from all fractions. In some experiments aliquots of the sample were placed directly on GF/A or GF-C filters, dried, and treated as above.

Preparation of T4D DNA. T4 DNA used as a sedimentation marker was prepared according to Frankel et al. (13). Phage particles labeled with [^{14}C]thymidine (0.20 μCi/ml of media) were obtained from a culture of *E. coli* B3 infected with T4D. After a 1- to 2-h incubation, the cells were lysed with $CHCl_3$ and treated with 40 μg of pancreatic DNase per ml. Cell debris was removed by centrifugation at 3,000 × *g* for 15 min, and the phage were collected at 14,000 rpm for 90 min. After the pellet was resuspended in M9 medium, 0.75% sodium lauryl sarcosinate (Geigy Chemical Co.) and 0.005 M EDTA were added, and phage heads were disrupted by heating at 65 C for 10 min.

Preparation of intracellular T4D DNA. The procedure utilized was a modification of Frankel et al. (13). Cultures of *E. coli* B3 at 2 × 10^8 cells/ml in M9 medium were infected with 3.2 phage/cell. Eight or ten min after infection, 20 μCi of [^{3}H]thymidine per ml was added. In some experiments a "chase" of thousandfold excess of cold thymidine was added 5 min later. Samples (3 ml) were removed and diluted in 2 ml of ice-cold media. After one min, 5 ml of ice-cold solution containing 2 mg of lysozyme per ml, 0.1 M KCN, and 0.005 M EDTA (pH 8.5) was added. After 15 min, sodium lauryl sarcosinate was added to a final concentration of 3%. Five minutes later the tube contents were gently mixed by rotating at 5 rpm for 10 min at 5 C. Samples were stored at 4 C.

Zone sedimentation analysis of intracellular DNA. By using a wide-mouth pipette, 0.2 ml of a DNA sample was gently layered onto 4.8 ml of a 20 to 70% linear sucrose gradient. The sucrose solutions contained 0.9 M NaCl, 0.01 M EDTA (pH 8.5). The tubes were centrifuged in an SW50 rotor of a Spinco model L3-50 ultracentrifuge at 32,000 rpm for 90 min at 10 C. Samples were collected dropwise through the bottom of the tube by using a tube-piercing device equipped with 20-guage needle onto 2.4-cm paper disks (Whatman no. 3MM). After drying, the disks were washed with cold 10 and 5% trichloroacetic acid (45 min each) and acetone (15 min) and counted for radioactivity as described earlier.

Zone sedimentation of T4 DNA through alkaline sucrose. Alakline sucrose gradient sedimentation was

conducted by the procedures of Kelly and Thomas (20). The sample was first denatured by the addition of an equal volume of 1 N NaOH. It was then carefully layered onto a 5 to 20% linear sucrose gradient (4.8 ml) made up in 0.9 M NaCl, 0.1 M NaOH. The tubes were centrifuged in an SW50 rotor of a Spinco ultracentrifuge at 32,000 rpm for 90 min at 18 to 20 C. Samples were collected by drops and counted for radioactivity as described above.

Thin-section electron microscopy. Procedures used for fixation and embedding were modified from those described by Kellenberger et al. (19). In procedure I, 5 ml of a culture sample was immediately mixed with 5 ml of 2% OsO_4 in Michaelis buffer, pH 6.0. Casamino Acids (Difco) were added to a final concentration of 2%, and the suspension was left overnight at room temperature. The cells were then pelleted, embedded in 1% agar (Difco Noble agar), and prestained for 1 h with 2% uranyl acetate (distilled water), followed by dehydration by repeated soaking in acetone and by two 0.5-h washes in propylene oxide. The dehydrated pellets were then embedded in Epon 812.

For procedure II (partial lysis), a 10-ml sample was chilled and pelleted by centrifuging at 10,000 × *g* for 5 min. The pellet was resuspended in 1 ml of chilled PO_4 buffer, followed by the addition of 1 ml of 0.1% OsO_4 in veronal-acetate buffer (pH 7.2) to cause partial lysis. After 3 min at room temperature or when some clearing of the solution occurred, 0.5 ml of 5% glutaraldehyde was added to stop lysis. The sample was pelleted by centrifuging at 10,000 × *g* for 10 min, and the pellet was incubated overnight at room temperature in a solution of 1% OsO_4-2% Casamino Acids in veronal-acetate buffer, pH 7.2. After fixation in OsO_4, the pellet was prestained with 2% uranyl acetate (distilled water) and dehydrated in a graded series of ethanol washes, finishing with two 0.5-h washes in propylene oxide. The dehydrated sample was then embedded in Epon 812.

Samples were sectioned on an MT2-B Sorvall Porter-Blum ultramicrotome, using glass knives made with a Sunkay Messer knifemaker or a Dupont diamond knife. Sections were mounted on grids carrying carbon-coated collodion films and stained with 20% uranyl acetate in methanol for 20 min. Sections were also stained for 10 min with a 1:10 dilution in 0.1 N NaOH of Reynolds lead citrate (33).

DNA spreading for electron microscopy. For visualization of intracellular T4 DNA, a sample was prepared as described above, with one modification. A 40-ml sample of the infected cells was pelleted, the pellet was resuspended in 5 ml of chilled M9 medium, and lysis and preparation was as above. The sample was stored at 4 C until used. The spreading shadowing of the DNA was by the procedure of Davis et al. (7). An aliquot of the prepared sample was diluted 100-fold into "spreading solution," which consisted of 0.1 mg of cytochrome *c* per ml, 0.1 M Tris, and 10 mM EDTA (pH 8.5) in 40% formamide (Matheson, Coleman and Bell). After gentle mixing, 0.05 ml of the suspension was carried up in a Teflon tube connected to a Hamilton syringe and slowly layered on to a hypophase solution of 10 mM EDTA (pH 8.5) in 10% formamide prepared immediately before use. The layering was aided by a glass-slide "ramp" placed in the hypophase solution. After 30 s to 1 min, the DNA was picked up on a 1-day-old collodion-coated grid by touching the grid to the surface of the solution. After staining for 30 s in 5×10^{-5} uranyl acetate in 90% ethanol and rinsing for 10 and 90% ethanol, the grid was air dried. The dried grids were then rotary shadowed at an angle of 8° with platinum (80%)-paladium (20%) in an Edwards vacuum coating unit equipped with a rotating stage. The prepared grids were examined in the electron microscopy as described above.

RESULTS

During the terminal stages of T4 head maturation a full complement of phage DNA is packaged inside the capsid, resulting in a filled head structure which is stable to lysis of the infected cells (9, 10, 26, 28). We have been interested in delineating the steps of this terminal process by characterizing the intermediate head structures formed when specific gene products involved in these stages are blocked. Based on in vitro complementation experiments, the products of T4 genes 13 and 14 have been postulated to function at this final step in head packaging (9, 16). Our previous results studying intermediates in gene 13 mutant infections have demonstrated that there are at least two classes of T4 head precursors: stable-filled heads (10 to 20%) and fragile-filled heads (80%), each of which can be efficiently converted to viable phage upon restoration of the missing or altered gene product (16). These earlier studies extensively utilized mutant *ts*N38, which was specifically selected from a collection of several mutants in gene 13 since it most efficiently led to rescue of a wild-type burst of phage after a temperature shift-down of the mutant-infected cell culture (16). Evidence from this earlier study, as well as from the present one, has confirmed that the phenotype of *ts*N38-infected cells was gene rather than mutant specific. That is (i) these cells and *am*E609-infected cells exhibited the same type and number of head structures in lysates prepared by three different procedures (16); (ii) *ts*N49, another gene 13 mutant, showed a similar redistribution as *ts*N38 of label from unfilled and filled heads into phage (D. Hamilton, unpublished data); (iii) *am*E609 and *am*NG329 gave the same profile of label at 0, 20, and 40 min post-shift-down, as found with the *ts*N38 heads at 0-min shift-down (15; D. Hamilton, unpublished data); and (iv) *ts*N38 and *am*NG329 showed the same intracellular DNA pools for 24 min postinfection at 41.5 C (Fig. 4, 5). We have now extended these earlier studies, again using

*ts*N38 as well as other gene 13 mutants, to obtain an estimate for the level of complexity of this assembly process. Specifically, we have investigated: (i) what is the size of the pool of phage precursor structures in these cells when grown under nonpermissive conditions; (ii) what, if any, metabolic requirements are necessary for complete rescue of the precursors; and (iii) what is the state of the intracellular DNA pools inside the gene 13-defective cells? In these latter studies we found that the intracellular DNA pool showed a 50-50% distribution between 63*S* and 200*S* DNA. This was unexpected since, assuming a constant specific activity for the [^{3}H]DNA in these two forms and assuming that fragile-filled heads were associated with the 200*S* DNA, we would have expected a 20 to 80% DNA ratio, corresponding to the ratio of stable- to fragile-filled heads that had been found in these cells (16). This result, therefore, led us to conduct a more careful examination of (iv) the in situ state of gene 13-defective heads and the effect of different methods of cell lysis on increasing the stability of head structures present in gene 13-defective infected cells. Finally, to integrate our results with previously studied intermediate steps in the pathway (26, 28), we have (v) compared the different classes of gene 13-defective head structures with those found in gene 49-defective infected cells (gene 49 presumably controls the step preceding gene 13 in T4 head assembly [28]), before and after treatment of infected cells with FUdR, an inhibitor of DNA synthesis. The results are presented below.

