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Studies Related to the Head-Maturation Pathway of Bacteriophages T4 and T2: I. Morphology and Kinetics of Intracellular Particles Produced by Mutants in the Maturation Genes

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Mutants in the genes governing the maturation of the head of bacteriophage T4 and in gene 24 were studied by electron microscopy of thin sections. We define morphologically: black particles, comprising mature, stable heads and immature, fragile heads, which break down upon lysis; grizzled particles, which apparently are partially filled or partially emptied; empty large particles without DNA or core which are all the same size as normal heads; empty small particles without DNA and without core which are of the size of the τ particle, which is the prehead of phage T4.

The study of single and double mutants of the maturation genes demonstrates that the phenotypes are only different by the proportions of the different particles made except for 17^- where only empty small and empty large particles accumulate. The mutants in gene 24 are epistatic on all other mutants. Mutants in gene 17 are epistatic on the remaining ones.

The results are consistent with the hypothesis that the products of several of the maturation genes act on DNA to render it competent for packaging while the others act directly on the particle. By this uncoupling, bypasses and abortive pathways can result.

Key words: bacteriophage precursors, bacteriophage maturation, bacteriophage growth kinetics, abortive pathways, fixation sections

Abbreviations: bp – black particles: particles of different species but containing condensed DNA; gr – grizzled particles of specific patchy appearance, particularly distinct after lead staining; esp – empty small particles; elp – empty large particles; ep – empty particles, comprising esp and elp; pfu – plaque forming units; m.o.i. – multiplicity of infection; BSA – bovine serum albumine; TCA – trichloroacetic acid; PPO – 2,5-diphenyloxazole; POPOP – 2-(5-phenyloxazole)benzene; CAP_p – chloramphenicol pulse treatment; SDS – sodium dodecyl sulfate; FoOs – prefixation with formaldehyde 3% and main fixation with OsO_4 1%; Os – both prefixation and main fixation in OsO_4 1%; PL – partial lysis induced on T4 by OsO_4 0.01%; UG – uranyl acetate/glutaraldehyde fixation after centrifugation; Glu-Os/UAc – glutaraldehyde-OsO_4/ uranyl acetate fixation according to Simon (44)

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Complex DNA phages such as T-even, P22, and λ assemble first a prehead into which the phage DNA complement is then packed during maturation into the final head. During maturation one or several proteins of the core are removed or processed (see review by Casjens and King, Ref. 4).

The prehead of phage T4 is the membrane-attached τ particle, for which a conservative in situ transformation of the major head protein gp23, into the cleaved form, gp23*, was demonstrated (3). Laemmli and Favre (28) have investigated the pathway of the prehead into the final head by following a short pulse label in its flow through different particles separated on a sucrose gradient. They give evidence that from the 3 precursors which they identified, the first (prohead I) is likely to be a τ particle (29, 32).

Prohead II is postulated to be an empty capsid made of gp23* (28). The available experimental evidence does not exclude that this extracellularly observed empty intermediate is a breakdown-product of a particle which was intracellularly still full. The finding that in lysates the DNA content of prohead III – another partially filled intermediate – is found to be larger when gentle lysis procedures were employed and/or chemical fixatives were used, supports the latter viewpoint (28, 32).

Prohead III is a partially filled intermediate for which it was demonstrated that its DNA content can indeed be completed in vivo at later times (32).

In the present series of papers we will give experiments designed to characterize the different successive precursor particles (intermediates) by electron microscopy. We know that most of the intermediate particles in T-even phage head morphogenesis are very fragile and barely survive lysis of the cell without a breakdown into substructures and/or components. For T2 wild-type phage about 20% of the particles found in lysates are empty, while in thin sections the relative number of empty particles is less than 1% (18). With a gentle procedure, such as in situ lysis, values in between those of lysates and thin sections were found (17). This clearly demonstrates that at least one class of head-related particles exists which is full in sections and empty in lysates. Other experiments suggest for T2 and T4 the existence of a full particle, which upon lysis does not give recognizable breakdown products (20). This particle was initially interpreted as a "condensate" of DNA without a shell (20); suggestive evidence for a shell was given later (21). Thin sectioning of fixed infected cells apparently represents the least damaging preparation method for such fragile particles. This has been confirmed by Granboulan et al. (15) in a study of mutants in the T4 head maturation genes.

