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Studies Related to the Head-Maturation Pathway of Bacteriophages T4 and T2: II. Nuclear Disruption, Protein Synthesis and Particle Formation With the Mutant $43^{-}\cdot30^{-}\cdot46^{-}$

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We describe the aberrant phage multiplication of the triple conditional lethal mutant 43^{-}(polymerase)$\cdot 30^{-}$(ligase)$\cdot 46^{-}$(exonuclease) of bacteriophage T4D in which phage DNA replication is arrested but some late protein synthesis occurs (33). The nuclear disruption is indistinguishable from wild type. Forty-five empty small and empty large particles are assembled per cell when the multiplicity of infection (m.o.i.) is 100. This number corresponds closely to the 38 phage equivalents of cleaved major head protein determined biochemically. By reducing the m.o.i. the number of observable particles decreases, reaching 1–5 per cell at an m.o.i. of 5(+5).

The total synthesis of phage related proteins is not significantly dependant on the m.o.i. The synthesis of late proteins is about 10% of that of wild type at high m.o.i. and decreases with the m.o.i. The different early and late proteins do not show the same relative proportions as in wild type and respond differently to an increased m.o.i. These and other results are discussed with respect to the role of phage DNA in prehead assembly and head maturation.

Key words: late bacteriophage proteins, regulation phage proteins, bacteriophage maturation, bacteriophage head precursors

From what is known on phage morphogenesis it becomes obvious that we should have more information on the role of the vegetative phage DNA in the process of head assembly and head maturation.

The prehead of phage T4, the τ particle (3, 4, 38), is fragile. Among others, this is probably due to the fact that this prehead is still attached to the membrane. Attempts to quantitatively isolate functionally and morphologically intact preheads were only recently successful (Onorato and Showe, personal communication) and in vitro experiments designed to package DNA in vitro were completely unsuccessful (Bijlenga and Hohn, unpublished). Hence we choose the approach through in vivo experiments.

In phage T4, the onset of late functions is dependent on prior phage DNA synthesis (5, 10, 26, 29). When phage DNA synthesis is inhibited or slowed down, either by chemicals or as a consequence of mutations, the synthesis of late proteins appears also to be stopped or slowed down. The first attempts to study the involvement of vegetative phage

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DNA on assembly did not give conclusive results because they were not designed for very quantitative measurements (21, 2).

In the triple mutant in genes 43$^-$(DNA-polymerase), 30$^-$(ligase), and 46$^-$(exonuclease) phage DNA is not replicated, but late proteins can be synthesized to some extent (33). Therefore, we decided to use this triple mutant (G) to study the role of DNA in phage head assembly. We confirmed and extended the observations of Riva et al. (33) that the amount of late proteins produced depends on the multiplicity of infection (m.o.i.). It reaches a maximum of 10% of that of wild type with very high multiplicities of 50–100. Work under these conditions demands an adequate experimental approach which allows for sufficient precision and accuracy. Since the number of the infecting parental phages is at least as large as the highest expectable number of head-related particles produced per cell, only observations on thin sections could be used, for which a distinction between intracellular and extracellular particles is no problem. Furthermore, only a combined approach, joining counts on thin sections with those of biochemically determined phage equivalents, could provide observations of some significance.

In this paper we give the results obtained on this triple mutant using the approach developed and the results achieved previously (43, 44). We will show that at the highest multiplicities of infection about 40 empty particles composed of gp23* are produced per cell. We extend the preliminary results obtained by Anderson and Notani (2). The empty particles obtained in the triple mutant (G) are very similar to those produced by an amber mutant in gene 17. This interesting similarity will be discussed and we propose that lack or incompetence of vegetative phage DNA misleads the maturation pathway to an abortive side track.

MATERIALS AND METHODS

1. General

The bacterial strains, media, and general procedures for phage experiments are those described in the first paper of this series (44).

Unless stated, cultures were grown in M9A (containing 1% casamino acids) with vigorous air bubbling. For experiments with low multiplicities of infection, we applied the usual procedure of infecting and superinfecting cultures at 2.2×10^8 cells/ml as described in the previous paper of this series; this is indicated by m.o.i. 5(+5). For multiplicities of 20 and higher, superinfection is no longer necessary for producing lysis inhibition.

2. Phage Strains

The T4D wild type was the same as used in all papers of this series. (43, 44) T4·43(*ts*P36)·30(*am*H39X)·46(*am*N130) was obtained from Dr. Geiduschek. In this triple mutant we exchanged the *ts* mutation in 43 against 43(*am*B22) by genetic crossing and selecting by complementation tests (G_b). Another triple *am*, 43(*am*E4301)·30(*am*H 39X)·46(*am*N130), was provided by Dr. Cascino (G_n). Since the 2 *am* mutations in 43 were suspected to show some physiological differences, at first we used both G_n and G_b; our results showed no differences between the 2 strains. Most of the experiments were then made with G_b. In what follows we shall not distinguish between the 2 different triple *am* mutants and shall refer to them both as G. We constructed the following quadruple mutants: G·17(*am*N56), G·20(*am*N50), G·21(*am*N90), G·23(*am*B17), G·24(*am*N65), and G·24(*ts*L90).

A triple *ts* mutant 43(*ts*P36)·30(*ts*A80)·46(*ts*L166) was constructed in our laboratory by R. van der Broek.

3. Techniques for Using High Multiplicities of Infection

In order to get maximum particle production we had to use multiplicities of infection of 50–100. This necessitates particular care to avoid lysis-from-without (8).

Since we observed that bacterial cultures growing under suboptimal conditions are much more susceptible to lysis-from-without than exponentially well-growing cells, we always infected vigorously air-bubbled cultures. A second important parameter is the quality of the phage stocks. Freshly prepared stocks were used only for 3 days, and only after checking the killing titer and finding it to be identical to the plaque forming titer. With older stocks, lysis-from-without occurred within the first minutes after infection.

Phage stocks were prepared by infecting a bacterial culture of 4×10^8 cell/ml with a multiplicity of 0.1. Lysis inhibition occurs in the second cycle of phage growth and leads to high titer stocks. Every freshly prepared stock of the G mutant was checked in a complementation test (9). The occurrence of lysis was checked by counting infected cultures at different times after infection in the Petroff-Hausser counting chamber using a phase contrast microscope with a 40X objective. Only "black" cells were counted knowing that cells which are harmed in permeability ("leakage") decrease their refractive index and therefore appear pale under phase contrast observation. In all experiments reported lysis or leakage did not affect more than 10% of the cell population.

It should be mentioned here that the quadruple mutant G·17$^-$ always produced lysis-from-without at multiplicities of 20 and higher. Extensive backcrossing to wild type did not improve this situation.

4. Electron Microscopy

4.1. Fixation and embedding. We mainly used prefixation of the infected culture with 3% formaldehyde, followed by a main fixation in OsO_4 under RK-conditions (18). This method (FoOs), described in Materials and Methods in the first paper of this series, was found to give the most reproducible results for particle counts (28, 43, 44).

In cases where the shape of the bacterial nucleus is relevant, prefixation with aldehydes cannot be used. In these cases aldehyde prefixation is placed by a prefixation with 0.1% OsO_4 (Os). With T4D and its mutants, this prefixation induces leakage and lysis at times later than 20 min after infection.

A large number of experiments were performed with uranyl acetate/glutaraldehyde (UG) fixation (36) before we discovered that empty phage-related particles are not well preserved by this technique (44). Only few of the photomicrographs presented here are from cells fixed in this manner. We followed exactly the procedure described previously (36) which, summarized, consists of the following essential steps: The cells are sedimented and the pellet resuspended in Michaelis buffer, pH 6.12, containing 2.5% glutaraldehyde and 0.1% uranylacetate. This suspension is left overnight at room temperature and then after centrifugation, resuspended in melted agar as in the other fixation procedures.

After any of the fixation procedures given above, the agar blocks containing the unfixed cells are posttreated for 1 h in uranyl acetate 5% in distilled water for further fixation of DNA and staining. The blocks were then dehydrated in acetone and embedded either in Vestopal W or in Epon. The sections were always poststained by floating on 5% aqueous uranyl acetate.

4.2 Particle counts on thin sections. Particle counts were made on photomicrographs of thin sections of a given thickness (28, 37, 43, 44). The calibration practice is described in (43).

4.3 Global observation of the bacterial nuclei. The overall shape of the nuclear equivalents or nucleoids of E. coli were observed by preparing the bacterial cells by one of the variant procedures of the agar filtration method (14, 17) which is as follows: A liquid culture of cells is deposited on a collodium film which is supported by culture medium solidified with agar. The film is permeable to the liquid, which is absorbed into the agar, leaving the suspended particles on the surface. The supporting collodium film with the cells is then exposed for 10 min to OsO_4 vapors for fixation and afterwards floated off by immersing a cut-out agar block into distilled water.

4.4 In situ lysis. By adding 0.01% of OsO_4 (final concentration) to the culture, T4 wild type- and T4 mutant-infected cells are made ready to become lysed in situ on an electron microscopy supporting film when in the presence of negative stain (19). In combination with high multiplicities of infection this method is of limited usefulness, because of the numerous breakdown products (ghosts and others) resulting from the infecting parental phages.

4.5 Particle counts in lysates. Particle counts in lysates were made by the agar filtration method adapted to negative stain (17). After filtration the particles are fixed in vapors of formaldehyde for 10 min and then the film floated on 8% sodium phosphotungstate.

5. Radioactive Labeling of Cultures and Calculation of the Number of gp23* equivalents

Unless specially indicated, pulse-chase labeling was conducted using cultures grown in M9 to 2×10^8 cells per ml. Before infection, 1 μCi per ml of a ^{14}C-labeled amino acid mixture (CFB 104, Radiochemical Center, Amersham, England) was added to the culture. Two minutes later the pulse was terminated by adding 1% (final) unlabeled casamino acids.

The procedure for continuous labeling is described in detail elsewhere (43). It consists in short of the following: Prior to infection, cultures were grown to 2.2×10^8 cells/ml in M9a (containing 1 mg/ml casamino acids). The infected culture was then continuously labeled by adding an additional 1 mg/ml of a labeled amino acids mixture with a specific activity of 5 μCi/mg which was obtained by diluting the radioactive CEB 104 with an adequate amount of unlabeled casamino acids. Taking into account the actual concentration of amino acids remaining in M9a at the time of labeling, this leads to a specific activity in the medium of 2.7 μCi/mg of amino acids.

The processing of the samples for gel electrophoresis and autoradiographs was described previously (43, 44), where we also gave detailed procedures for determining the gp23* phage equivalents. This is in essence done by assuming, that in wild type-infected cells all DNA-containing ("black") particles counted on sections contain gp23*. This value is then related to the amount of gp23* determined by densitometry of autoradiographs of gels from SDS-polyacrylamide gel electrophoresis. Recently, more direct determinations at our laboratory (unpublished) have confirmed the value.

RESULTS

1. Early Morphological Events After Infection With the G Mutant

After T4 or T2 infection a typical morphological modification of the bacterial nucleus takes place (6, 20, 30) which is now called nuclear disruption (40). Snustad et al. (40) have shown that nuclear disruption is independent of the chemical breakdown of host DNA; the 2 phenomena are controlled by different genes (39). Cascino et al. (7) have shown that in the G mutant, as well as in $30^-\cdot46^-$, some host DNA synthesis still occurs,

although this DNA is of low molecular weight and rather unstable. We therefore asked the question whether in the G mutant the pattern of nuclear disruption is distinct from that in wild type and thus might give a key towards elucidating this curious process of host DNA replication.