(i) Estimate of the percentage of gene 13-defective phage precursors. We analyzed the number of filled head particles observed in thin sections of *am* mutant-infected cells grown under nonpermissive and permissive conditions. Amber, rather than temperature-sensitive, mutants were used to minimize recovery of the gene 13 function during specimen preparation. The particles observed in both gene 13$^+$ and gene 13$^-$ cells appeared to be almost all filled and nearly identical in appearance to T4D$^+$ (16; Fig. 7B). However, there were only about one-third as many intracellular filled head particles in gene 13$^-$- as in 13$^+$-infected cells (Table 2). The number of particles in 13$^+$-infected cells, namely, 18 phage particles per cell section, corresponded to a burst size of about 150 phage/cell (19) and is nearly equivalent to the average burst size of 160 phage obtained at 40 min after a shift-down of *ts*N38-infected cultures (16). Both of these values are similar to a wild-type burst of 175 ± 30 phage obtained for T4D$^+$-infected *E. coli* Bb cultured under identical conditions. These results strongly suggest that, under nonpermissive conditions, gene 13 mutant-infected cells accumulate approximately one-third of the normal number of head structures per infected cell. A nearly identical result had previously been shown for *ts*C9, a mutant in gene 49 that accumulates unfilled head intermediate structures (30).

TABLE 2. *Number of filled head particles observed in thin sections of amNG329 grown under nonpermissive and permissive conditions*

Phenotype[a] expressed	Total no. of intracellular filled head particles	Avg no. of particles/cell section
13$^-$	85	6
13$^-$	160	5.7
13$^+$	245	18

[a] 13$^+$ refers to growth of *am*NG329 in *E. coli* CR63 for 24 min. The particles per thin section are equivalent to a burst size of about 150 phage/cell (19). 13$^-$ refers to growth of *am*NG329 in *E. coli* B/S/6 for 24 min.

(ii) Metabolic requirements for rescue of gene 13-defective head precursors from tsN38-infected cells. We found that there was an apparent requirement of metabolic energy, which was related to both a need for macromolecular protein and DNA synthesis for this process. The requirements were assayed by using one of several metabolic inhibitors and then looking for the number of residual viable phage in the following type of experiment. A 20- to 30-ml culture of *E. coli* B3 was infected at 41.5 C as described in Fig. 1. At 21 min after infection, the metabolic inhibitor was added, followed 2 min later by the addition of an appropriate radioactive compound to measure the effectiveness of the inhibitor. The culture was shifted to 25 C at 24 min after infection, and small samples (0.1 ml) were subsequently removed to measure the incorporation of the radioactive compound as well as to assay for the production of viable phage. These results were compared to a similarly treated control, which lacked the presence of an inhibitor.

The first metabolic requirement tested was that for ATP energy. A potent inhibitor of this energy source is potassium cyanide, an uncoupler of oxidative phosphorylation which previously has been used to exhibit the need for energy metabolism at early steps of both bacteriophage λ (1) and T4 infections (28). However, a difficulty with using cyanide at late stages of a T4 infection is that it causes premature lysis

(8). This difficulty was overcome by constructing the double mutant (*am*tA3:*ts*N38). This mutant, when grown under restrictive conditions for the *t* gene, prevents cellular membrane breakdown, and premature lysis is prevented even in the presence of cyanide (18). The effect of 30 mM cyanide on phage production with this double mutant is shown in Table 3. The recovery of viable phage was reduced to 7% of the control, which indicates a block both in the rescue of "precursor" particles as well as in the "de novo" synthesis of phage; i.e., based on the results from Table 2, we would have expected a burst size about 30% of the control, if all the "precursor" particles were rescued. There was also a significant block in conversion of labeled gene 13$^-$ precursor particles to phage (Table 3, last column). These results suggest that ATP energy is required for conversion of >90% of gene 13$^-$-defective precursor structures found in *ts*N38-infected cells to viable phage. Whether such need for metabolic energy might be associated with a need for macromolecular protein, DNA, or RNA synthesis was then analyzed. Chloramphenicol was added to cultures (Fig. 1) at a concentration of about 50 μg/ml. This dose, which decreased amino acid incorporation to a 5% level of normal, was effective in reducing the assembly of phage in (*am*tA3:*ts*N38)-infected cells to 8% of that of the untreated control. Again, based on results of Tables 2 and 3, de novo protein synthesis therefore appears necessary for conversion of >70% of the gene 13$^-$-defective phage precurssors to viable phage in *ts*N38-infected cells. Whether this indicates a need for new gene 13 product or the need for some short-lived late protein(s) that may be involved in an undetermined step of morphogenesis is unknown.

The necessity of DNA synthesis for assembly of gene 13-defective heads was determined by the addition of FUdR, an inhibitor of T4 DNA synthesis (6, 31), to the *ts*N38-infected culture 3 min before the shift to 25 C. Since the gene 13-defective "precursor particles" appear filled in thin sections (16, Fig. 7B), it is unlikely that there is insufficient packageable DNA present for rescue of these particles. We found that the burst size was decreased in the presence of FUdR, yielding less than a third as many phage rescued as the untreated infected cell control (Table 3). Although this number could be accounted for by rescue of all the gene 13-defective heads, this appears unlikely, since only 30% of precursor label was converted to phage (Table 3). Thus, again there appears to be a block in the rescue of gene 13$^-$-defective precursor particles, as well as in the rescue of de novo synthesized phage precursors. Rifampin, an inhibitor of RNA synthesis in vitro (17, 32), did not have any effect on phage assembly in these experiments when added at a concentration of 200 μg/ml (D. Hamilton, unpublished data).

The above results indicated that conversion of head precursors to phage in *ts*N38-infected cells apparently required metabolic energy associated with both protein and DNA synthesis. Further, since chloramphenicol only slightly inhibits DNA synthesis and FUdR has relatively no effect on protein synthesis (Table 4), these results suggest that the protein and DNA synthetic requirements are independently involved in the recovery of gene 13-defective precursors from these cells. A similar independence of protein synthesis from the action of FUdR in T4 has been reported (23). One possible explanation is that protein synthesis is required for the production of P13 or a late protein(s) involved in gene 13 action and that this protein acts at the step where stable-filled, gene 13-defective heads are rescued. The ability of gene 13-defective, stable-filled heads to be complemented in vitro with a gene 23-defective extract (supplying P13 and other late proteins) is consistent with this interpretation. Our data with FUdR can then be explained by a mechanism whereby rescue of the

TABLE 3. *Effect of metabolic inhibitors on conversion of gene 13$^-$-defective heads to viable phage*

Inhibitor	Label	Relative incorporation of label after shift-down (%)	Viable phage[a] produced at 40 min post-shift (%)	% of precursor[b] label converted to wild-type phage
KCN (30 mM)	[^{3}H]arginine	9	7	2
Chloramphenicol (62 μg/ml)	[^{3}H]arginine	5	8	33
Fluorodeoxy-uridine (200 μg/ml)	[^{3}H]deoxyguanosine	30	31	33 ± 3

[a] Infection and incorporation of inhibitors into the culture is described in the text. Burst size at 40 min post-shift for the control was 110 ± 20 PFU/infective center.

[b] A description of this experiment is given in the text and in Fig. 2.

other gene 13-defective heads, namely, fragile-filled heads, requires DNA synthesis. As described above, these contentions were supported by experiments in which the fate of labeled head precursors in the presence of potassium cyanide, chloramphenicol, or FUdR was observed (Table 3, last column).