With sectioning techniques, only kinetics based on particle counts (43) can lead to significant conclusions. In such an approach considerations of the growth characteristics of bacteriophage are fundamental. Studies by Koch and Hershey (26) on the precursor pools of head proteins in phage T2-infected cells showed for the steady-state of phage formation about 28 head precursors per bacterium. In a further paper of this series, we will show that for T4 about 35 head precursor particles per cell are present in the steady-state. Since at least 3–4 distinct head intermediates seem to exist we roughly estimate that an infected cell contains an average of about 5–20 precursor particles of a given species. Recalling, that the number of particles counted per bacterial section corresponds only to one twentieth of the number present within a cell (43), it becomes clear that this number is certainly insufficient for defining morphologically a true precursor on thin sections of wild type-infected cells. This consideration is obviously also valid when attempting to follow the time sequence of the first appearance of precursors and later inter-

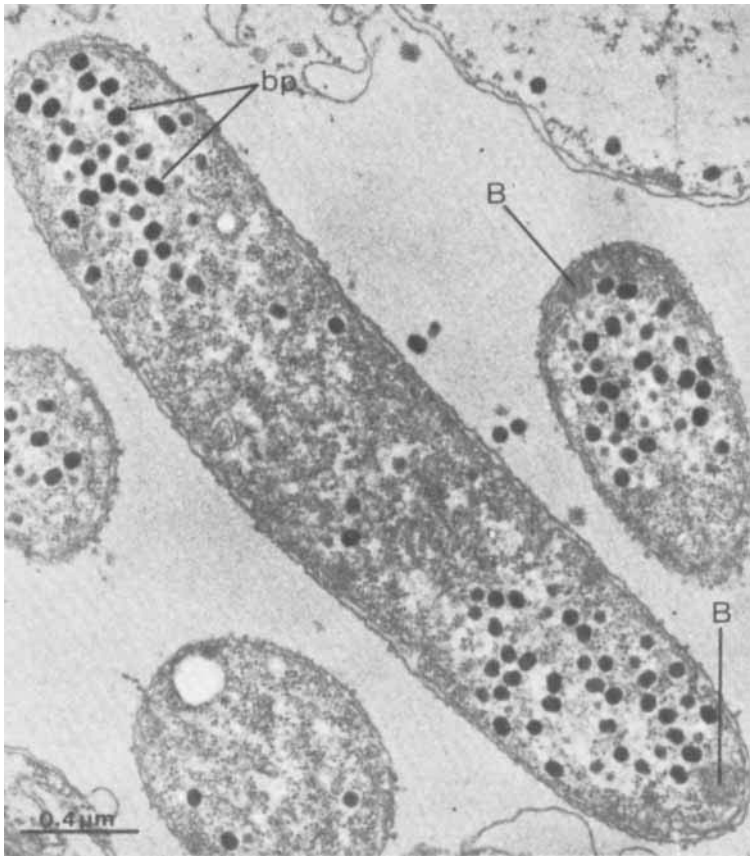


Fig. 1. Thin section of a wild type-infected and superinfected E. coli BE (m.o.i. 5; 37°C) at 60 min after infection showing only black particles (bp). Note the different levels of sectioning through the phage particles. Very often "lump-like bodies" (B) are found. These will be discussed in paper II of this series (47). Fixation: FoOs.

mediates in sectioned cells (44). We also have taken into account, that one species of particles gives rise to morphologically different cross sections depending on the level at which the particle is sliced (34).