To observe the patterns of nuclear disruption we have studied G- and wild type-infected cells up to 30 min after infection by the following methods: a) agar filtration technique for the global observation of nuclei (see Materials and Methods) and b) thin sections. The agar filtration technique gives results which are superposable with those obtained by phase-contrast light microscopy but which are attained with greater ease and with slightly more significant resolution. For thin sections we prefixed and fixed the cells in OsO_4 using RK-conditions (see Materials and Methods). This fixation procedure was chosen because prefixation with OsO_4 is necessary for the preservation of the nuclear shape (as checked by phase contrast microscopy of living cells). It is known that aldehyde prefixation profoundly modifies this shape (36, 42).

For phage T4D, 0.1% OsO_4 prefixation works, however, only up to about 15–20 min after infection; after this time the cells become leaky (19) or even lyse. It should be noted that this is specific for T4 and not the case for phages T2 (20) and λ (27).

The patterns of nuclear disruption are illustrated by the results of the global observation in Fig 1, where we show wild type- and G-infected cells both at a multiplicity of infection of 5. We see that the pattern of the nuclear disruption is indistinguishable for the 2 phages. Immediately after infection the nuclei of growing cells are morphologically modified; they seem to move toward the margins of the cell (Fig. 1b and 1e). Between 5 and 9 min after infection we observe the "nuclear margination" which is very typical for T-even infection (Fig. 1c and 1f).

In thin sections the initital patterns of nuclear disruption are not as clear, because in each section we only observe about one tenth of a nucleus. Hence the variation due to the level of sectioning is much larger than the phage-induced changes. The typical margination, however, is very clear-cut as is seen on Fig. 2a for G and Fig. 2b for wild type.

At times later than 10 min after infection with wild type the marginal vacuoles are steadily replaced by the vegetative pool of phage DNA exactly as described for T2 (20). In contrast to the morphology of the nuclei of growing uninfected cells, the general morphology of the phage DNA pool appears not to depend on the fixation used. Aldehyde or OsO_4 prefixation together with Os main fixation or uranyl acetate/glutaraldehyde fixation, give phage DNA pools which do not show the same "compact" delimitation as a nucleoid; the DNA-containing areas appear as if "disrupted" into fragments (Fig. 3a).

The marginal vacuoles of G-infected cells also disappear but no pools are built up; the cytoplasm has now a rather homogeneous appearance with the "polysomes" equally distributed.

Distinct helical structures are sometimes observed in these cells with "diluted" ribosomes (Fig. 3b). They resemble the helical form of polysheath (16), an aberrant assembly of the tail-sheath protein (gp18) of T_4. We will see later (Table IV) that this gene product is present in amounts corresponding only to 5% of that in wild type-infected cells. With this low amount polysheaths would not be visible. We prefer to think that the helical structures are favorably oriented polysomes or crystalline arrangements of ribosomes. On close inspection of photomicrographs similar to Fig. 3b, one can indeed see "stretches" of ribosomes, mostly pointing from the cell membrane to the axis of the cell, which could correspond to the same helical structures viewed in a direction which is not strictly vertical to their axis. In order to check if such structures arise as a consequence of a reduced

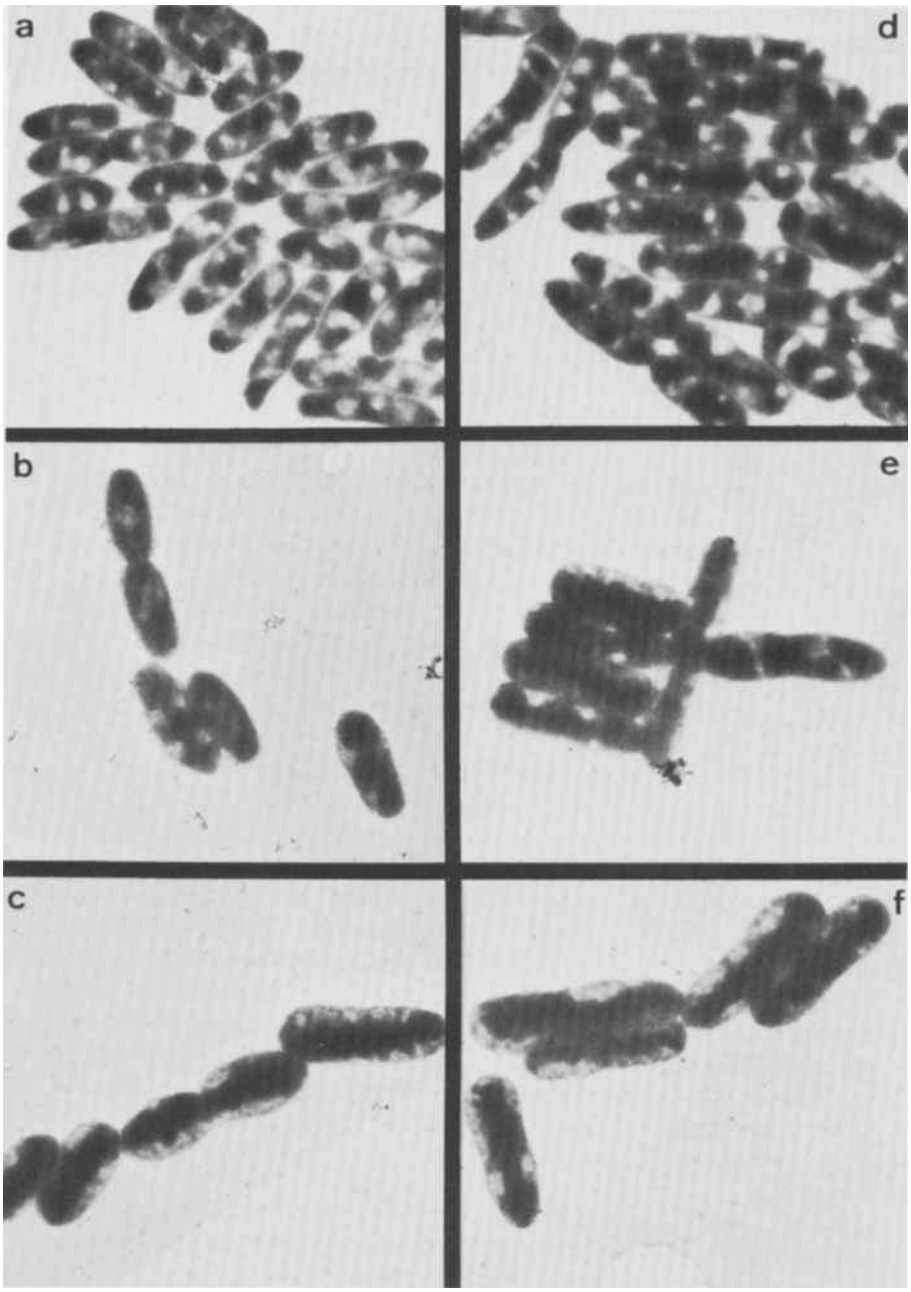

Fig. 1. Nuclear disruption in E. coli BE after infection at an m.o.i. 5(+5) with wild type and the G mutant at 37°C. Global observation of samples prepared by agar filtration. a) Uninfected; b) 2 min after infection with wild type; c) 5 min after infection with wild type; d) 0 min after infection with G; e) 5 min after infection with G; f) 9 min after infection with G. The nuclei move towards the margins of the cells (b and e), and between 5 and 9 min after infection with both wild type and G the typical "nuclear margination" is observed (c and f).

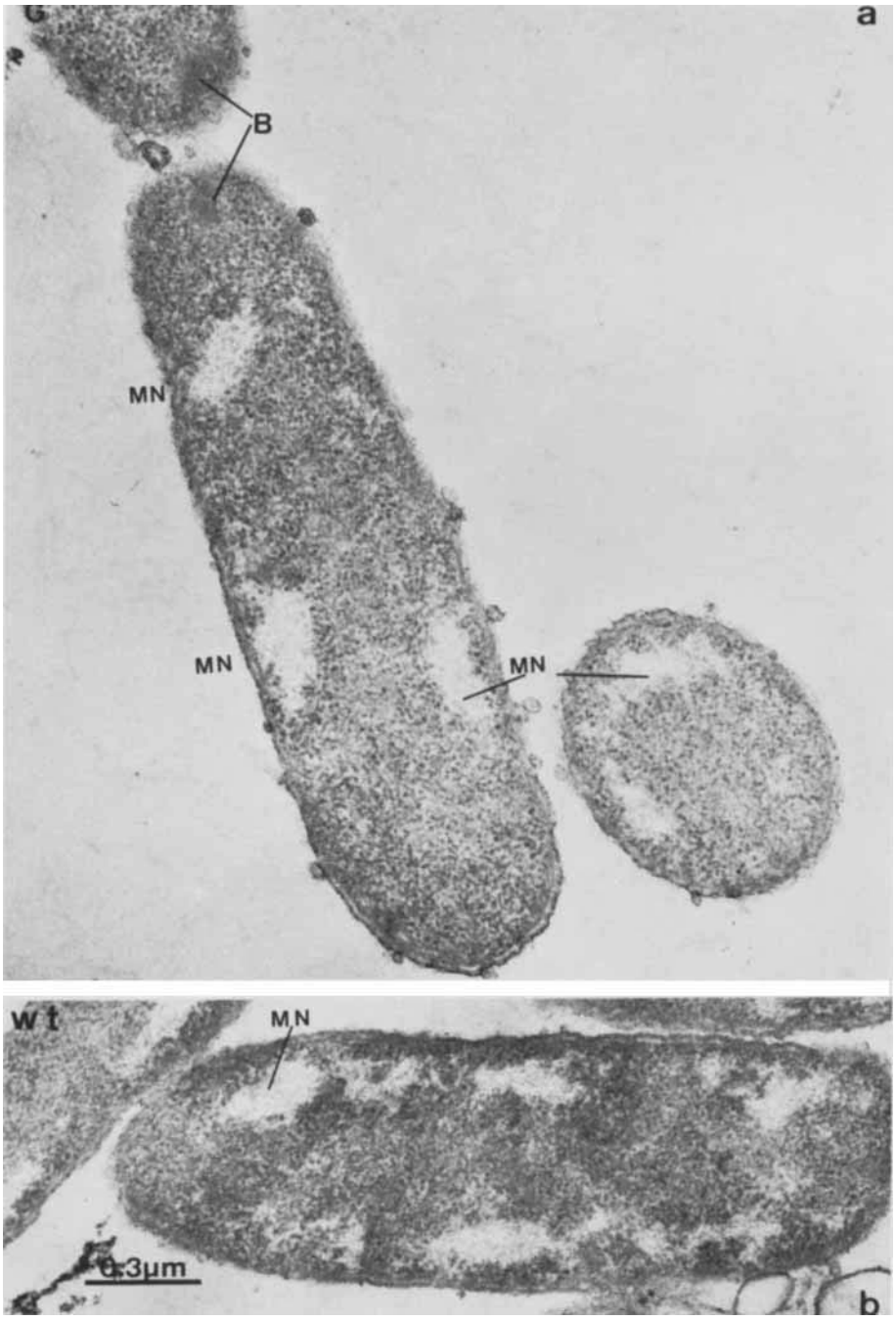

Fig. 2. "Nuclear margination" in E. coli BE after infection with T4 wild type (wt) and the G mutant as observed on thin sections. FoOs fixation, embedded in Vestopal W. The typical marginal nuclei (MN): a) 7 min after infection with G; b) 9 min after infection with wild type. Note the "lump-like bodies" (B) of unknown nature, which are discussed in the text.