Such experiments were conducted as described in Fig. 1, with the following modifications. At 21 min after infection of *E. coli* B3 with (*am*tA3:*ts*N38), the inhibitor (potassium cyanide, chloramphenicol, FUdR, respectively) was added to one-half of the culture, the other half being used as a control. Two min later, [^{3}H]arginine or [^{3}H]thymidine, respectively, was added to the appropriate inhibited culture and its control to measure the effectiveness of the inhibition. The results are shown in Fig. 2 and have already been quantified in Table 3. Figures 2a and d show the distribution of label at the time of the shift (a) or at 40 min (d) after the shift, in both cases without inhibitor added. Figure 2b shows the distribution of label at 40 min after the shift to 25 C in the presence of FUdR; we noted here that in contrast to Fig. 2d only about 30% of the total label has been converted to the 1,000*S* phage peak, whereas almost none of the label is found in the 1,200*S* filled head peak. In Fig. 2c the distribution of counts in the absence of protein synthesis is observed. The redistribution is about the same as with FUdR, except for the noticeable 1,200*S* filled head peak. Our interpretation of these experiments is that, in the case of DNA synthesis inhibition, all gene 13-defective, fragile-filled heads that are converted to gene 13-defective, stable-filled heads are apparently then converted to viable phage. It is always possible that the FUdR inhibition may be the result of a secondary effect; however, we believe this unlikely since (i) FUdR does not affect the incorporation of ^{14}C-labeled amino acids into protein (Table 4), (ii) FUdR does not interfere with the conversion of stable-filled heads to phage, and (iii) in a previous study the ability of FUdR to inhibit maturation of another head precursor structure, e.g., gene 49 unfilled heads, was shown to be paralleled by the action of nalidixic acid, another inhibitor of phage DNA replication (29). In the case of a lack of protein synthesis, little P13 or a protein necessary for P13 to act is produced. Thus the structure for which this protein is required, i.e., stable-filled heads, is prevented from being converted to phage, and the block on assembly results in the presence of both stable- and fragile-filled heads.

(iii) Nature of intracellular DNA in gene 13-defective infections. Our results above have indicated that DNA synthesis is required for maturation of greater than 70% of phage precursor particles in gene 13-defective infections. Similar requirements for normal DNA synthesis in the morphopoiesis of the capsid have previously been reported (23, 29). One approach for

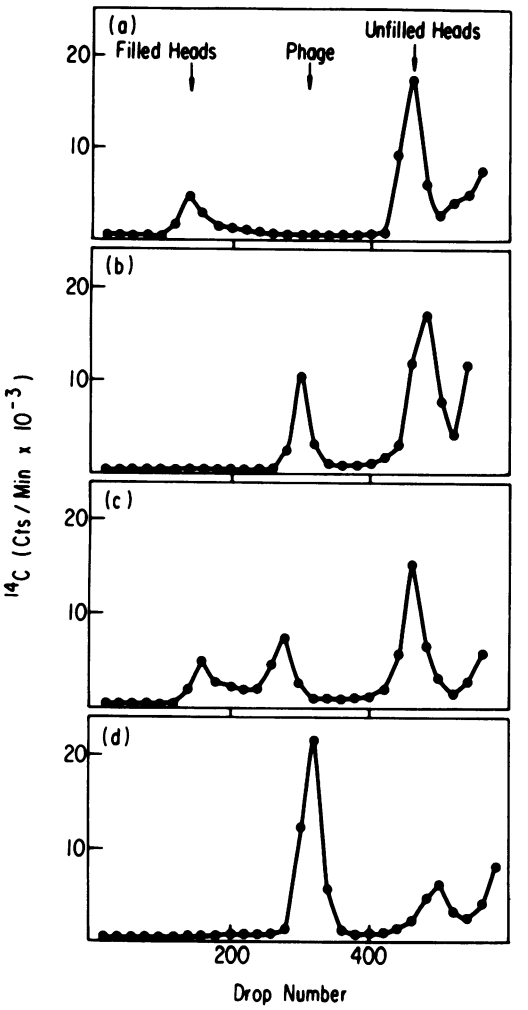

FIG. 2. *Distribution of labeled phage and phage precursors after rescue of the gene 13 function in the presence of either chloramphenicol or FUdR. Pulse-chase, temperature shift experiments were conducted as described for Table 2. Aliquots were removed from an (amtA3:tsN38)-infected culture after a 40-min recovery period at 25 C and prepared for 20 to 40% sucrose gradient analysis as described in Fig. 1. (a) and (d) Distribution of label at the time of recovery and after 40 min at 25 C, respectively, in the absence of any inhibitors. (b) Label distribution when recovery is inhibited by FUdR. (c) Distribution when recovery is inhibited by chloramphenicol.*

TABLE 4. *Effect of a single inhibitor on various metabolic functions*

Inhibitor[a]	Relative incorporation (%) after shift-down of:		Viable phage produced at 40 min post-shift (%)
	[^{3}H]deoxyguanosine	[^{14}C]lysine	
Chloramphenicol (62 μg/ml)	83	7	8
FUdR (200 μg/ml)	30	90	31

[a] Infection and utilization of the inhibitors is described in the text.

obtaining additional information on how such a requirement is coupled to maturation of the gene 13$^-$ head precursors is to examine the intracellular DNA pool. This approach was used in the case of gene 49$^-$ head precursors where the same need for maintenance of DNA synthesis during maturation had been shown (28). Gene 49-defective infected cells possessed an intracellular T4 phage DNA pool in the form of a greater than 1,000*S* concatenate (13; F. R. Frankel and M. Batcheler, Bacteriol., Proc., p. 162, 1969). To determine the nature of the intracellular DNA concatenate in gene 13-defective infected cells, we used the procedure of Frankel et al. (13) and compared the sedimentation profiles on sucrose gradients of the intracellular DNA of *ts*N38 or *am*NG329 (gene 13 mutants) cells infected under restrictive conditions with those of mature T4 phage DNA, intracellular DNA of *am*B17-infected cells (200*S* concatenate), and intracellular DNA of *am*E727-infected cells (>1,000*S* concatenate). The results are shown in Fig. 3. In comparison to the controls, *am*NG329-infected cells contained intracellular T4 phage DNA in two forms, 200*S* and 63*S* (Fig. 3). This is consistent with previous results (16), which have indicated the existence of two classes of head structures, namely, stable- and fragile-filled heads. The 63*S* DNA probably arises in part from DNA found in the stable-filled heads of *am*NG329-infected cells, whereas the 200*S* DNA may represent a precursor pool for the fragile-filled heads. There are several reasons to believe that this is the case. First, alkaline sucrose gradients (Fig. 4) indicate that the 200*S* DNA (Fig. 4b) is composed of greater than phage-size pieces (Fig. 4a) and, therefore, resembles the 200*S* wild-type, vegetative DNA precursor pool (Fig. 4c) described by Frankel (12). Secondly, pulse-chase DNA label experiments conducted using *ts*N38-infected cells also supported the precursor nature of the 200*S* pool in these cells. These experiments were conducted essentially as described in Fig. 1, except that [^{3}H]thymidine was added 10 min after infection, followed 5 min later by the addition of a thousandfold excess of cold thymidine. At the time of the shift and

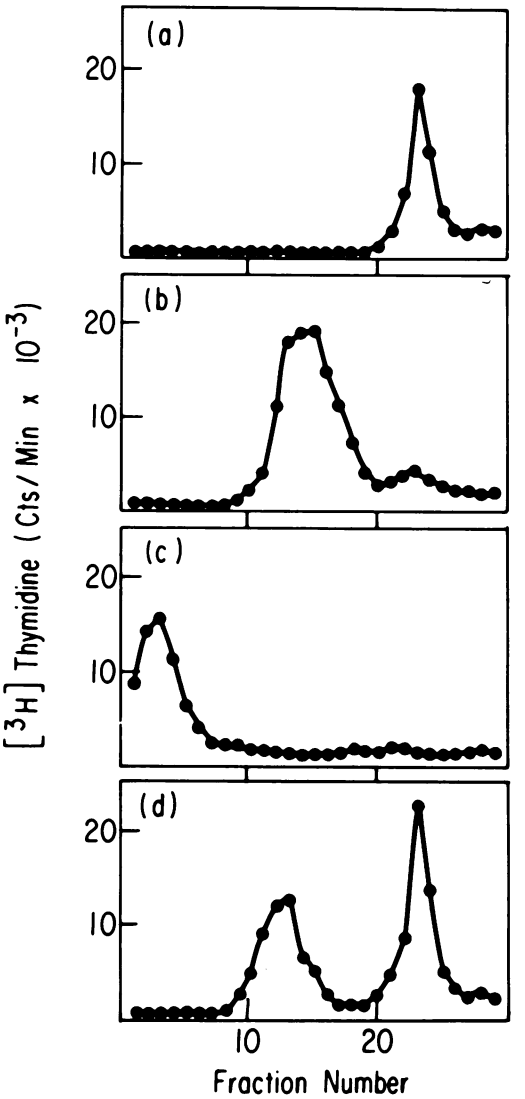

FIG. 3. *Sedimentation profiles of intracellular T4 phage DNA isolated from cells infected with mutants in T4 phage head maturation. The intracellular DNA was isolated, and sucrose gradient analysis was performed as described in Materials and Methods. The peak in (a) represents mature T4 DNA (63S) extracted as described earlier; in (b) the sedimentation profile is that of intracellular DNA (200S) isolated from amB17 (gene 23)-infected cells; (c) is >1,000S intracellular DNA from amE727 (gene 49)-infected cells; and (d) shows the profile of 200S and 63S DNA isolated from amNG329 (gene 13)-infected cells.*