Two approaches are available for possibly overcoming this dificulty: 1) By using mutants in maturation genes, the precursor particle preceding the action of the considered gene might accumulate. We encounter an accumulation of precursors – with only very few exceptions – in the assembly pathway of the T4 tail (25, 4). In the assembly and maturation pathway of the T4 head, precursors and intermediates are frequently deviated onto abortive sidetracks instead of accumulating in a functionally unaltered form. Morphological variants are thus produced, as for instance polyheads instead of preheads (16, 30). We will see in these papers that similar situations of abortive side tracks seem to occur with some mutants in the maturation genes (2). If the steady-state of phage growth is slightly or temporarily unbalanced, an enlargement of a given precursor particle pool might occur and the abor-

tive side track might possibly be avoided. In following papers of this series, we will see that this was indeed the only successful procedure for uncovering true intermediates. An unbalance of steady-state growth occurs certainly at very early times of particle formation. As we will show elsewhere our experimental conditions do not result in an unbalance which is favorable for following the first appearance of particles. Their number is so low that significant values would have required counts on thousands of bacterial sections. We thus did not attempt to confirm the results of Simon (44), which we can explain only by assuming that he happened to have a particular situation of unbalance at early times, such that the number of particles observed at 12 min after infection was already on the order of some 100–200 per cell [Fig. 1 of Simon (44)]. In our experiments at 37°C, the amount of cleaved and uncleaved gp23 allows for a maximum of 100 particles per cell at 20 min after infection. Extrapolation back to 8 min, when gp23 starts to be synthesized, would lead to 30 particles per cell at 12 min, that is, to a maximum of 1.5 immature particles of any species per sectioned cell, when all of the gp23 would be in an organized form.

In this paper we first compare different fixation methods in order to select the one method which is most reliable for particle counts. We then describe the different morphologically defined classes of head-related particles which we observe in single and multiple amber mutants in what we call the head maturation genes (X genes of Edgar and Lielausis, Ref. 9; see Table I) and gene 24.

At present not much is known about the T4 head maturation genes. No precise function can be assigned to either of the maturation gene products. All mutants were found to produce head-related structures which are ineffective or only of low efficiency in the in vitro complementation test (8, 9, 15). Electron microscopy studies (15, 37) revealed a whole spectrum of fragile phage-related particles ranging from full to empty both with and without tails attached. In these studies the relative amounts of full and empty particles were shown to depend very much on whether sections, in situ lysis, or lysates were used. This indicates again that during maturation of the prehead into phage, assemblies can occur which are much more fragile than complete mature phages. Besides characterizing morphologically the head-related particles which appear with these mutants, we studied the particle formation kinetically and comparatively to wild-type phage.

By using double mutants in different combinations of head maturation genes and gene 24, we then studied the epistatic behavior. A mutant allele in gene A is epistatic on another mutant allele in another gene B, if the phenotype of the double mutant, $A \cdot B$ is the same as A. As we shall see here and elsewhere (46) studies of this kind are helpful in elucidating the mode of action of gene products. By this approach the form-giving action of the products of the assembly genes was defined (12, 30).

Based on the relative amounts of particles observed by electron microscopy and on the relative amounts of cleavage of gp23, we classify the phenotypes into some main classes. From these we select a few genes or gene combinations for further studies (Refs. 46, 47, and following papers of this series), where we correlate kinetics established by particle counts on thin sections with those based on biochemically determined particle-equivalents of gp23 and gp23*. Combined with some other experimental approaches these counts allow us to demonstrate whether or not some of the particles observed are made of gp23*; evidence for the precursor nature or abortiveness will also be provided in some cases.

Finally, we will investigate in paper II of this series (47) particle formation and late protein synthesis in the absence of phage DNA replication, using methods previously described (46).