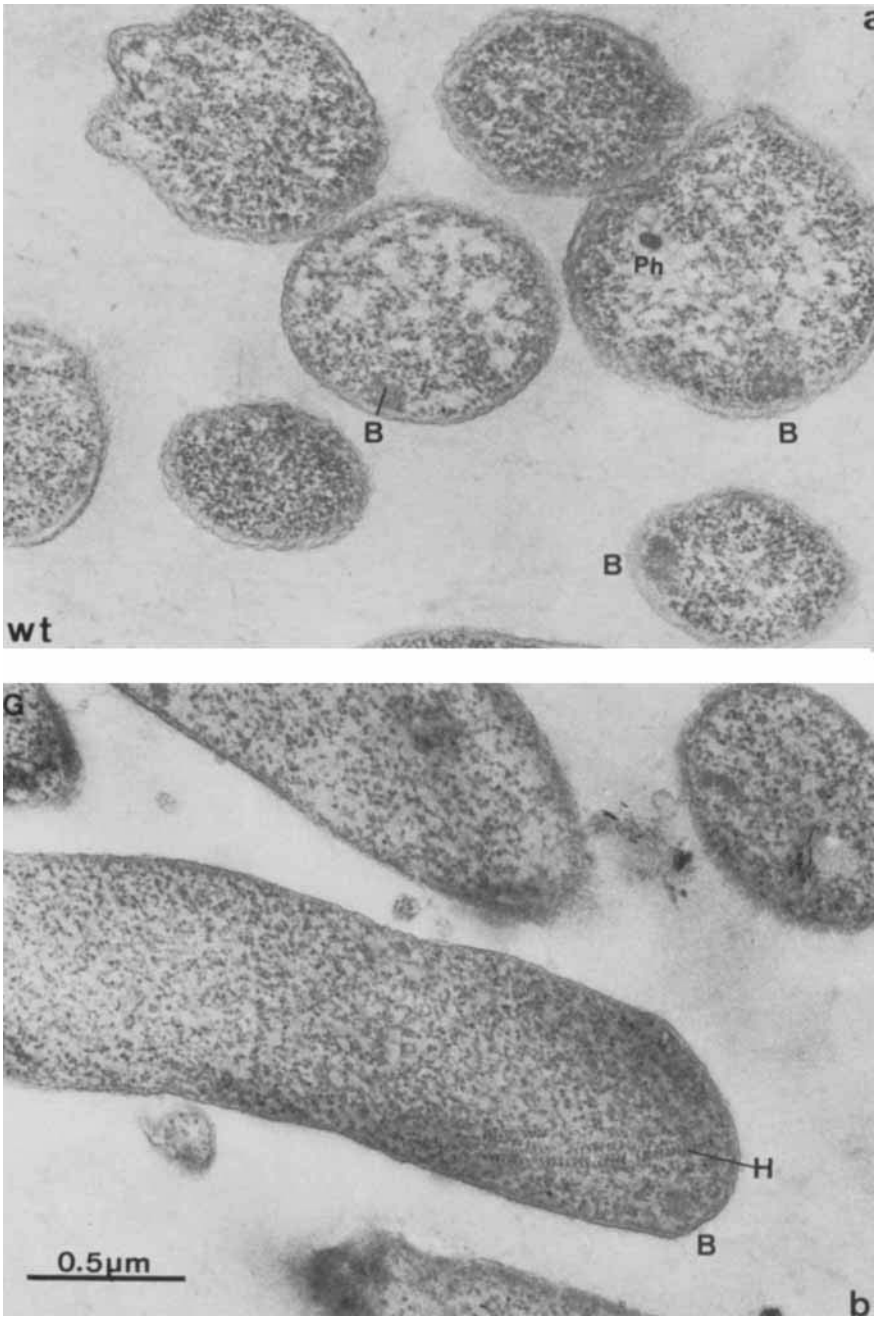

Fig. 3. Thin sections of T4 wild type- and G-infected E. coli BE at 20 min after infection at an m.o.i. of 5(+5). UG fixation, embedded in Vestopal W. a) wild-type(wt): The pool of phage DNA appears "disrupted" into fragments. Some phage particles (Ph) can be observed; b) G: note the homogeneous appearance of the cytoplasm, in which the "polysomes" are equally distributed and sometimes appear as helices (H). In both cases (a and b) many "lump-like bodies" (B) are found (see text).

amount of mRNA, we observed uninfected bacterial cells treated with rifampycin (50 μg/ml). We did not find similar structures.

With both methods of fixation (Os, UG) we found "lump-like bodies" (B) in wild type as well as in G (Figs. 2a, 3a). As we will discuss later, they are probably of different types, and only a few of them are likely to be gp23 lumps (25, 38). Indeed, we find "lump-like bodies" 7 min after infection at low multiplicity (Fig. 2a), when practically no gp23 is as yet synthesized. About 20 min after infection, some phage particles can be observed in wild type (Fig. 3a), but no head-related material in G (Fig. 3b).

In conclusion: Up to 20 min after infection, G- and wild type-infected cells do not show striking morphological differences, except that in G no phage DNA pool is formed.

2. Production of Phage-Head-Related Material in G-Infected Cells at High Multiplicity of Infection

As we shall see in Results, section 3, the number of phage-related particles increases with the multiplicity of infection. We have also found that under our conditions of phage growth protein synthesis is continuous up to 3 or even 4 h after infection when lysis starts to occur (Fig. 8). In the following we describe the particles found 90 min after infection at high multiplicity with G, G·21$^-$, G·24$^-$, G·23$^-$, G·17$^-$ and compare them with those produced by 21$^-$ and 24$^-$ (τ particles), 20$^-$ (polyheads), and 17$^-$ (empty particles); gene 23 codes the major head protein.

2.1. In G-infected cells we observe a few empty particles in the cytoplasm (Fig. 4b). Two types can be distinguished by their morphology and size (Table I). They correspond exactly to the 2 types of particles (empty small particle, esp, and empty large particle, elp) found in 17$^-$ (Fig. 4a; see also Fig. 7 in paper I of this series (44)]. It is interesting to note that G-infected cells which are fixed by the UG procedure do not show distinct particles, but sometimes have structures which could be "blown-up" or swollen empty capsids. As discussed in the first paper of this series (44), the greatest number of 17$^-$-type particles is found with the FoOs fixation. With F. Eiserling we had previously studied 17$^-$-infected cells fixed in UG. We found only empty large particles. This suggests that these types of empty particles are not very stable against deformation or dissociation. This will be further substantiated in Results, section 4, and in forthcoming papers.

In Fig. 4b and Fig. 7 of paper I (44), we see 2 types of differently preserved 17$^-$-infected cells; in the one, the capsids of esps and elps are clearly defined, while in the other, we find only clearly delimited holes of uniform size. In 17$^-$-infected bacteria, a completely homogeneous response of the population towards fixation was never achieved. This might be a property related to the abnormal lysis behavior of 17$^-$ mutants which we have mentioned already. In G, we do not find the 2 types of response of the infected cells.

2.2. G·21(*am*N90) and G·24(*am*N65) both produce indistinguishably a few τ particles, which are always attached to the membrane (Fig. 5). They correspond in size and aspect to the τ particles of 21$^-$ and 24$^-$ described in paper I of this series (44). The particles are visible in both UG and FoOs fixation.

2.3. As a control, we observed G·23(*am*B17) at high multiplicity of infection. As expected we found no head-related particles in either FoOs- or UG-fixed cells. (No example is shown, because they appear in all respects, including the "lump-like bodies," the same as G-infected cells at high multiplicity of infection, except that they lack empty particles.)

TABLE I. Size of Phage-Related Particles in the G Mutant*

Particle	Mutant	Number of particles counted	Length outside Å	Width outside Å	Length inside Å	Width inside Å
esp	G	13	924 ± 29	727 ± 44	640 ± 67	488 ± 47
	$16^-, 17^-$	64	–	–	653 ± 23	472 ± 38
elp	G	9	1,122 ± 49	849 ± 51	–	–
	$16^-, 17^-$	9	1,132[a]	894[a]	907 ± 57	676 ± 26

*The particles were measured as described in paper I of this series (44, Materials and Methods 4.5). The experimental reproducibility is indicated by the standard deviation. The values for mutants 16^- and 17^- are taken from Table IV of paper I. All measurements were made on sections of well-preserved bacteria, with the exception of the values in the last line, which are from partially lysed cells. The distances measured are inserted in Fig. 3 of paper I (44). esp) Empty small particle; elp) empty large particle.
[a]Only two particles measured.

2.4. G·17(*am*N56) could not be observed at high multiplicities of infection, because, as already mentioned (Materials and Methods) most cells of the population lyse. In the few remaining cells, or at lower multiplicities of infection, very few empty particles are found, and they are morphologically indistinguishable from those produced by G.

2.5. The mutants in 24^- produce τ particles and also an approximately equal number of polyheads (4). In sections of the quadruple mutant G·24^- we observed only very few polyheads. This is not unexpected because of the low number of particles per cell and the requirement of finding a polyhead lying nearly parallel to the section. In order to make sure that polyheads can be made without phage DNA replication we studied G·20(*am*N50) by in situ lysis (Fig. 6). We see that polyheads are found in numbers compatible with the biochemical determinations described in section 9 of Results.

2.6. Besides the specific types of particles described above, the following particular features were observed with all G and G-derived multiple mutants in bacteria 90 min after infection at high multiplicities: a) A central, well-delimited area containing very dilute, fine-stranded material is observed (Figs. 4b, 5b, and 5c). The precise nature of this area – which we interprete as a DNA-plasm – is undecided. Nothing indicates whether it contains parental phage DNA without or with some replication (G. Garcia and G. Mosig have evidence for some 10% phage DNA replication with 43(*am*B22) on E. coli B, they determined it by density labeling; personal communication) or the abnormal, short and fragile host type of DNA reported by others (7). b) "Lump-like bodies" (B) are found in numbers and sizes which are not significantly different for different mutants or different multiplicities of infection. Such bodies are found also in G·23(*am*B17). For further consideration refer to Discussion.

3. Particle Production as a Function of the Multiplicity of Infection

On numerous photomicrographs of thin sections we counted the number of head-related particles formed per cell. The results given in Table II can be summarized as follows: 1) As expected from general experience, the yield of phage or phage-related particles in wild type is not significantly increased with high multiplicities of infection; in contrast to this the G mutant, and all studied combinations thereof with mutants in assembly genes, shows a clear-cut increase of yield with increased multiplicity of infection. 2) The number of τ particles found with G·$24^-$and G·21^- is about the same as that of esps and elps found for G.