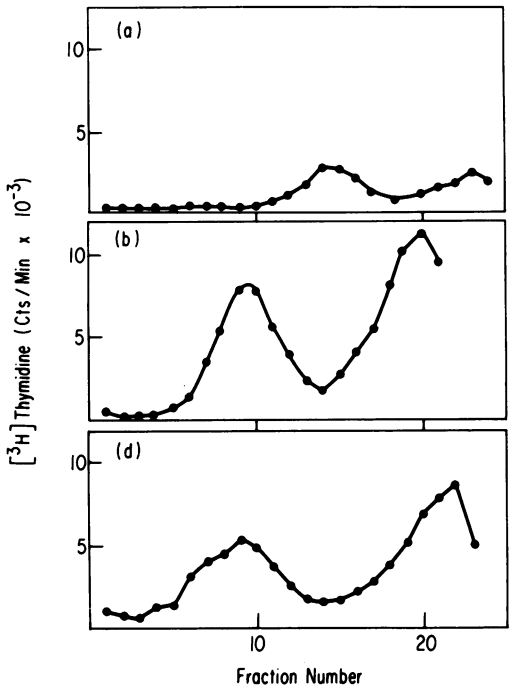

FIG. 4. *Comparison of sedimentation profiles of intracellular DNA from amNG329- and amB17-infected cells and mature T4 phage DNA in alkaline sucrose gradients. Preparation of DNA samples was as described in Fig. 3. Alkaline sucrose gradients were prepared and centrifuged as described in Materials and Methods. (a) Distribution of alkaline-denatured mature T4 DNA; (b) distribution of intracellular DNA from amNG329-infected cells; (c) distribution of intracellular DNA from amB17-infected cells.*

intervals thereafter, 3-ml aliquots were removed from the infected culture and poured immediately into 2 ml of ice-cold media. The cells were lysed and intracellular DNA was analyzed as previously described. The results in Fig. 5 indicate that the 200*S* DNA is being converted to 63*S* DNA. From an initial distribution of label of approximately 50% (200*S*) to 50% (63*S*) at the time of shift (Fig. 5a), the distribution goes to approximately 25% of the counts in 200*S* DNA and about 75% in 63*S* DNA (Fig. 5c).

It is of interest to note that this intracellular pool of DNA for gene 13$^-$-defective fragile-filled heads, namely, 200*S*, differs from the sedimentation profile of DNA from gene 49$^-$-defective infected cells, namely, >1,000*S*. These differences in sedimentation coefficient may be correlated indirectly with the arrangement of DNA inside the two types of defective head structures, since thin sections of infected cells show mostly filled heads in the former case, whereas in the latter case they are mostly unfilled (16).

To learn more about these intracellular DNA pools, we prepared Kleinschmidt spreads of the intracellular DNA concatenates and examined them in the electron microscope. Our isolation procedure of the DNA was similar to that described above, except to have a sufficient concentration of DNA concatenates 40 ml of an infected culture (instead of 5 ml) was used. The cells were pelleted by centrifugation and resuspended in 5 ml of ice-cold media; lysis and subsequent isolation of DNA was then identical

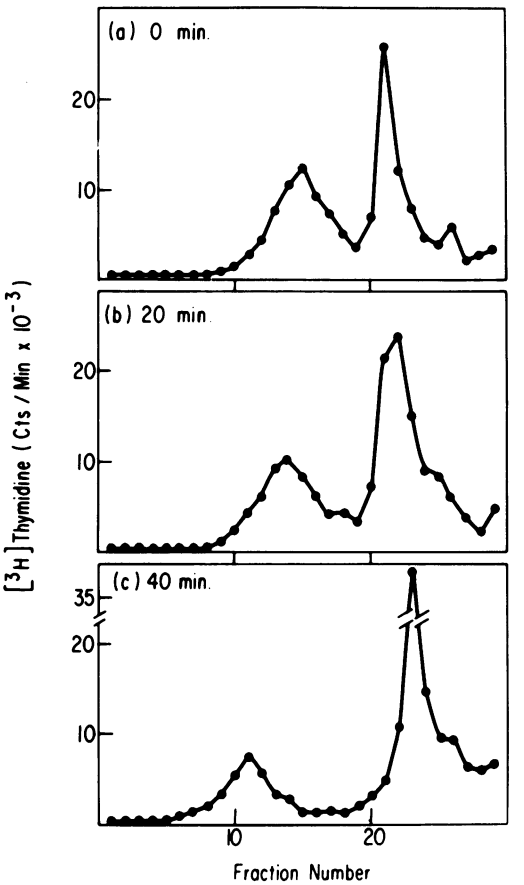

FIG. 5. *Analysis of the intracellular DNA pool in tsN38-infected cells during the recovery of viable phage production. A pulse-chase, temperature shift experiment such as that described in Fig. 1 was conducted, except 20 μCi of [^{3}H]thymidine per ml was added at 10 min postinfection, followed by a chase of about a 1,000-fold excess of "cold" thymidine 5 min later to observe the fate of intracellular T4 DNA. Aliquots were removed, and intracellular DNA was prepared and analyzed on 20 to 70% sucrose gradients as described in Fig. 3. The distribution of intracellular DNA after 0, 20, and 40 min at 25 C is shown in (a), (b), and (c), respectively.*

to the procedure previously described. For electron microscope preparations, an aliquot was removed and diluted 100-fold in spreading solution. Spreading and shadowing were done as described in Materials and Methods. The results are shown in Fig. 6. The major observation to be noted is the variation in the dispersal of 49-defective and 13-defective intracellular DNA. The >1,000*S* DNA of gene 49-defective infected cells (Fig. 6a) is very compact, with a dense central region comprising the major part of the structure. With gene 13-defective infected cells (Fig. 6b), the 200*S* DNA has a less dense core and a more disperse array of DNA strands. Since Frankel et al. (13) indicated that both the 1,000*S* and 200*S* DNA concatenates are comprised of about the same total amount of DNA, the difference in sedimentation coefficients between these forms probably reflects the tighter massing of DNA in the center of the 1,000*S* form. The more numerous array of strands around the 200*S* DNA may be a result of leakage from the fragile-filled heads, whereas the fewer number of strands present around the periphery of 1,000*S* DNA may be due to the lower DNA content of gene 49$^-$ unfilled heads. However, further studies in which we can compare the morphology of the DNA concatenate when the two types of head precursors are stabilized on the concatenate are required before we can be convinced of this argument.

(iv) Head structures observed with different cell lysis procedures. As mentioned earlier, if we assume a constant specific activity for the [^{3}H]DNA in the 63*S* and 200*S* forms, there is an apparent anomaly between the results reported above, namely, showing a 50%-50% distribution for DNA pools inside gene 13-defective infected cells, and our earlier results (16), in which we had found the distribution of stable (containing 63*S* DNA)- to fragile (expected to be primarily associated with the 200*S* DNA pool inside the cell)-filled heads was 20 to 80%. This may indicate that the state of the intracellular DNA pool in gene 13-defective infected cells under nonpermissive conditions is unrelated to the stage of capsid assembly and/or that the greater number, namely, an additional 30%, of fragile-filled heads observed is an artifact resulting from our chloroform-DNase lysis procedure. The results reported below comparing several conditions and methods of lysis support the latter possibility; i.e., we found that the larger number of fragile-filled heads observed is largely attributable to the $CHCl_3$-DNase lysis of the cells.

From the previous study (16), we knew that our lysis procedure (yielding 70% unfilled heads) was causing a loss of DNA from the majority of the gene 13-defective heads, since under normal fixation and embedding procedures (see Materials and Methods) the gene 13$^-$ head precursors appeared to be predominately full in situ. To analyze the sensitivity of the gene 13$^-$ heads to lysis, we performed the experiments described below. First, we examined thin sections of whole cells under conditions where the cells were still essentially intact, but most of the small macromolecules of the cytoplasm had leaked out, namely, the partial lysis procedure (19). A culture of *E. coli* B3 infected with *am*NG329 and incubated for 30 min at 25 C was split into two equal fractions, one of which was immediately fixed without lysis occurring and the other of which was first partially lysed in the presence of 0.1% OsO_4 and then fixed. Both samples were embedded, sectioned and stained in an identical manner. Sections were examined in the electron microscope, and intracellular phage particles were counted and scored as follows. Densely stained particles were counted as full; less densely but uniformly stained particles were counted as probably full; and particles lacking in central density were denoted as unfilled (see Fig. 7 for examples of each). The result are given in Table 5. In the case of the partially lysed sections, threefold more head particles (20 versus 7%) appear unfilled than in the unlysed samples. These results indicate that some filled gene 13$^-$ heads are so sensitive to lysis that a loss of DNA from the head capsid can occur even while the heads are still inside the cell. It would thus be expected that, in any lysis procedure in which we are attempting to isolate gene 13$^-$ filled heads for further study, a certain percentage (15%) of the heads would lose their DNA even before the cells were lysed. By use of the $CHCl_3$-DNase lysis method we are presumably greatly increasing this percentage of heads that will lose their DNA upon isolation, probably as a result of the drastic shock of rapidly breaking down the entire cell membrane. Thus, to optimize the yield of 1,200*S* filled head precursors, we have investigated other cell lysis procedures, which would either break cells in a more gentle manner or would lead to a greater head stability by formation of protein-protein cross-links. Two additional methods of lysing *am*NG329- or *am*E609-infected B3 cells were compared to the $CHCl_3$-DNase method, i.e., lysis by lysozyme-DNA treatment (which would punch holes in the membrane, and thus not lead to as drastic a breakdown as that caused by $CHCl_3$) and Laemmli's partial fixation lysis with lysozyme-detergent and DNase treatment