TABLE I. List of T4D Mutants Used

Gene	Mutant	Remarks
24	*am*N65	a
17	*am*N56	a
17	*am*N56 × 1	backcrossed
16	*am*N66	a
16	*am*N66·*am*N87	
64	*am*C86	a
50	*am*A458	a
2	*am*N51	a
4	*am*N112	a
49	*am*E727	a
49	*am*E727 × 1	backcrossed
17	*ts*CB80	
17	*ts*L51	
17	*ts*L2	
17	*ts*N20	
17	*ts*N21	

[a]In our experiments these mutants are frequently abbreviated to X^-. All the double mutants indicated as $X^- \cdot Y^-$ are derived from these same mutants. Mutant 65(*am*E348) included by Edgar and Lielausis (9) in the x mutants was not used in our studies because – in spite of a normal reversion rate – it shows such a high leakiness that complementation tests could not be used for selecting double mutants with other genes. According to Granboulan et al. (15) its phenotype is comparable to 64^-. The mutants stem from the collection of Epstein et al. (11) and Edgar and Lielausis (9). The double mutant in gene 16 was constructed in our former laboratory in Geneva by E. Terzaghi.

MATERIALS AND METHODS

1. Bacteria

E. coli BE was used as nonpermissive and E. coli CR63 as permissive strain for amber mutants (11).

2. Phage

$T4D^+$ was used as wild-type strain. All mutants used are derived from this strain; they are listed in Table I. All possible double mutant combinations between the head maturation genes and the head maturation genes with 24^- were constructed in our laboratory.

3. Phage Techniques

General techniques were as described by Adams (1) and Eisenstark (10). Special techniques for the conditional lethal mutants were as described by Epstein et al. (11). Bacteria were grown on M9 medium (1) supplemented with 1% (M9A) or 0.1% (M9a) casamino acids. Generation times and phage yield are identical on both of these media (see Ref. 46). In some cases (see Materials and Methods 4.1.2.) tryptone broth (1% Bacto tryptone, 0.5% NaCl) was used. For experiments, E. coli BE was grown in an air-bubbled culture at 37°C up to 2.2×10^8 cells per ml. Growth was followed in a Coulter Counter Z_B using a 50-μm orifice (Coulter Electronics, Dunstable, England). Details of the procedure have been previously described (46). Infection was performed at a multiplicity of 5,

followed by superinfection with the same multiplicity at 8 min after infection, to ensure lysis inhibition. We determined the number of surviving bacteria at 4 min after infection and found it to be less than 2%, corresponding well to the expected value of the Poisson distribution ($e^{-m.o.i.}$).

4. Electron Microscopy Techniques

4.1. Fixation methods. 4.1.1. Formaldehyde – OsO_4. [Abbreviation: FoOs.]

Prefixation was performed by adding formaldehyde (35%) to a final concentration of 3% to the culture. After careful mixing, the sample was centrifuged rapidly with a swinging-bucket type table centrifuge (Christ) at 3,800 rpm for 3 min. The pellet was resuspended in 1 ml OsO_4 (1%) in RK-buffer (Michaelis buffer, pH 6.1, containing 0.12 M NaCl and 0.01 M $CaCl_2$) with tryptone broth added to a final concentration of 0.1%. This is referred to as RK conditions (19). After 90–120 min (the sample being brownish), the bacteria were sedimented again as above. The well-drained pellet was mixed with 1 drop of melted agar [2% wt/vol Bacto agar in RK-buffer containing 1% wt/vol of Bacto tryptone as source of amino acids (A. Ryter, personal communication)] using a microsyringe (22). As little as 1–2 ml of 2×10^8 bacteria per ml are sufficient for this procedure. From the solidified agar small pieces were cut which were then fixed overnight in 1% OsO_4 in RK-buffer (without adding tryptone, the tryptone being present in the agar). The whole fixation procedure was carried out at room temperature. The bacteria are visible as brown regions within the agar. Postfixation was done for 2 h in 0.5% uranyl acetate in RK-buffer.