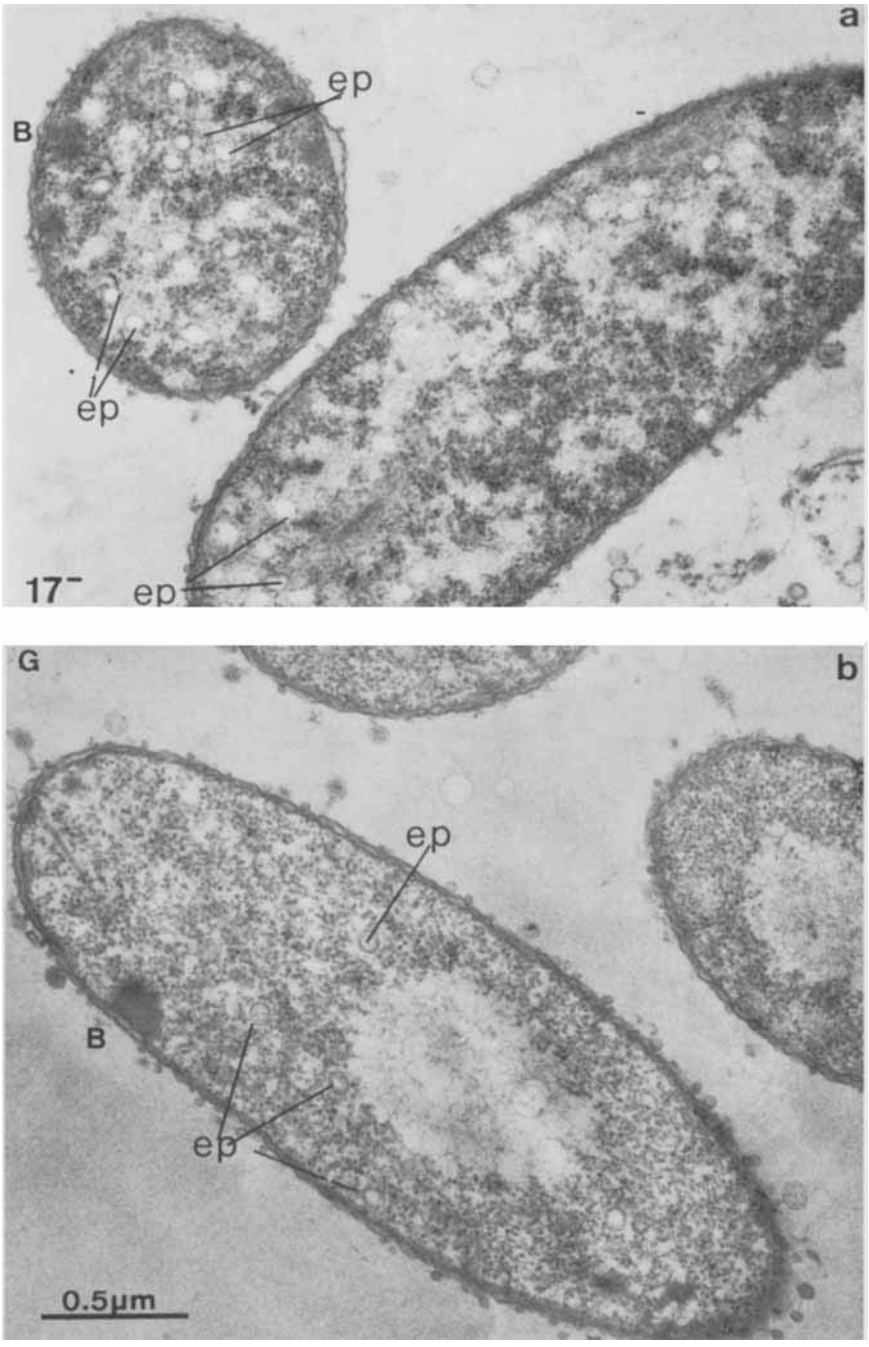

Fig. 4. Thin sections of G- and 17⁻-infected cells. FoOs fixation, embedded in Epon. a) 17(*am*N56)-Infected E. coli BE, 90 min after infection (m.o.i. 5(+5]. The empty particles (ep) appear as holes; sometimes a membrane can be distinguished. In the lower right corner, part of a lysed bacterium is seen with some empty particles. Note the "lump-like body" (B). b) G-Infected E. coli BE, 90 min after infection (m.o.i. 100). The same type of empty particles (ep) is found as in 17⁻. Note the central area of dilute, fine-stranded material, the nature of which is unknown as discussed in the text.

TABLE II. Number of Phage-Related Particles in G and Combinations Thereof With Assembly Mutants as a Function of the Multiplicity of Infection*

m.o.i.	Phage-related particles	Number of phage-related particles in: w.t.	G	G·21⁻	21⁻	G·24⁻	24⁻	G·17⁻	17⁻
5(+5)	τ	0.8	0	0.35	300	0	156	0	13.7
	bp + gr	240	0	0	0	0	0	0	1
	cp	0	4.3	2.4	0	1.1	1.1	3.2	196
	total	240.8	4.3	2.8	300	1.1	157.1	3.2	210.7
20	τ	–	0	6.6[a]	–	–	–	–	–
	bp + gr	–	0	0	–	–	–	–	–
	ep	–	6[a]	4	–	–	–	–	–
	total	–	6	10.6		–	–	–	–
50	τ	–	–	–	–	21	–		–
	bp + gr	–	–	–	–	0	–	lysis	–
	ep	–	–	–	–	6.8	–		–
	total	–	–	–	–	27.8	–		–
100	τ	3	0	55	–	48	–		–
	bp + gr	346	0	0	–	0	–	lysis	–
	ep	0.7	45	6.5	–	3	–		–
	total	349.7	45	61.5	–	51	–		–

*The experiments were carried out at 37°C. Aliquots of the cultures were taken at 90 min for fixation with the FoOs method, with the exception of those marked [a], which were fixed with UG. bp) Black particles, gr) grizzled particles, ep) empty particles (for definition of the types of phage-related particles see paper I, section 2, of this series (44)). Counting of particles was done as described in the same paper (Materials and Methods, section 4.5).

4. Attempts to Transform Preheads Into Empty Particles

In the first paper of this series (44), we have shown that mutants in gene 24 are epistatic on those of gene 17. Since the particles found in G are morphologically similar to those in 17⁻, we made an attempt to quantitatively transform preheads into empty particles by using *ts* mutants and temperature shifts. By similar experiments a quantitative transformation of preheads into heads was demonstrated by Bijlenga et al. (3). If the empty particles represent a true intermediate, a transformation of preheads into empty particles should be at least equally quantitative. In order to test this prediction we designed the following experiments with 17(*am*N56)·24(*ts*L90) and G·24(*ts*L90): Infected cells were grown at nonpermissive temperature (40.5°C) for 90 min and then shifted to permissive temperature (30°C). Samples were taken before and after shift for particle counts on thin sections. Experiments were done with and without chloramphenicol added shortly before shift. The results are given in Table III. In both cases the transformation is not quantitative. In the G·24*ts* we clearly lose particles, even without chloramphenicol added. (See note added in proof at the end of this paper).

These results suggest that the accumulated empty heads are not true intermediates in either case and, furthermore, that they are not very stable in contrast to finished capsids. In order to check this stability we lysed 17(*am*N56)-infected cells at 30 min after infection by either chloroform or 0.01% OsO_4. In Fig. 7 we show a photomicrograph of the particles obtained after OsO_4-induced lysis. We observe 2 types of empty particles which are defined by a different "roughness" of the surface structure and by the degree of flattening. We think that they represent the empty small and empty large particles as were

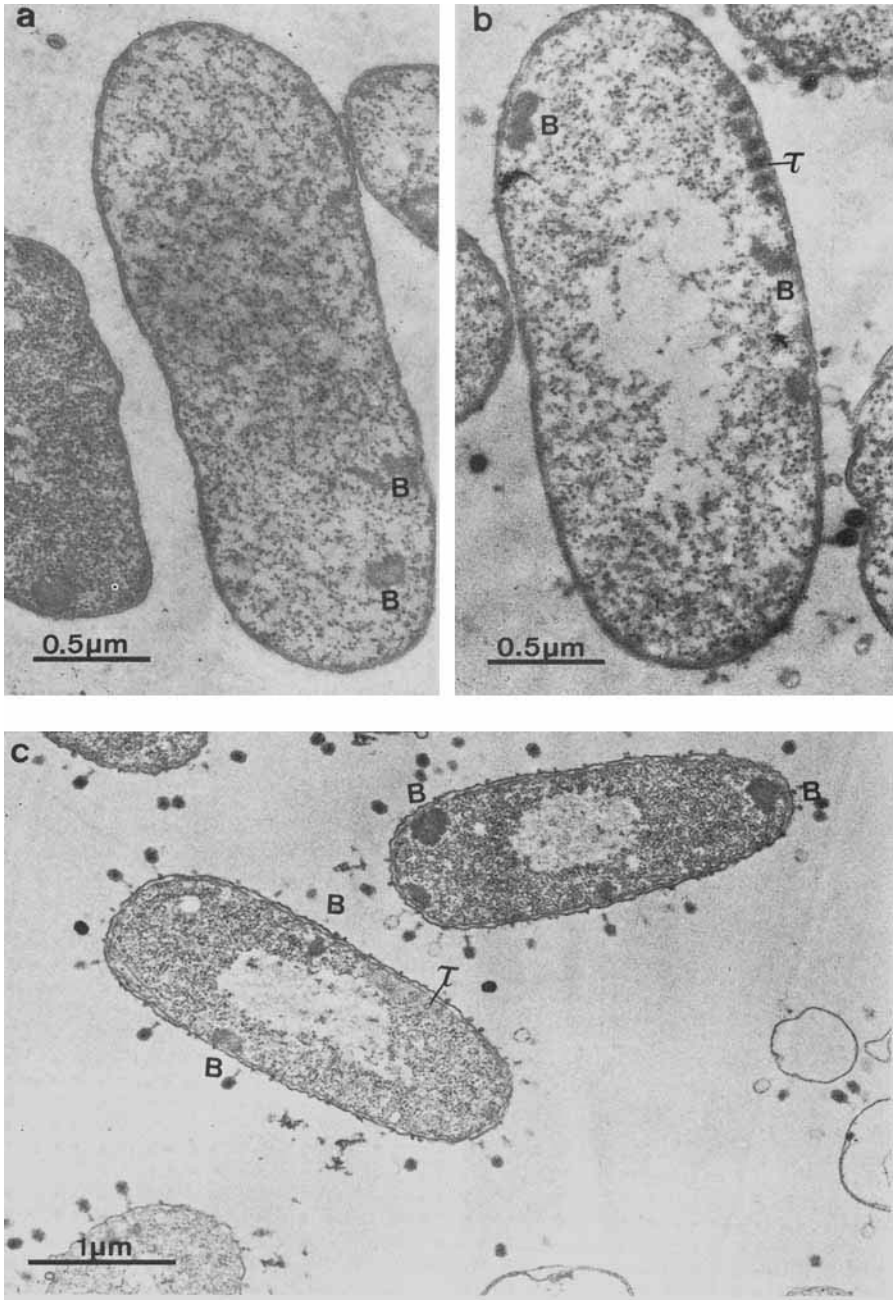

Fig. 5. τ Particles found on thin sections of G·21$^-$, m.o.i. 5(+5), 90 min after infection. No phage-related particles are visible (UG fixation, embedded in Vestopal W). b) G·21$^-$, m.o.i. 100, 90 min after infection. τ Particles are found, which are membrane-attached and indistinguishable from the τ particles found in the 21$^-$ single mutant. UG fixation, embedded in Vestopal W. c) G·24$^-$, m.o.i. 100, 90 min after infection. τ Particles are found as the only head-related structure. Note the central area of fine-stranded material. FoOs fixation, embedded in Epon. "Lump-like bodies" (B) are found with both fixation methods.