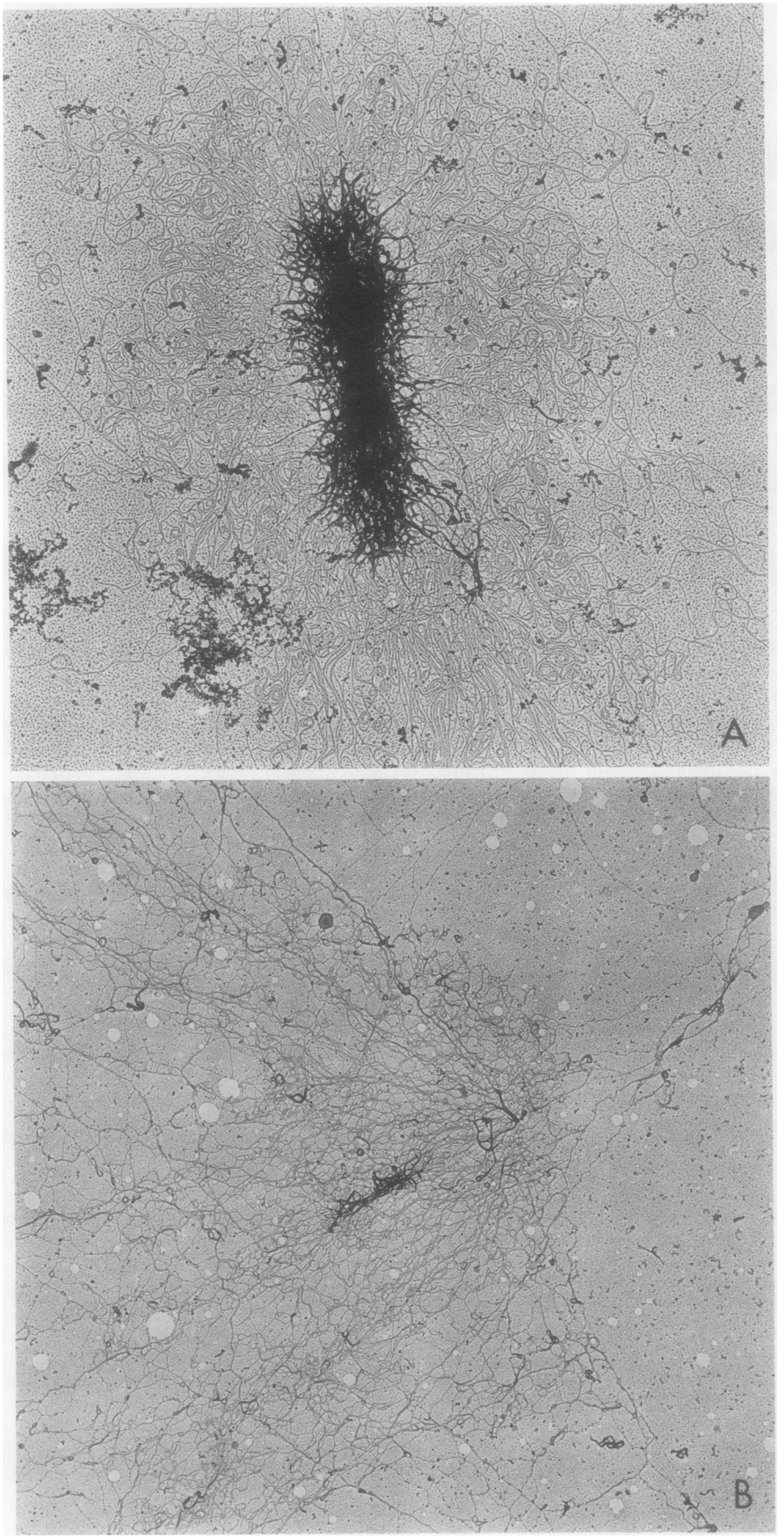

FIG. 6.

in glutaraldehyde (24). The results are shown in Fig. 8. The first method gave a similar sucrose gradient profile of ~20% of the counts in the 1,200*S* peak and 80% in the 300*S* peak to that found with $CHCl_3$-DNase. The partial fixation technique, however, gave a different profile. In this latter case, 50% of the label was found in a homogeneous 1,200*S* peak. This 1,200*S* peak is apparently homogeneous relative to filled heads obtained by the $CHCl_3$-DNase lysis procedure, namely, the [^{3}H]thymidine-^{14}C-labeled amino acid) ratios were approximately the same for both peaks (Table 6).

These results indicate that approximately 50% of the gene 13-defective precursor heads can be isolated in a filled state when precautions are taken to stabilize the head protein. This result is now consistent with the result of Fig. 3d; namely, 50% of the intracellular DNA exists as mature 63*S* pieces.

(v) Comparison of packaging gene 13$^-$- and gene 49$^-$-defective head structures. As we have seen in Fig. 2, de novo DNA synthesis is required for conversion of the gene 13$^-$ fragile-filled heads to stable-filled heads. Also, de novo DNA synthesis is required for the conversion of the 300*S* unfilled mature phage in gene 49$^-$-defective infections (28, 29). Since both the gene 49$^-$ and gene 13$^-$ 300*S* heads appear identical in morphology, sedimentation rate, and in content of pancreatic DNase-resistant DNA upon isolation, one may interpret the de novo DNA synthesis requirement seen in the case of the gene 49$^-$ precursor to be only a reflection of the need for DNA synthesis at the level of the gene 13 block. We can test this hypothesis by a detailed examination of the classes of gene 49$^-$ head particles observed in ultrathin sections of infected cells after blocking DNA synthesis. Specifically, if de novo DNA synthesis was required at the level of packaging for gene 49$^-$ heads, a large number of the unfilled head particles seen in thin sections would remain unfilled- or empty-appearing in the absence of DNA synthesis. If, however, DNA synthesis were not required until the final steps of packaging, the unfilled head particles would become filled in the absence of DNA synthesis and would assume the in vitro appearance of gene 13$^-$ heads. Experimentally, we split a culture of *E. coli* B3 cells infected with *ts*C9 (a gene 49 temperature-sensitive mutant) at 43 C into two equal parts. One part was treated with FUdR 21 min after infection; the other was untreated. Three minutes later both aliquots were shifted to 25 C for an additional 30 min. Thin sections were then prepared of all cell pellets (Table 7). Forty percent of the total particles counted in the *ts*C9-infected culture remained unfilled- or empty-appearing even after FUdR treatment (Table 7). This indicates that the de novo DNA synthesis requirement for packaging of gene 49$^-$ unfilled heads is not a reflection of the need for DNA synthesis seen at the level of gene 13 blockage. Rather, it appears that DNA synthesis is needed for packaging the gene 49$^-$ unfilled head intermediate, as well as for the completion of packaging DNA into the gene 13$^-$ fragile-filled head intermediates.

DISCUSSION

In our earlier studies with gene 13 we reported the existence of two classes of gene 13$^-$ head precursors, which existed in mutant-infected cells grown under nonpermissive conditions (16); one class was isolated as a filled head and could be complemented in vitro, and another, which was fragile to isolation, retained only 18 to 32% of a head full of DNA. Since thin sections of cells infected with *am* or *ts* gene 13 mutants under restriction conditions showed only "full"-appearing phage heads, we termed the above precursors stable- and fragile-filled heads, respectively. These heads were distributed on the average as 20% stable and 80% fragile.