4.1.2 OsO_4. [Modification of the method used by Okinaka (37).] [Abbreviation: Os.] Infected bacteria were mixed 1:1 with 2% OsO_4 in RK-buffer containing a twofold concentration of $CaCl_2$. If the bacteria are grown in M9, a heavy precipitation occurs immediately. To avoid this we used tryptone broth in all experiments in which this fixation was included. No additional tryptone was added to the fixation mixture. As soon as the samples turned brown, they were put in agar and processed as described in 4.1.1.

4.1.3. Glutaraldehyde-OsO_4/uranyl acetate (44). [Abbreviation: Glu-Os/UAc.] We exactly followed the procedure which Simon (44) refers to as Method I, which consists of prefixation with 0.5% glutaraldehyde in the cold and main fixation with a mixture of 1% OsO_4 and 0.5% uranyl acetate at room temperature.

4.1.4. Uranyl acetate/glutaraldehyde (42). [Abbreviation: UG.] We exactly followed the procedure described, using a fixation mixture of 0.1% uranyl acetate and 2.5% glutaraldehyde in RK-buffer.

4.2. Embedding. Dehydration with acetone was performed following standard practice (30, 50, 70, 90 and twice 100%, 30 min each) to get minimal and reproducible shrinkage. Impregnation with acetone-Epon 812 was done stepwise (3:1, 1:1, 1:3; 30 min each) followed by Epon 812 (1 h twice). Polymerization was carried out in two steps: 12 h at 50°C and subsequently 2–3 days at 70°C.

4.3. Thin sections. Thin sections were made with a constant nominal thickness of 250 Å with a LKB microtome (Ultratome III) using a diamond knife. The sections were put on collodium-carbon coated grids and stained with 5% uranyl acetate in water, followed or not by staining with Pb-citrate (38) (see 4.5.).

4.4. Electron microscopy. We used a Philips EM 300 and a Zeiss EM 10, both at an acceleration voltage of 80 kV. Photomicrographs were made on Kodalith films (Kodak).

4.5. Counting and measuring of particles. Counting was done at a magnification of 21,600× directly in the microscope. In those preparations which had no homogeneous response only intact bacteria showing no partial lysis were counted. Samples fixed with

FoOs which had more than 10% of lysed cells were repeated. The number of phage-related particles per bacterium are calculated from the number of particles per sectioned bacterium by multiplication with a factor F (43, 34). The factor F depends on the thickness of the section and the size and nature of the particle. We redetermined F and found it to be 20 for our conditions (46, 34). Counting was always done by 2 different persons on at least 2 different thin sections. By numerous experiments we confirmed the statement that the visual selection of sections of a given thickness is very reproducible. The relative deviation was less than ± 5%. For every determination we counted 100 ± 10 bacteria of the same thin section. This counting procedure is not designed to determine quantitatively the number of τ particles. These can only be counted reliably on photomicrographs.

Measurements of particle sizes were done on negatives taken with a Zeiss EM 10 at a magnification of 21,600X which were enlarged 10X with a distortion-free projector constructed in our laboratory. Only equatorially sectioned particles clearly showing a distinct "rim" were measured. For size determination we only used poststaining with uranyl acetate, whereas counting was done on sections both poststained with uranyl acetate alone and combined with Pb-citrate; they yielded the same numbers.

5. Determination of the Ratio of gp23* to (gp23 + gp23*)

Cultures in M9a were labeled continuously with ^{14}C-labeled amino acids (final specific activity 2.7 μCi per mg amino acids) as described elsewhere in detail (46), where also the preparation and electrophoresis of the samples is given as well as the procedures used for determining the ratio gp23* to (gp23 + gp23*), called "gp23* ratio."