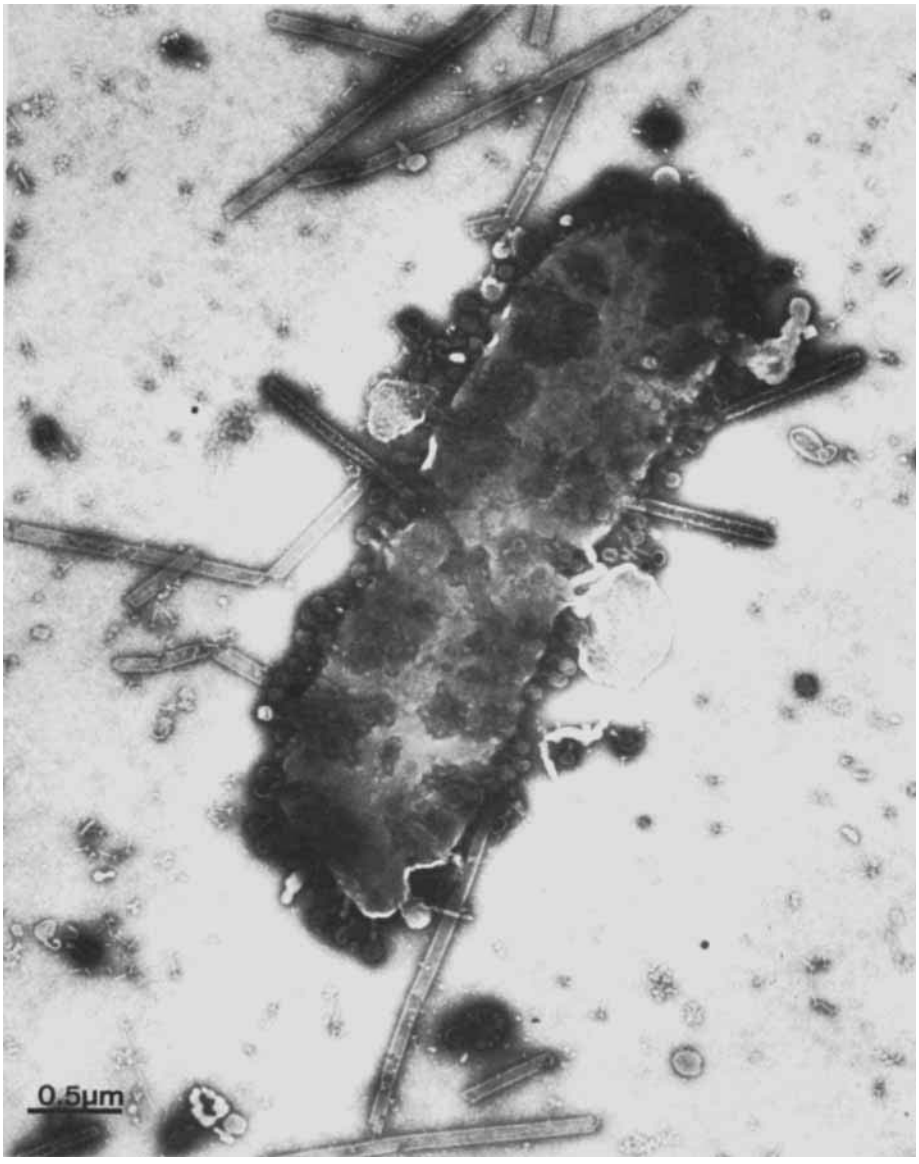

Fig. 6. In situ lysis of a G•20⁻-infected cell, m.o.i. 100. Polyheads made in the absence of phage DNA replication are visible. Note the remainders of the infecting parental phages.

defined on thin sections. The rough particles (Fig. 7), which are in the form of disks are likely to correspond to the esps. They can have tails attached or not and they always occur together with "normal" empty phages and capsids. These latter are smoother and flattening leads to a typically wrinkled appearance. They would correspond to the empty large particles seen on thin sections.

When comparing the particle counts after the 2 types of lysis (Table IV) it becomes obvious that the "rough" particles survive the OsO_4-induced lysis nearly 5 times better than after the chloroform-induced lysis, thus demonstrating their lability. (See note added in proof at the end of this paper).

TABLE III. Incomplete Transformation of Preheads Into Empty Particles*

Mutant	Time after infection, min	Time at 40.5°	Time at 30°C	Particles per bacterium without CAP τ	without CAP ep	with CAP τ	with CAP ep
24(*ts*L90)•17(*am*N56)	90	90	0	33	0	–	–
m.o.i. 5(+5)	130	130	0	34	< 1	18	0
	110	90	20	26	29	16	6
	150	90	60	22	77	13	14
24(*ts*L90)•G	90	90	0	29	2	–	–
m.o.i. 100	150	90	60	3	8	–	–

*E. coli BE was grown in M9A and infected and superinfected as usual and grown at 40.5°C. At 90 min after infection part of the cultures was shifted to permissive temperature (30°C), whereas the rest stayed at 40.5°C for control. For 24⁻ (*ts*)•17⁻(*am*) we made 2 aliquot cultures to one of which we added 25 μg chloramphenicol (CAP) per ml at 90 min after infection. At the times indicated, aliquots were taken for fixation with FoOs. Counting of the phage-related particles on thin sections was done as described in paper I [Materials and Methods, section 4.5 (44)]. ep) Empty particle.

5. Attempts to Mature Empty Particles Into Phage

If the empty particles accumulating in G were true precursors, they should mature into active phage when phage DNA becomes available. We therefore constructed the triple *ts* mutant 43(*ts*P36)•30(*ts*A80)•46(*ts*L166) and repeated, as a control, particle counts on thin sections of cells infected with this mutant at high multiplicity of infection and grown at nonpermissive temperature. The usual rigorous checks were applied as described before: less than 10% of cells lysed even at an m.o.i. of 100, and the cell population was homogeneous and showed no more leakage than the triple *am* mutant. Unfortunately this G-triple *ts* mutant behaves differently from the G-triple *am* mutant in that it does not produce countable numbers of empty particles (less than 10 per cell at 120 min). The results obtained after a temperature shift from 40.5°C to 30°C at 30 min after infection, were accordingly: less than 1 plaque-forming unit per cell was found even at 90 min after shift. Controls showed that the cells were lysing neither totally nor partially. We have to conclude that in this triple *ts* the metabolism of phage replication already is severely and irreversibly hampered after 30 min of growth at nonpermissive temperature.

6. Protein Synthesis in G- and Wild Type-Infected Bacteria

The total protein synthesis in G- and wild type-infected cells was compared using continuous label conditions. We find (Fig. 8) that during the first 15 min after infection, both of them have synthesized the same amount of proteins. After 20–30 min the G mutant levels off to a plateau, whereas in wild type synthesis continues linearly for 3–4 h after infection. With the G mutant we performed the following checks of continuous labeling: We measured the uptake of label when continuous label was started at different times after infection. The curves obtained are superposable (Fig. 8). As a further control, we determined the rate of protein synthesis in G mutant-infected cells by pulse-labeling the culture at different times after infection. We used the same medium conditions as for continuous label experiments. If we compare (Fig. 9) the experimental values to the values obtained by calculation from the continuous label data of G in Fig. 8, we find that they are in good agreement.

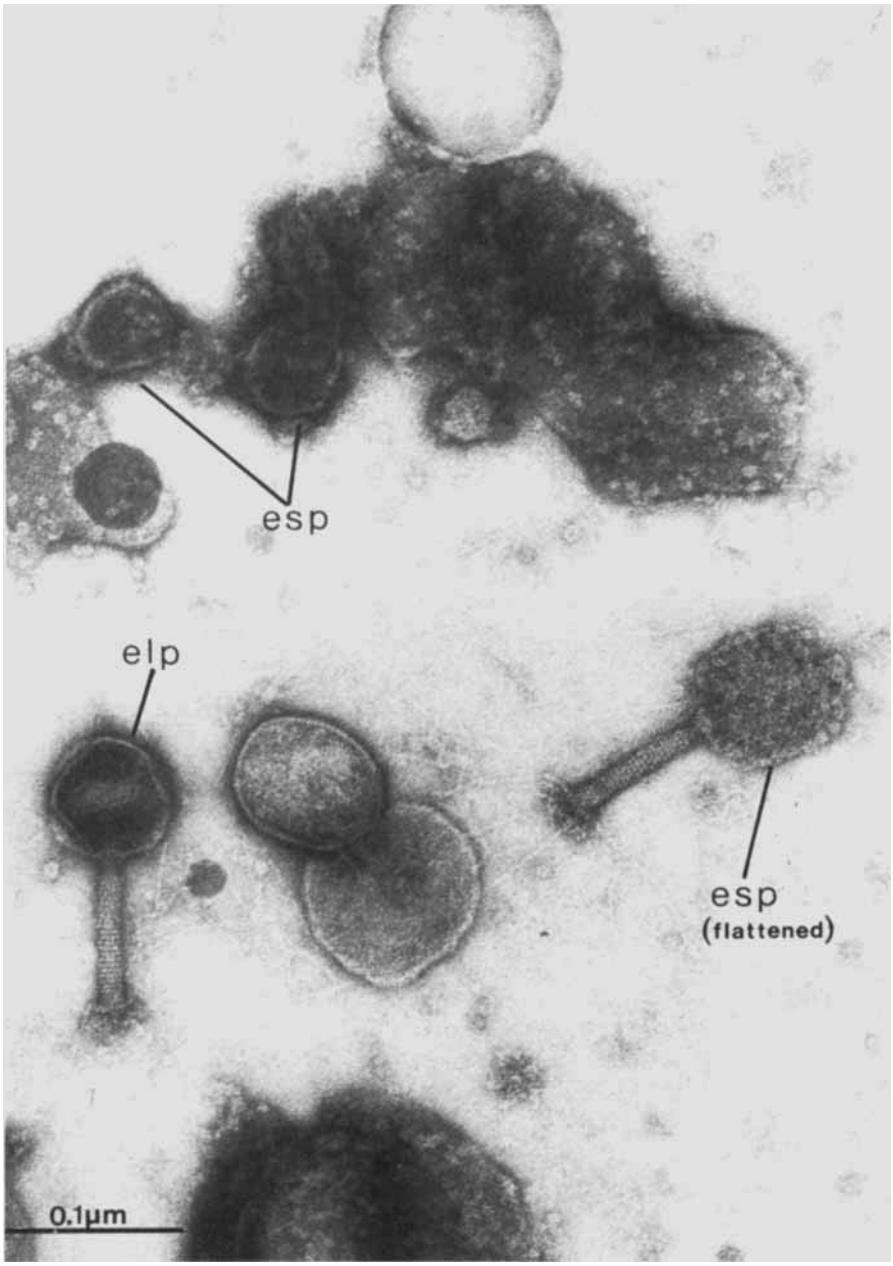

Fig. 7. Electron microscopy of a 17(*am*N56) lysate. Cells were lysed at 30 min after infection by addition of 0.01% OsO_4 to the infected culture. A sample was prepared for electron microscopy by adhesion and negative staining with NaPT 2%. Besides normal looking empty phages (elp), we find a large number of completely flattened particles, which show a "rough" surface (esp) and often have tails attached. Very rarely esps are found which are not completely flattened and retain some negative stain (upper left).