In this report we have expanded on this earlier study. Studies of the metabolic requirements necessary to convert the two precursors into viable phage particles yielded evidence toward explaining how both structures fit into the morphogenetic process. We found that both de novo DNA and protein synthesis were necessary for recovery of viable phage after shifting a gene 13 mutant (*ts*N38)-infected culture to a permissive temperature. We also observed, by the distribution of labeled precursors on sucrose gradients (Fig. 2), that the absence of protein synthesis blocked conversion of stable-filled heads and fragile-filled heads to viable phage, whereas

FIG. 6. *Electron micrographs of intracellular DNA concatenates from amNG329- and amE727-infected cells. A 40-ml culture of E. coli B3, infected with either amNG329 (gene 13) or amE727 (gene 49), was incubated for 24 min at 41.5 C. The culture was then pelleted, the pellet was resuspended in 5 ml of cold medium, and the intracellular DNA was isolated as previously described (Fig. 3). Preparation of samples for electron microscopy is described in the text. (B) Typical example of the 200S concatenate found in amNG329-infected cells. The more tightly massed concatenate of DNA seen in (A) is typical of the >1,000S concatenate of phage DNA found in amE727-infected cells. Magnification, × 16,000.*

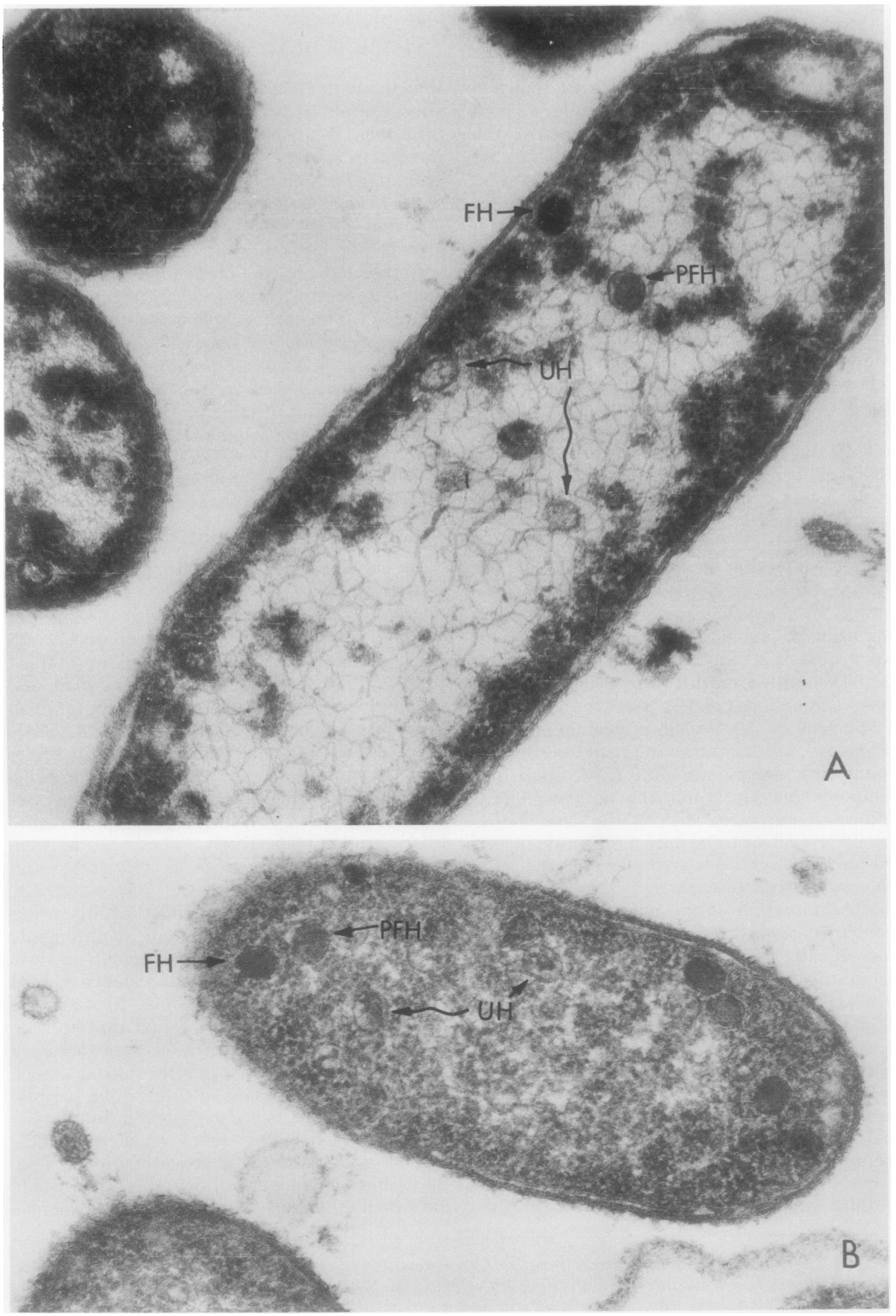

FIG. 7. *Thin sections of partially lysed and unlysed E. coli B3 infected with amNG329. (A) Representative thin section of a partially lysed cell; (B) an unlysed cell. UH, PFH, and FH refer to examples of unfilled heads (light electron density), probably or partially filled heads (intermediate to dense), and filled heads (greatest density), respectively. These are empirical classifications of observations based on at least three experiments (eliminating staining or fixation artifacts as sources of error in determining relative densities of the heads). Magnification, × 108,000.*

TABLE 5. *Effects of partial lysis on in vitro gene 13⁻-defective head particles*

Procedure[a]	Gene sample	No. of head structures (%)		
		Filled heads	Probably filled	Unfilled or empty
Partial lysis	13⁻	37 (43.5)	31 (36.4)	17 (20)
	13⁺	149 (61)	90 (37)	6 (2)
No lysis	13⁻	91 (63)	44 (30)	10 (7)
	13⁻	54 (34)	91 (57)	15 (9)
	13⁺	150 (77)	44 (23)	2 (1)

[a] Each line represents a separate, independently conducted experiment.

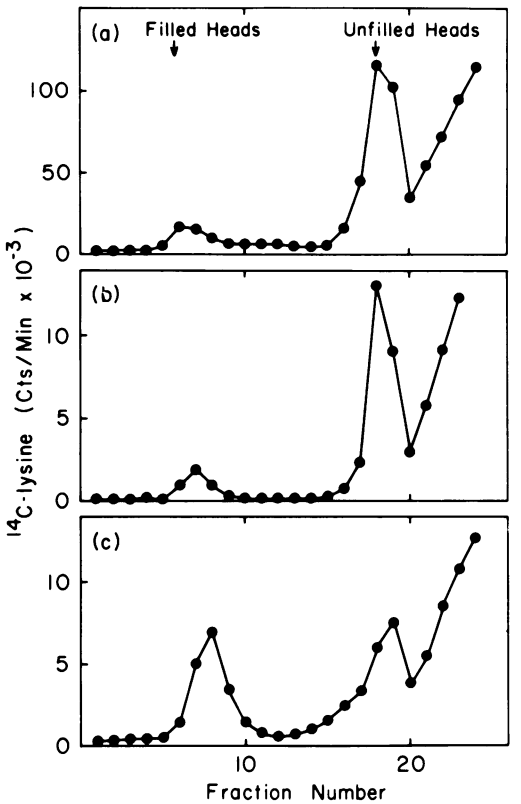

FIG. 8. *Distribution of amNG329 (gene 13) head particles isolated under various lysis conditions. Infection and labeling was as described in Fig. 1. Samples were removed 24 min after infection and lysed as follows: (a) by $CHCl_3$-DNase, (b) lysozyme-DNase as described by Frankel (12), or (c) lysozyme-detergent lysis in glutaraldehyde as described by Laemmli and Favre (24).*

the absence of DNA synthesis blocked only fragile-filled head conversion. We interpreted these results to indicate: (i) protein synthesis was necessary for production of functional gene 13 product (P13) to act on stable-filled heads prior to tail attachment, and (ii) DNA synthesis was necessary for conversion of fragile-filled heads to stable-filled heads.

We then examined the intracellular pool of phage DNA in gene 13 mutant-infected cultures. There were two classes of DNA: (i) 63*S* DNA, which sediments as mature size DNA and presumably comes from stable-filled heads; and (ii) a 200*S* concatenate of DNA, which is largely converted to 63*S* DNA during recovery of viable phage in *ts*N38-infected cells (Fig. 5) and is presumably associated with the fragile-filled heads. These two classes of intracellular T4 DNA were found to be distributed equally. This was in contrast to the 20% stable-filled and 80% fragile-filled heads that had been isolated by $CHCl_3$-DNase from the infected cells.