6. Nomenclature and Abbreviations Used

The amber mutant N56 and the temperature sensitive mutant L80 in gene 17 of T4D, respectively T4D·17(*am*N56), and T4D·17(*ts*L80), are abbreviated to 17(*am*N56) and 17(*ts*L80). For convenience a conditional lethal mutation is frequently abbreviated to 17$^-$. Double mutants are designated with a point in between, e.g., 16(*am*N66·*am*N87) is a double mutant within gene 16, and the double mutant 16(*am*N66)·17(*am*N56) has a mutation each in gene 16 and gene 17. For convenience of expression this is mostly abbreviated to 16$^-$·17$^-$ (see also Table I). We adopted King's nomenclature gp17 for the gene product of gene 17 (4). A cleaved gene product is normally designated by a star, e.g., gp23*. If an uncleaved form is discovered after the cleaved form, then the uncleaved, (native) protein or gp is designated by adding n, e.g., protein B_1 and protein B_1n.

Mutants in maturation genes are sometimes abbreviated to "maturation mutants."

When talking about "phenotypes" of mutants, we understand in this paper the particle population observation on thin sections of bacteria infected with these mutants.

RESULTS

1. Comparison of Different Fixation Methods

The choice of a peculiar fixation procedure depends mainly on the subject of interest. As we were particularly concerned with the fixation of fragile structures, we compared some current fixation procedures for qualitative and quantitative reproducibility We tested these fixation procedures both on wild type and on some mutants in maturation genes which were suspected to produce fragile head-related structures. As the essential criteria we chose: equally good preservation of each bacterium of a population as judged

from the general aspect of the bacteria, the detectability of a maximum number of particles, and last, but not least, reproducibility of the counts. Furthermore, a rather practical aspect was considered, namely, the formation of precipitations which renders work with small quantities of culture impossible. The results of these comparative studies are summarized in Tables II and III. We found that formaldehyde-OsO_4 (FoOs) and OsO_4 (Os) fixation are most adequate for our purpose, the preservation and topology of T4 particles being the same with both methods (Fig. 1). The Os method has the major disadvantage that it cannot be used for cultures in media based on M9, which we employed for radioisotope labeling experiments, because the chelating of Ca^{2+} by phosphates prevents adequate DNA fixation (19). Therefore we decided to use the FoOs method as basic procedure in our investigation.

From a theoretical point of view the uranyl acetate/glutaraldehyde (UG) fixation would be much more adequate for the fixation of labile DNA-containing structures than a prefixation with formaldehyde because the UG fixes DNA rapidly, whereas osmium under RK-conditions only fixes late, and aldehydes do not fix at all (41, 42). As the culture medium contains phosphate, the UG fixation cannot be used directly in the culture and a centrifugation has to be made prior to fixation. Centrifugation in turn is known to cause uncontrollable leakage of the cells; we confirmed this in our experiments. Therefore we preferred to prefix with formaldehyde, which probably also induces some leakage but which is reproducible when done under standard conditions. Needless to say, results obtained from thin sections of fixed material always have to be interpreted with the necessary precautions (34).

The classes of phage-related particles (see Results, section 2) were the same in all fixation methods tested; only quantitative differences were found (Table III). In all mutants studied, except 16^- and 17^-, the populations of infected bacteria were rather homogeneous with respect to the aspect of the cell and the relative amounts of the different phage-related particles. In 16^- at early times and in 17^-, however, where only empty particles were present, the population was very inhomogeneous and the individual cells could be situated in between two extremes (see note added in proof). In one the particles appeared as holes whereas in the other clearly distinguishable large and small empty particles were present (see Results, section 2c, d). The latter type of response was also found when cells were partially lysed. We will come back to this phenomenon later.