TABLE IV. Number of Phage-Related Particles in Lysates of 17(*am*N56)*

Lysis procedure	empty phage (with tail)	capsids	"rough" particles (with tail)	"rough" particles (without tail)
Chloroform	213	332	47	62
0.01% OsO_4	214	371	106	369

*Two samples of 5 ml each were taken from a 17⁻-infected culture at 30 min after infection. One sample was centrifuged directly (5 min, 3,800 rpm, room temperature) and resuspended in 0.5 ml phosphate buffer (0.1 M), pH 6.0, saturated with chloroform. The other sample was treated with 0.01% OsO_4 just prior to centrifugation; the pellet was resuspended in 0.1 M phosphate buffer, pH 6.0. With this procedure lysis is induced in the pellet and during resuspension. Both samples were prepared by agar filtration adapted to negative staining (Materials and Methods). Aliquots of a wild type lysate were added for calibration. "Rough" particles are shown in Fig. 7.

7. Global Protein Synthesis for Different Multiplicities of Infection

As we have seen in section 3 of Results, the number of phage-related particles produced in G-infected cells is augmented significantly with increased multiplicity of infection. We therefore asked if the protein synthesis is multiplicity of infection dependent and could thus explain the multiplicity of infection dependent particle formation. We established the kinetics of protein synthesis for different multiplicities of infection [5(+5), 20, 50, and 100) for wild type and G. In Fig. 10, we present the protein synthesis of wild type for multiplicities of infection of 5(+5) and 100. The results show no significant multiplicity of infection dependence of the global protein synthesis. In Fig. 11 the kinetics of protein synthesis of G is shown for several multiplicities of infection. Here also, the global protein synthesis is not significantly dependent on the multiplicity of infection. Differences which are smaller than 10% could, however, not be detected by this procedure.

8. Multiplicity of Infection Dependence of Individual Early and Late Proteins

In the previous section we have shown that within 10% the global protein synthesis in G is independent of the multiplicity of infection. Knowing that the number of phage-related particles produced is multiplicity of infection dependent, we investigated a possible positive or negative multiplicity of infection dependence on some individual early and late proteins.

In a first approach we estimated the rate of synthesis of different proteins. For this purpose, we analyzed samples obtained with different multiplicities of infection, which were pulse-labeled at different times after infection. The gel patterns are given in Fig. 12, where we compare cells infected with G at several multiplicities of infection with wild type. On the wild type gels we identified early and late proteins according to previous data of the literature or that available in our laboratory.

As an essential qualitative difference we find that in the G mutant the synthesis of early proteins is not turned-off at some 8 min after infection as happens in the wild type-infected cells. The synthesis of early proteins continues, although for some of them at altered rates which also depend strongly on the multiplicity of infection. When we compare cells infected with G at low multiplicity of infection (5+5) with those infected at high multiplicity of infection (100) we find that the rate of synthesis of most early proteins is practically unaltered (rII A, gp39, gp52, gp33) while some others clearly show a

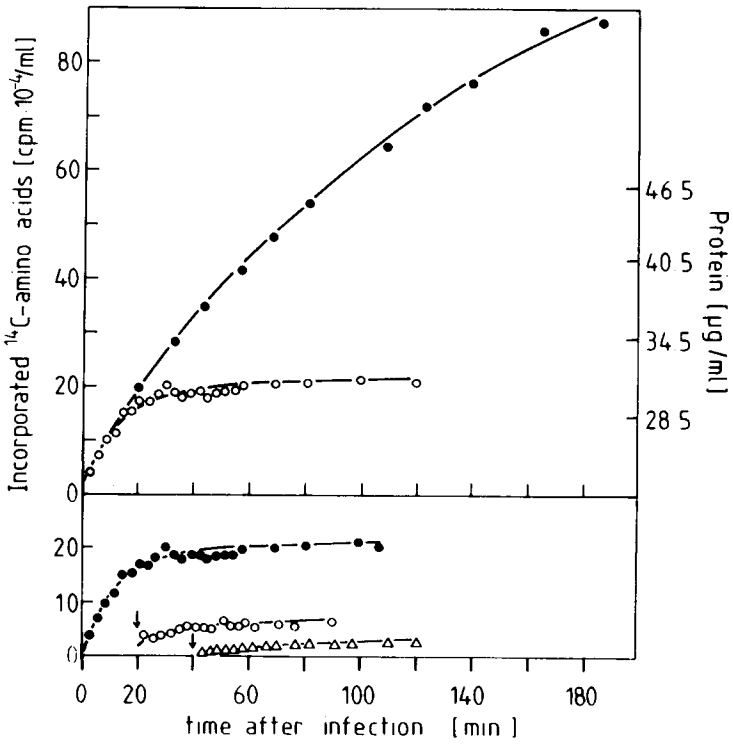

Fig. 8. Kinetics of protein synthesis in G- and wild type-infected bacteria at 37°C. Cultures of E. coli BE, infected and superinfected with T4 wild type or G, both at a multiplicity of infection of 5, were continuously labeled with ^{14}C-labeled amino acids (see Materials and Methods). The scale on the left side represents the amount of phage-related protein, measured as incorporated ^{14}C- labeled amino acids contained in a TCA precipitate. The scale on the right side indicates the amount of protein expressed in grams, as determined by calibration with the Lowry method (43). No lysis could be detected during the time of the experiment. •—•) Wild type; ○—○) G. The lower part of the figure represents protein synthesis in G-infected cells under the same conditions as above, but labeling was started at: •—•) 1 min after infection; ○—○) 20 min after infection; △—△) 40 min after infection.

decreased rate at high m.o.i. (gp63 and "293"). The rate of synthesis of some identified late proteins (gp23, gp7, gp37, gp18, gpwac, gp23*) is greater at the high multiplicity of infection than at the low one. Most of these rates increase with the time after infection while in wild type they are nearly constant. Only extremely few proteins eventually reach rates which become comparable to those of wild type (e.g., gp18, gpwac), while many others stay smaller or even decrease (gp34, gp37). Some bands which are present in wild type are completely lacking in G. These results seem to contradict those reported in the previous section, which demonstrated that the global protein synthesis in G is – within 10% – independent of the multiplicity of infection.

From pulse-labeled experiments we cannot make statements on the absolute amounts of early and late proteins synthesized. We thus analysed samples of continuously labeled cultures infected with G at multiplicities of infection of 5(+5) and 100 and with wild type at 100. The results are summarized in Table V. We analyzed 36 protein bands which were resolved by gel electrophoresis (Fig. 13). Proteins b, gp52, and gp63 are produced in

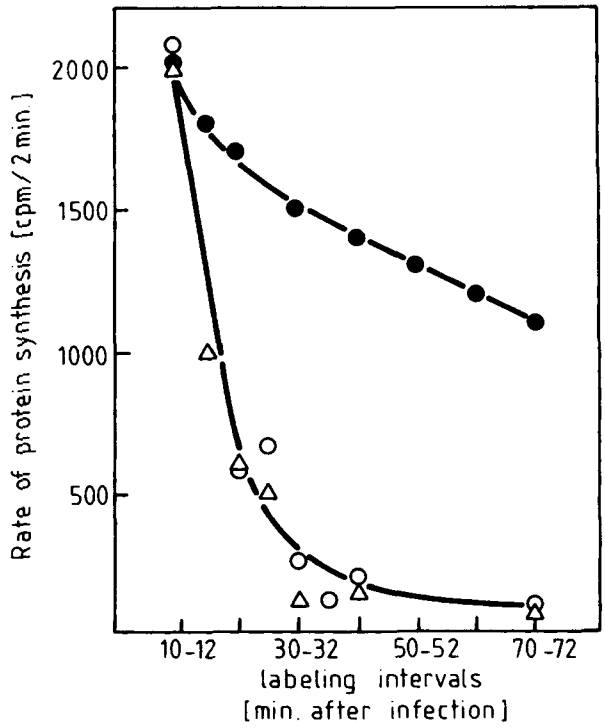

Fig. 9. Rate of protein synthesis as a function of time in G- and in wild type-infected bacteria at 37°C. ○—○) G-Infected cells, pulse-chase experiment under the same growth conditions as used for continuous labeling. E. coli BE was grown in M9a, infected and superinfected as usual with an m.o.i. 5(+5). From this culture 1-ml aliquots were pulse labeled by addition of 2 μCi of ^{14}C-labeled amino acids (specific radioactivity: 2.7 μCi per mg), at the times indicated on the abscissa. Immediately after addition of the label, 0.1 ml were precipitated with TCA. The radioactivity of the precipitate was counted in a scintillation counter to yield the background value. Two minutes after pulse the label was chased by addition of 1% (final) cold casamino acids. Fifteen minutes after the pulse 2 samples of 0.1 ml each were precipitated by addition of TCA. After deducing the background, the 2 measurements are averaged and plotted (○—○). Values calculated from the continuous labeling data in Fig. 8 are also plotted: △—△) for a G-infected culture; •—•) for a wild type-infected culture.

smaller amounts with wild type than with the G mutant. All other early proteins are present in similar amounts in wild type-infected cells and in G-infected cells at both multiplicities of infection. With G at a multiplicity of infection of 5(+5), the amount of late proteins synthesized is less than 5% of that of wild type. With G at a multiplicity of infection of 100, the amounts of most of the identified late proteins are increased over those obtained at a multiplicity of infection of 5(+5). It is interesting to note that not all of the late proteins are increased by the same factor: i.e., gp34 and gp7 reach no more than 5% of the levels found in wild type, whereas protein c attains about one third of wild type. Protein h is the only one which is produced in large amounts in G at a multiplicity of infection of 5(+5), but not at 100 and not in wild type. From these data we conclude that the results of this section are not at all contradictory to the ones obtained in the previous section: The global protein synthesis in G at high multiplicities of infection is slightly increased comparatively to that at low multiplicities of infection. But as the increase is at the most 10%, it could not be detected by measuring the global amount of protein.

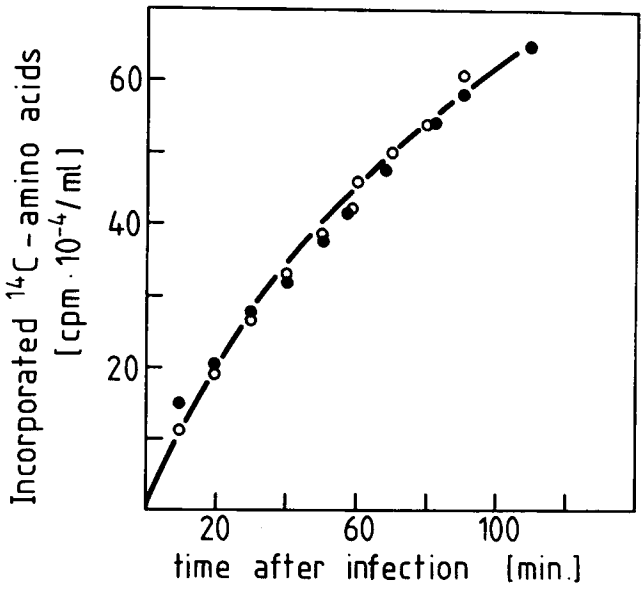

Fig. 10. Kinetics of protein synthesis for wild type-infected bacteria at multiplicities of infection of 5(+5) and 100. Incorporation of ^{14}C-labeled amino acids was determined by continuous labeling (see Materials and Methods). Lysis was checked throughout the experiment and found not to exceed 5%. •—•) m.o.i. 5(+5); ○—○) m.o.i. 100.