To understand the above anomaly in particle distribution, we could say that no direct relationship existed between the distribution of head precursors and the distribution of intracellular DNA; however, the lack of a large number of empty-looking heads in thin sections of gene 13⁻-infected cultures led us to consider that perhaps the method by which these cells were lysed affected the distribution of head precursors. Therefore, we attempted to stabilize the heads by using partial fixation with glutaraldehyde as described by Laemmli and Favre (24). By this technique we found that 30% of the precursor heads we thought were associated with the 200*S* DNA had a full complement of cleaved, 63*S* DNA. Thus, we can

TABLE 6. *DNA content of 1,200S head particles*

Procedure	Label		
	^{14}C	^{3}H	^{3}H:^{14}C
$CHCl_3$-DNase lysis	72,200	1,108,800	15.3:1
Lysis in glutaraldehyde	6,200[a]	90,600[a]	14.6:1

[a] Sum of counts per minute when adjusted for volume differences between procedures is equivalent.

operationally define three classes of precursors: (i) a stable head (20%), (ii) a fragile head requiring fixation for isolation in a stable form (30%), and (iii) a fragile head presumably attached to the intracellular DNA concatenate, which under present techniques can only be isolated as unfilled (50%). This latter class of fragile-filled heads found in gene 13 mutant infections is similar in some ways to the 300*S* unfilled heads found in the absence of a functional gene 49 product. (i) Both structures are intermediate in head assembly; (ii) after isolation by the $CHCl_3$-DNase treatment, both heads appear empty by negative-stain electron microscopy but actually contain between 12 to 32% of a phage equivalent of DNA; and (iii) both head structures in vivo require DNA synthesis for conversion to viable phage. They differ in that fragile-filled heads from gene 13^--defective infections appear filled by thin-section electron microscopy examination (although to what extent cannot be determined), whereas gene 49^- defective unfilled heads appear empty, and they differ in their associated precursor pools of intracellular DNA, 1,000*S* in gene 49 mutant infections and 200*S* in gene 13 mutant infections. Taking into account these similarities and differences between gene 49 unfilled heads and gene 13 unfilled heads, and those between these structures and gene 13 stable-filled head precursors, we have proposed the following pathway for the terminal stages of T4 head maturation (Fig. 9). The salient feature is that gene 13 unfilled and fragile-filled heads are intermediate forms between gene 49 unfilled heads and gene 13 stable-filled heads.

In particular, (a) depicts the precursor structure described by Luftig et al. (30), which accumulates in the absence of a viable gene 49 product. This structure is presumably attached to the >1,000*S* DNA concatenate described by Frankel et al. (13). (b) (c) and (d) are the three potential precursor forms that may accumulate in the absence of gene 13 product. Precursor (b) would still be attached to the intracellular pool of DNA, can be isolated only as a partially full head, and requires DNA synthesis to be converted to (c). (c) is unique in that it can be isolated as unfilled or filled depending upon the isolation conditions. A likely explanation for this property is that partial fixation may cross-link proteins at the base

TABLE 7. *Effect of FUdR on packaging of gene 49⁻ (tsC9)-defective heads in vivo*

Inhibitor	Temp (C)	No. of head structures (%)		
		Filled heads	Probably filled	Unfilled or empty
None	43	13 (10.7)	20 (66.4)	89 (72.9)
None	25	143 (52)	86 (32)	44 (16)
FUdR[a] + CAM	25	61 (38.6)	31 (19.6)	66 (41.8)

[a] FUdR (200 μg/ml) was added 3 min prior to the shift to 25 C. Since we wanted to follow what had happened to the head structures present at the times of the addition of FUdR, chloramphenicol (CAM; 62 μg/ml) was added to prevent accumulation of additional new head structures.

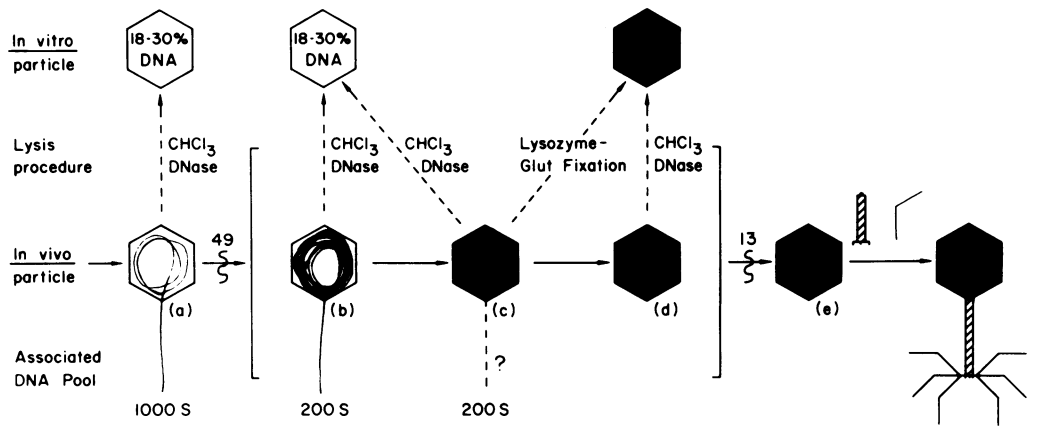

FIG. 9. *Diagrammatic representation of the final stages of head assembly. Numbers at various steps indicate the point at which the absence of the corresponding gene product blocks further assembly. See text for details.*

of the head so as to preclude DNase from entering the head and/or prevent DNA from escaping out of the head. (d) is the stable-filled gene 13$^-$ head structure, which with the addition of gene 13 product can be converted to (e), a stable-filled, modified head. The (e) head can now accept a tail structure and tail fibers and this completes the morphogenetic pathway. During conversion of (a) and (b) to (c) and (d), DNA synthesis is required. Whether this synthesis is necessary to drive the packaging process in some manner or whether it is necessary for altering the tertiary structure of the DNA pool is unknown at this time. Another unresolved problem of this pathway scheme is how would the absence of functional P13 lead to blocks at three different steps. One plausible explanation would be that, after a certain number of stable-filled heads accumulate, these filled heads, by not being released from a template assembly site, physically block further stabilization of fragile-filled heads. Another possibility which would explain the presence of two filled head precursors is that a limited amount of a head stabilizing factor is involved in assembly. The function of this factor would be to temporarily stabilize filled heads after completion of DNA packaging. After P13 interacts with the stabilized filled heads, the stabilizing agent would be released for use on other fragile-filled heads. As candidates for such a stabilizing factor are a large number of T4 genes whose phenotypes suggest a defect in head completion, namely, genes 2, 4, 50, 64, and 65, and gene 14. However, studies on the role of these gene products is largely in a nascent stage; perhaps some of these genes are primarily involved in the protein cleavage reactions in the early stages of capsid formation (26) and only secondarily exhibit effects at the later stages of head filling.

It should be emphasized at this point that an alternative interpretation of the model in Fig. 9, based on similarities between the terminal stages of head maturation in the *Salmonella* phage P22 and T4, is that precursors (b), (c), and (d) represent a single class of unstable particles, which decay slowly, causing some to survive lysis in a stable form and the remainder to be unstable. In the P22 system, there is strong evidence for such a single class of defective filled particles at the terminal stages of maturation. Using P22 gene 10$^-$ and 26$^-$-infected cells, it was shown that the lysates accumulate empty phage heads, whereas freshly lysed cells, as well as thin sections of infected cells, show substantial numbers of full heads (4, 22). Further, concentrated extracts from P22 10$^-$ and 26$^-$ cells contained heads that could be reconstituted in vitro into viable phage. Similar in vitro reconstitution studies have been carried out with T4 gene 13$^-$-defective extracts. Also, in both T4 and P22, if glutaraldehyde was added to these defective lysates described above, an increased number of full heads was observed (4, 22).

However, there are also differences between T4 gene 13$^-$ heads and P22 gene 10$^-$ and 26$^-$ heads. (i) The P22 gene 10$^-$ and 26$^-$ unstable heads are apparently empty of DNA after isolation (22), whereas the T4 gene 13-defective heads contain at least an average 18% piece of pancreatic DNase-resistant DNA (16); (ii) lysis of gene 13-defective infected cells yields about 18% stable-filled heads, which can be purified as 1,200*S* structures and complemented in vitro (9, 16); and (iii) in gene 13-defective, phage-infected cells, 50% of the intracellular DNA remains in the 63*S* mature form and 50% in the 200*S* concatenate, when the cells are maintained under nonpermissive conditions. In contrast, for P22, gene 10-defective infected cells, the concatenate DNA appears to be completely converted to mature DNA even under nonpermissive conditions (4). Based on these differences, we believe it is more likely that for T4 there are several classes of defective heads, as described above for Fig. 9; namely, some gene 13-defective heads have a head-full of 63*S* DNA, and some have a head-full or less of DNA but remain associated with the 200*S* concatenate.

In addition to allowing us to formulate the above schemes for head precursors, our results can be of importance in permitting us to understand the role of the intracellular DNA pool in the process of head assembly. Specifically, we are referring to the electron microscope comparison of the 200*S* concatenate of intracellular DNA in gene 13-defective infections with that of the >1,000*S* form found in gene 49-defective infected cells. Frankel et al. (13) speculated that perhaps the >1,000*S* form was the replicative form of DNA in vivo and that the 200*S* form was merely a result of uncontrolled action of gene 49-dependent function during lysis. Our observations showed a difference between the 200*S* and 1,000*S* forms that could account for variations in their sedimentation properties. Since these variations are assayable by Kleinschmidt-type preparations, it may prove feasible to observe the effect of limited amounts of functional P49 on the tightly massed DNA concatenate and gain an understanding of its tertiary structure. Such studies may determine the true nature of the

intracellular pool of DNA in a wild-type infection.