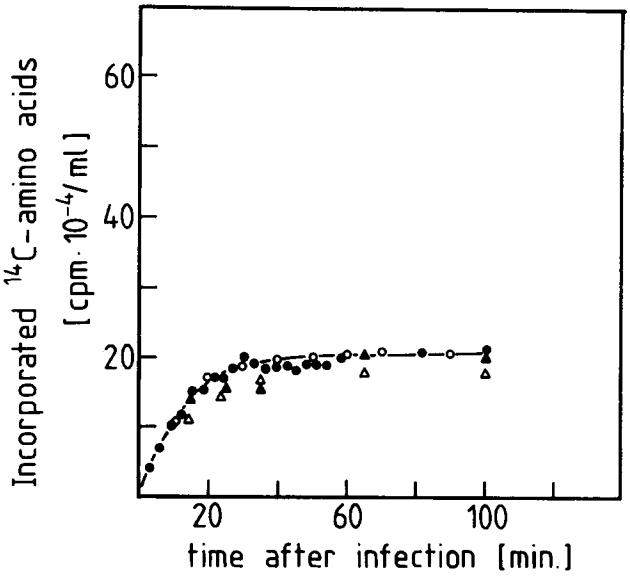

Fig. 11. Kinetics of protein synthesis for G-infected bacteria at different multiplicities of infection. Incorporation of ^{14}C-labeled amino acids was measured by continuous labeling as described (see Materials and Methods). Lysis did not exceed 10%. •—•) m.o.i. 5(+5); △—△) m.o.i. 20; ▲—▲) m.o.i. 50; ○—○) m.o.i. 100.

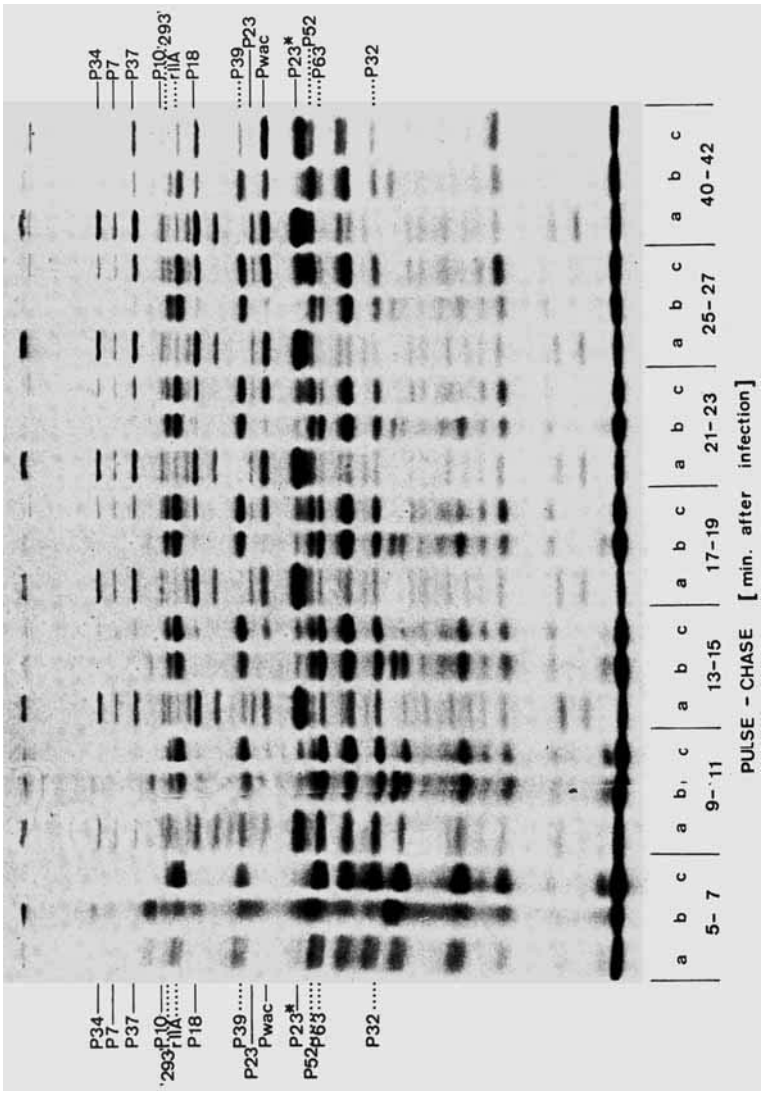

Fig. 12. Rates of synthesis of early and late proteins in wild type- and G-infected cells at 37° C. E. coli BE was grown in M9 up to 2×10^8 bacteria per milliliter. The culture was divided into 3 parts, each of which was infected with one of the listed phages and multiplicities: a) wild type, m.o.i. 5(+5); b) G, m.o.i. 5(+5); c) G, m.o.i. 100. From each culture aliquots (1 ml) were taken, which were pulse-labeled with ^{14}C-labeled amino acids (1 μCi/ml and 5 μCi/ml for wild type and G, respectively) at the times indicated in the figure. A chase was performed 2 min later by addition of casamino acids (1% final concentration). At 45 min after chase the samples were centrifuged (3,800 rpm, 5 min, room temperature) and the pellet resuspended in 0.2 ml 0.0625 M Tris-HCl, pH 6.8, saturated with chloroform to lyse the cells. "Sample buffer" (0.1 ml; 3 × conc.) (22), were added and the samples heated immediately by immersing in a boiling water bath for 5 min. 20,000 cpm were applied per sample. The samples were run on a 10% polyacrylamide gel [for details see (43)]. ....) Early protein; —) late protein. Note: Instead of the new symbol "gp" for gene product, the old one "P" is used in this figure.

TABLE V. Amounts of Early and Late Proteins as Determined From Continuously Labeled Material From Wild Type- and G-Infected Cells*

Protein[a]	Amount of protein [peak area, arbitrary units] wild type m.o.i. 100	G m.o.i. 100	G m.o.i. 5(+5)	Remarks
gp34	57	2	1	late
gp7	23	1	1	late
gp37	79	9	2.5	late
gp10	33	4.5	3	late
"293"	10.5	8.5	11.5	early
rIIA	14	25.5	19	early
gp6	29	3	2.5	late
gp18	107	15.5	5.5	late
B1	35	2.5	1.5	late
gp20	15	1.5	1.5	late
a	6.5	2.5	3.5	?
b	7.5	38.5	35	early
gp39	37	37	16	early
gp23	84	13	4.5	late
gpwac	47.5	3	3	late
gp23*	383[b]	28	5	late
c	71.5	22.5	9	late
d	41	6	7	late
e	48.5	32.5	36	late
gp52	36	55.5	54	early
gp63	34.5	73.5	82	early
f	52.5	25	23	?
g	20.5	13.5	11.5	?
h	7	6.5	35	?
gp32	44	31.5	39	early
j	30	10.5	9.5	?
k	22	8.5	8.5	?
l	8	5.5	9.5	?
m	23	5.5	4	?
n	16	9	9.5	?
o	29	23	28.5	?
p	27	10.5	10	?
q	35.5	10.5	10.5	?
r	37	33.5	29	?
s	61.5	2.5	3.5	?
t	4.5	3.5	4.5	?
u	48.5	2	2	?
v	7.5	8.5	9	?
Total	1,673.0	594.5	550.5	

*At 90 min after infection aliquots were taken from continuously labeled cultures for gel electrophoresis. The amount of protein was determined from autoradiograms which had been exposed for 9 days.

[a]The identification of bands is based on Vanderslice and Yegian (41), O'Farrell and Gold (31) and O'Farrell et al. (32) and results from our own laboratory.

[b]This peak was too large for the standard settings. It was therefore calculated from less exposed gels (24 and 72 h).

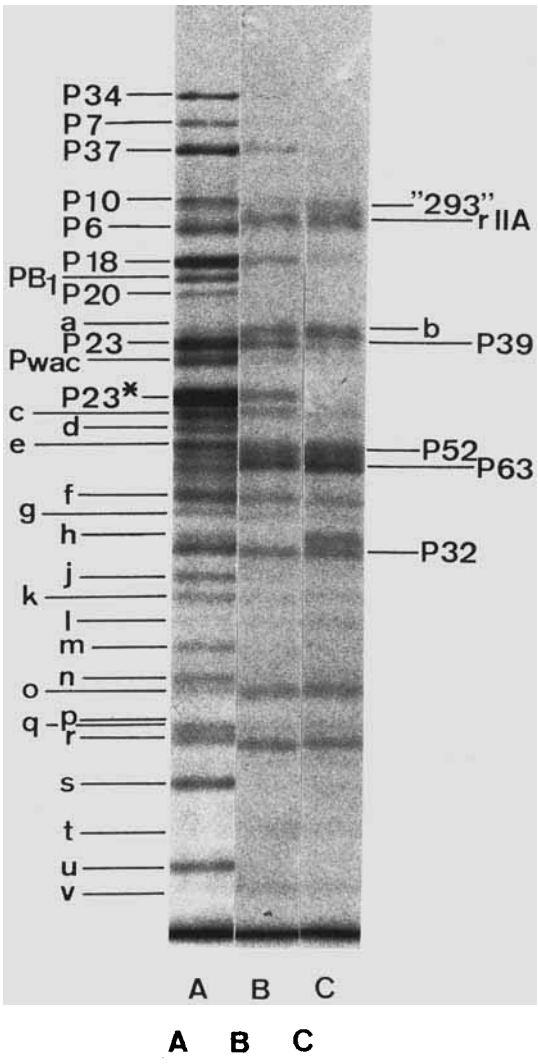

Fig. 13. Analysis of proteins present in cells 90 min after infection with wild type and G. A) wild type, m.o.i. 5(+5). B) G, m.o.i. 100; C) G, m.o.i. 5(+5). Continuous labeling was performed with ^{14}C-labeled amino acids as usual. At 90 min after infection, samples were taken for gel electrophoresis (43). Samples were run on 10% polyacrylamide gels. Note: Instead of the new symbol "gp" for gene product, the old one "P" is used in this figure.

In Table V we also give the sum of all proteins found 90 min after infection with G and with the wild type at low and high multiplicities of infection. We find that the amount of proteins produced by G at either multiplicity of infection is about one third of wild type, which corresponds to our measurements of the global amount of proteins reported in Fig. 8.

TABLE VI. Number of gp23* Equivalents as a Function of the Multiplicity of Infection in G- and Wild Type-Infected Cells*

Phage	m.o.i.	gp23* Equivalents per bacterium at 90 min after infection	Phage-related particles per bacterium at 90 min after infection
wt	5(+5)	449	440
	100	434	–
G	5(+5)	4.6	5
	20	12.6	–
	50	26.5	–
	100	37.7	45

Phage-related particles were counted on thin sections as described in paper I of this series [Materials and Methods, section 4.5 (44)]. The number of gp23 equivalents was determined as described in (43).