In studying the intracellular DNA of gene 13-defective infected cultures, alkaline sucrose gradient analyses were conducted and allowed us to verify the similarity of gene 13-defective, 200*S* DNA with the 200*S* structure reported by Frankel et al. (13). One surprising aspect of these studies was the absence of a large 73*S* peak in our preparations, which we would have expected as a result of the presence of mature size (63*S*) DNA. Several possibilities exist to explain the absence of 73*S* single-stranded DNA in alkaline gradients. First, the mature 63*S* DNA in gene 13-deficient heads may accumulate nicks as a result of nuclease susceptibility of the tailless heads. Precedence for this possibility comes from the case of lambda heads whose DNA is susceptible to micrococcal nuclease attack (3). A second possibility may be that mature 63*S* DNA is susceptible to limited nuclease attack during lysis and storage, resulting in nicks in the strands. The replicating 200*S* concatenate would be immune to this action. Evidence supporting this latter possibility comes from the results of Frankel et al. (13), who concluded that sites of nuclease attack on mature DNA may not be present or accessible in the replicating DNA. Our present data cannot offer any explanation or confirm at this time which, if any, of the above explanations is correct.

ACKNOWLEDGMENTS

This research was supported by Public Health Service research grants CA-15573 and CA-12708 from the National Cancer Institute.

LITERATURE CITED

1. Barnhart, B. J., and C. T. Gregg. 1967. Effects of uncouplers of oxidative phosphorylation on the infection of *Escherichia coli* K12 by phage λ-DNA. Virology **32**:687-691.
2. Bijlenga, R. K. L., R. Vander Brock, and E. Kellenberger. 1974. The transformation of τ-particles into T4 heads. J. Supramol. Struct. **2**:45-59.
3. Bode, V. C., and F. D. Gillin. 1971. The arrangement of DNA in lambda phage heads. I. Biological consequence of micrococcal nuclease attack on a portion of the chromosome exposed in tailless heads. J. Mol. Biol. **62**:493-502.
4. Botstein, D., C. H. Waddell, and J. King. 1973. Mechanism of head assembly and DNA encapsulation in Salmonella phage P22. I. Genes, proteins, structures and DNA maturation. J. Mol. Biol. **80**:669-695.
5. Chao, J., L. Chao, and J. F. Speyer. 1974. Bacteriophage T4 head morphogenesis: host DNA enzymes affect frequency of petite forms. J. Mol. Biol. **85**: 41-50.
6. Cohen, S. S., J. G. Flaks, H. D. Barner, M. R. Loeb, and J. Lichtenstein. 1958. The mode of action of 5-fluorouracil and its derivatives. Proc. Natl. Acad. Sci. U.S.A. **44**:1004-1012.
7. Davis, R. W., M. Simon, and N. Davidson. 1971. Electron microscopy heteroduplex methods for mapping regions of base sequence homology in nucleic acid, p. 413-428. *In* L. Grossman and K. Moldave (ed.), Methods in enzymology, vol. 21. Academic Press Inc., New York.
8. Doermann, A. H. 1952. The intracellular growth of bacteriophages. I. Liberation of intracellular bacteriophage T4 by premature lysis with another phage or with cyanide. J. Gen. Physiol. **35**:645-656.
9. Edgar, R. S., and I. Lielausis. 1968. Some steps in the assembly of bacteriophage T4. J. Mol. Biol. **32**:263-276.
10. Edgar, R. S., and W. B. Wood. 1966. Morphogenesis of bacteriophage T4 in extracts of mutant-infected cells. Proc. Natl. Acad. Sci. U.S.A. **55**:498-505.
11. Epstein, R. H., A. Bolle, C. M. Steinberg, E. Kellenberger, E. Boy de la Tour, R. Chevalley, R. S. Edgar, M. Sussman, G. A. Denhardt, and A. Lielausis. 1963. Physiological studies of conditional lethal mutants of bacteriophage T4D. Cold Spring Harbor Symp. Quant. Biol. **28**:375-394.
12. Frankel, F. R. 1968. DNA replication after T4 infection. Cold Spring Harbor Symp. Quant. Biol. **33**:485-493.
13. Frankel, F. R., M. L. Batcheler, and C. K. Clark. 1971. The role of gene 49 in DNA replication and head morphogenesis in bacteriophage T4. J. Mol. Biol. **62**:439-463.
14. Fujisawa, H., and T. Minigawa. 1971. Genetic control of the DNA maturation in the process of phage morphogenesis in bacteriophage T4. Virology **45**:289-291.
15. Granboulan, P., J. Sechaud, and E. Kellenberger. 1971. On the fragility of phage T4-related particles. Virology **46**:407-425.
16. Hamilton, D. L., and R. B. Luftig. 1972. Bacteriophage T4 head morphogenesis. III. Some novel properties of gene 13-defective heads. J. Virol. **9**:1047-1056.
17. Haselkorn, R., M. Vogel, and R. D. Brown. 1969. Conservation of the rifamycin sensitivity of transcription during T4 development. Nature (London) **221**:836-838.
18. Josslin, R. 1971. Physiological studies on the t-gene defect in T4-infected *Escherichia coli*. Virology **44**: 101-107.
19. Kellenberger, E., F. A. Eiserling, and E. Boy de la Tour. 1968. Studies on the morphopoiesis of the head of phage T-even. III. The cores of head-related structures. J. Ultrastruct. Res. **23**:335-360.
20. Kelly, T. J., Jr., and C. A. Thomas, Jr. 1969. An intermediate in the replication of bacteriophage T7 DNA molecules. J. Mol. Biol. **44**:459-475.
21. King, J. 1968. Assembly of the tail of bacteriophage T4. J. Mol. Biol. **32**:231-262.
22. King, J., E. V. Lenk, and D. Botstein. 1973. Mechanism of head assembly and DNA encapsulation in Salmonella phage P22. II. Morphogenetic pathway. J. Mol. Biol. **80**:697-731.
23. Kühl, P. W., and P. H. Hofschneider. 1969. Studies on the morphopoiesis of the head of phage T-even. 6. Maturation of T4 polyheads and T4 phage under conditions affecting protein-, DNA-, or ATP-synthesis. Eur. J. Biochem. **7**:353-359.
24. Laemmli, U. K., and M. Favre. 1973. Maturation of the head of bacteriophage T4. I. DNA packaging events. J. Mol. Biol. **80**:575-599.
25. Laemmli, U. K., J. R. Paulson, and V. Hitchins. 1974. Maturation of the head of bacteriophage T4. Supramol. Biol. **2**:276-301.
26. Laemmli, U. K., N. Teaff, and J. D'Ambrosia. 1974. Maturation of the head of bacteriophage T4. III. DNA packaging into preformed heads. J. Mol. Biol. **88**: 749-765.
27. Loomis, W. F., and F. Lipmann. 1948. Reversible inhibition of the coupling between phosphorylation and oxidation. J. Biol. Chem. **173**:807-808.

28. Luftig, R. B., and C. Ganz. 1972. Bacteriophage T4 head morphogenesis. II. Studies on the maturation of gene 49-defective head intermediates. J. Virol. **9**:377-389.
29. Luftig, R. B., and N. P. Lundh. 1973. Bacteriophage T4 head morphogenesis. V. The role of DNA synthesis in maturation of an intermediate in head assembly. Virology **51**:432-442.
30. Luftig, R. B., W. B. Wood, and R. Okinaka. 1971. Bacteriophage T4 head morphogenesis. On the nature of gene 49-defective heads and their role as intermediates. J. Mol. Biol. **57**:555-573.
31. Matthews, C. K. 1966. Deoxyribonucleic acid metabolism and virus-induced enzyme synthesis in a thymine requiring bacterium infected by a thymine requiring bacteriophage. Biochemistry **5**:2092-2100.
32. Reid, P., and J. Speyer. 1970. Rifampicin inhibition of ribonucleic acid and protein synthesis in normal and ethylenediaminetetraacetic acid-treated *Escherichia coli*. J. Bacteriol. **104**:376-389.
33. Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. **17**:208-212.
34. Simon, L. D. 1972. Infection of *Escherichia coli* by T2 and T4 bacteriophages as seen in the electron microscope: T4 head morphogenesis. Proc. Natl. Acad. Sci. U.S.A. **69**:907-911.
35. Snustad, D. P. 1968. Dominance interactions in *Escherichia coli* cells mixedly infected with bacteriophage T4D wild type and amber mutants and their possible implications as to type of gene-product function: catalytic vs. stoichiometric. Virology **35**:550-563.
36. Steinberg, C. A., and R. S. Edgar. 1962. A critical test of a current theory of genetic recombination in bacteriophage. Genetics **47**:182-208.
37. Streisinger, G., J. Emrich, and M. M. Stahl. 1967. Chromosome structure in phage T4. III. Terminal redundancy and length determination. Proc. Natl. Acad. Sci. U.S.A. **57**:292-295.