9. Number of gp23* Equivalents

At 90 min after infection we determined the number of gp23* equivalents present in wild type- and G-infected bacteria at different multiplicities of infection, and compared it to the number of phage-related particles per bacterium counted on thin sections. We followed exactly the procedures described in (43). The data are summarized in Table VI. The number of phage-related particles which contain gp23* increases significantly with increasing multiplicities of infection and reaches about 40 at a multiplicity of infection of 100. Within the accuracy of this determination virtually all phage-related particles observed at 90 min after infection can be accounted for by the gp23* present At this time the number of uncleaved gp23 equivalents is less than 5 per bacterium independent of the multiplicity of infection.

DISCUSSION

The results of our studies of the synthesis of phage-coded proteins in cells infected with the triple mutant $43^{-} \cdot 30^{-} \cdot 46^{-}$(G) can be summarized as follows.

In wild type-infected, lysis-inhibited cells the total protein synthesis proceeds sublinearly up to at least 3 h after infection with a slightly decreasing rate; in the G-infected, lysis-inhibited cells the protein synthesis starts to level-off at 18 min after infection and in some 10 min reaches a plateau which corresponds to about one third of the total of phage proteins present in wild type at 90 min. A possible multiplicity of infection dependence is in both cases within the experimental accuracy of some 10%. When studying the protein synthesis for individual early and late proteins by gel electrophoresis a rather complicated pattern emerges which we can describe only qualitatively. In G-infected cells the rapid switch-off of early proteins, which occurs in wild type-infected cells between 7 and 9 min, is replaced by a slow decrease of the rate of synthesis; the switch-on of late proteins is replaced by a slow increase of their rates of synthesis. This general picture describes the real situation only poorly: Indeed the individual early or late proteins behave very differently and, in addition, have individually different responses to the multiplicity of infection effect. High multiplicities of infection with G lead, on the average, to a faster

switch-off of early and switch-on of late proteins. But again, some of the proteins are strongly dependent, others not, and a few respond negatively. The proportions of late proteins present are thus very different from those found in wild type-infected cells. Not being able as yet to extract a senseful hypothesis, we have to refer the reader to Table V and Figs. 12 and 13 for data on the responses of the individual proteins.

When studying quantitatively the particle formation by electron microscopy of sections, we find here also a strong multiplicity of infection dependence. With the triple mutant G and the quadruple mutants G·21$^-$, G·24$^-$, and G·17$^-$ we find 1–5 particles per cell at 90 min after infection with low multiplicities of infection; we observe 40–60 particles per cell when we use a multiplicity of infection of 100.

When the amount of gp23 and gp23* is determined by continuous label, and expressed in phage equivalents, we find that the number of gp23* equivalents eventually reaches 38 per bacterium (at the most). This number corresponds within the experimental accuracy to the 45 empty particles per cell counted on thin sections which were made in the same experiment. The number of uncleaved gp23 equivalents is very small and corresponds to about 4–6 per cell. We thus conclude that the empty particles found in the G-infected cells are in the majority composed of gp23*. The low amount of uncleaved gp23 can be in the form of membrane-associated or free cytoplasmic subunits, or τ particles; it is too low for any significant correlation with structures observed on thin sections.

The above finding of the small amount of gp23 makes it unlikely that all of the observed "lump-like bodies" are composed of gp23. We find "lumps" throughout the whole replicative cycle of the G mutant, even at times where the amount of gp23 is chemically undetectable. In 23(*am*B17) where the 20% *am* fragment is known to remain attached to the membrane (25), we also find "lump-like bodies" in numbers and sizes which appear to be similar to wild type. This observation is however of limited value as long as we do not know if the number of translations is the same for the unaltered gene product or *am* fragments. Considering the different morphological aspects of the "lump-like bodies" it might very well be that we are dealing with several species of lumps. Membrane associated gp23 and lumps were discovered in 31$^-$-infected cells (15); it was later confirmed that in these cells the gp23 stays strongly associated with the envelope fraction of disrupted cells (25). A direct proof that these lumps are made of gp23 is still lacking. Attemps for providing it failed, because under the present conditions we could not reproduce the high reversibility which is claimed (25, 38) for the lumps produced by 31(*ts*A70). Morphologically comparable "lumps" are produced in uninfected E. coli after treatment with canavanine, and analogue of asparagine (34). Electrophoresis of the envelope fraction containing such lumps revealed no accumulation of a specific protein (1). Amino acid substitutions in a protein are expected to lead to conformational changes and thus possibly to a loss of solubility. Lumps might thus be simply accumulations of proteins which lack the ability either to stay in solution in the cell sap or on the lipid interphase of the cytoplasmic membrane. Without further careful studies nothing can presently be concluded about the nature of the observed lump-like bodies in G-infected cells.

Let us now consider the possible roles of DNA in T4 phage maturation. If everything would happen according to the hypothesis that in the absence of vegetative phage DNA the head maturation proceeds up to the exact step at which DNA-packaging occurs, then either the empty small or the empty large particles which occur in the G-mutant would represent this precursor. This is in agreement with the postulated existence of a "truly" empty prohead II in the maturation pathway of Laemmli and Favre (23). We tried to prove the existence of this postulated precursor by using a triple *ts* 43$^-$·30$^-$·46$^-$ and

temperature shifts. We attempted to accumulate empty particles during growth at non-permissive temperature and to fill them with DNA which is produced after shift to permissive temperature. Unfortunately, we found that this triple *ts* does not produce countable numbers of particles. After shift to permissive temperature less than 1 active phage is produced per cell, both in the presence and the absence of chloramphenicol. This number is much too low for achieving significant results. For 17^-, where we find the same types of empty particles as in G, we have contributed additional experimental evidence (43) in favor of an abortiveness of these particles. We cannot prove, however, that all "truly" empty particles (those which are found to be empty on thin sections) are abortive. It could easily be that the abortiveness of the 17^- particles is specifically related to the mutated gp17. A comparable situation is indeed found for τ particles: both 21^- and 24^- lead to morphologically identical τ particles. Those produced by 21^- are abortive (24) while those of 24^- are maturable (3, 4).

In a previous paper (43) we presented results which do not preclude that the T4 prehead becomes packaged with DNA before gp23 is cleaved. We also discussed the lack of experimental evidence for demonstrating that prohead II, defined as an empty gp23* capsid in lysates (23), was already empty intracellularly. Taken together with other data discussed in (43), these results lead us to postulate tentatively that the "truly" empty heads composed of gp23* are likely to be abortive products of a side track of the maturation pathway (43). The following temperature shift experiments give results which are consistent with such an idea: In the experiments with 24*ts* the τ particles, accumulated at nonpermissive temperature, are quantitatively transformed into phage heads after shift to permissive temperature; in similar experiments with 24*ts*·17*am* and 24*ts*·G, the transformation of τ particles into empty small and empty large particles is very incomplete (see note added in proof). Furthermore, we have shown that the capsids of the empty small particles are unstable in lysates, in contrast to normal mature capsids, and the empty large particle.

It is interesting to compare the prehead maturation of T4 with that of λ. If DNA synthesis is very strongly inhibited, petit λ accumulate (13). A subspecies of these petit λ – the preheads – are competent for in vitro packaging with DNA, while other subspecies are not (12). In wild type-infected cells the proportion of incompetent preheads among the petit λ increases with time after induction (27). Hence, an abortive sidetrack seems also to occur in λ.

Our experiments do not allow us to distinguish an effect of parental DNA on late protein synthesis from a possible direct involvement of this DNA in particle formation. It is suggestive that the parental DNA, which is present in large amounts in cells infected at high m.o.i., could have some role in the assembly of preheads by replacing the replicating phage DNA of wild type phage. The amount of parental DNA entering and remaining in the cell is unknown; it is certainly only a fraction of the DNA of the absorbed phages. This is obvious from the observation on thin sections that, among the parental phages adsorbed to bacteria, many are found which still have a full head, not to speak of the known – but not understood – phenomenon of expulsion of the DNA of late injections (11). It should be noted also that 43(*am*B22) which we have used has some 10% of the phage DNA replicated, as indicated by a density shift in density labeling experiments (G. Garcia and G. Mosig, personal communication).

In conclusion we propose the following hypothesis for further experimental tests (43): If late proteins are available, but no or only incompetent DNA, the assembly pathway deviates into an abortive side track which terminates itself with empty small (esp) and empty large (elp) particles, both without a visible core. The deviation is likely to occur at the level of τ particles.

We readily admit that the study of the triple mutant ($43^{-} \cdot 30^{-} \cdot 46^{-}$) has not provided us with very useful results on the role of DNA in either the assembly of the prehead and/or the head maturation. More questions are raised than answered. But these studies provide us with numerous data which, together with those of previous papers (43, 44), have clarified some generally accepted oversimplifications or even misconcepts and thus provide starting points for new experiments.

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NOTE ADDED IN PROOF (November 22, 1977)

Several new facts have been discovered since the experimental work of this paper was terminated April 1976. 1) Hsiao and Black (Proc Natl Acad Sci USA 74, 3652, 1977) discovered empty particles on sections of bacteria infected with a cold sensitive mutant in gene 20, when grown at non permissive temperature. These particles mature into phage after shift to permissive temperature. These authors do not distinguish between empty small and empty large particles. 2) In studying the particles accumulating in 9-amino acridine treated, T4-infected cells, Schärli and Kellenberger (Experientia, in press, and paper V of this series, in preparation) found, besides full (black particles) and partially filled (grizzled) particles, a new species, the ϵ particle, which is maturable. The ϵ particle is not distinctly visible on sections of well preserved cells, but becomes easily recognizable as a more or less empty particle in cells which have undergone fixation-induced leakage. When isolated, this particle is morphologically indistinct from the empty small particle produced in 17^{-}. Empty small and ϵ particles are composed of gp23*. 3) When 17^{-}-infected cells are fixed under conditions of best preservation, then the number of visible empty particles decreases (F. Traub and E. Kellenberger, unpublished). 4) Carrascosa et al. isolated empty small particles and demonstrated that they are composed of gp23*. In buffer of low ionic strength they become expanded into empty large particles. Only the expanded particles are able to bind the minor head proteins soc and hoc (Carrascosa and Kellenberger, Experientia, in press, and paper III of this series, submitted). From the now available data we conclude that the following pathway occurs at least in some conditions of phage growth: The τ particle or prehead with its core (all non-processed proteins) become an ϵ particle by processing of both the shell and core proteins. These particles then become expanded and filled with DNA. In this pathway protein cleavage precedes both expansion and DNA packing. The results obtained also strongly suggest that the empty particles observed in sections and lysates are breakdown products of the ϵ particle, either artifactually induced by fixation, or, as the consequence of a physiological, abortive side tracks or both. The "invisibility" of ϵ particles on sections of well preserved cells would explain why, in the above reported experiments (Results, section 4), designed for demonstrating a maturation of τ particles into empty particles, we have a substantial loss of the amount of particles observed (Table III). An alternative pathway, which possibly occurs under other conditions is based on observations by Onorato and Showe (Experientia, in press, and paper in preparation), which suggest that expansion might occur before protein cleavage. The possibility that in some cases even DNA packaging occurs before gp23-cleavage (43) is not yet ruled out.

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