2. Morphological Characterization of the Different Classes of Head-Related Particles Found in the Maturation Gene Mutants

The formaldehyde-OsO_4 fixation method was applied on wild type and the mutants 64^-, 2^-, 50^-, 4^-, 17^-, 16^-, 49^-, and 24^-. All phage-related particles were characterized by their morphology and size. Measurements were done as described (Materials and Methods 4.5.), chosing only equatorial cross sections with clearly visible rims. The distances measured are indicated in Fig. 2. The values obtained for the different classes of particles are summarized in Table IV. We can distinguish the following classes of head-related particles:

a) Black particles (Fig. 2a). They appear as homogeneously dense particles of polyhedral shape of which the density is dependant on the sectioning level. As expected from the random orientation of the particles within the section, only rarely is a thin, membranous structure seen surrounding the particle.

b) Grizzled particles (Fig. 2b). They appear as if less tightly packed with DNA than the black particles, and show an inhomogeneous "patchy" density. This aspect is particu-

TABLE II. Comparison of Different Fixation Methods*

Fixation method	Culture medium	Population of bacteria	General aspect of bacteria	Reproducibility of particle counts[a] bp	ep	Precipitations inside bacteria	outside bacteria	Remarks
FoOs	M9a or tryptone	homogeneous	shape well preserved normal concentration of ribosomes DNA plasm fibrillar "formol nuclei"[d]	good	good	no	no	Figs. 1,2,4,5, 6,7,9
Os	M9a	homogeneous			b	(yes)	yes	
	tryptone	homogeneous	shape well preserved normal concentration of ribosomes DNA plasm fibrillar, clearly separated from cytoplasm	good	good	no	no	
Glu-Os/UAc	M9a or tryptone	inhomogeneous	in some cases heavily deformed, cytoplasmic membrane often damaged DNA plasm fibrillar, sometimes rather coarse		b	yes	yes	
Glu-Os/UAc modified[c]	M9a or tryptone	inhomogeneous		good	bad	(yes)	no	
Os without prefixation	M9a or tryptone	homogeneous	as Os, at later times much lysis	good (unless lysis occurs)	good	no	no	
UG	M9a	inhomogeneous	bacteria heavily deformed, cytoplasm rather coarse DNA plasm fibrillar	good (unless lysis occurs)	bad	no	yes	see (47) Figs. 3, 5a, 5b.

*The bacteria were grown, infected and superinfected as described in Materials and Methods. The following phages were used: T4 wild type, 16^-, 17^-, and 49^-. Aliquots (2 ml) of the cultures were fixed with the fixation methods indicated in the table at different times after infection (30, 40, and 150 min). bp) Black particles; ep) empty particles.

[a]Depends on the visibility of the particles; the numbers are summarized in Table III.

[b]Numbers of bacteria per thin section are too small for significant counts.

[c]After prefixation in the cold the bacteria were sedimented and washed twice by resuspending in cold RK-buffer containing 0.3 M sucrose before they were resuspended in 1% OsO_4 + 0.5% uranyl acetate.

[d]Formaldehyde prefixation does not preserve the overall shape of the nuclei of uninfected cells; this is discussed in more detail in paper II of this series (47).

Fixation methods: (for details see Materials and Methods 4.1.) FoOs – formaldehyde-OsO_4; Os – OsO_4; Glu-Os/UAc – glutaraldehyde-OsO_4/uranyl – acetate; UG – uranyl acetate/glutaraldehyde

TABLE III. Influence of Fixation on the Visibility of Phage-Related Particles*

Phage	Fixation method	Bacteria grown in M9a bp+gr/bact. I	bp+gr/bact. II	ep/bact. I	ep/bact. II	Bacteria grown in tryptone bp+gr/bact. I	bp+gr/bact. II	ep/bact. I	ep/bact. II
T4 wild	FoOs	172	182	0	0	–	–	–	–
type	Os	–	–	–	–	140	147	0	0
	Glu-Os/UAc	178	175	0	0	–	–	–	–
16⁻	FoOs	0.6	0.6	75	74	0	0	55	60
	Os	–	–	–	–	0	0	50	50
	Glu-Os/UAc	0	0	34	20	0	0	27	29
17⁻	FoOs	0	0	68	63	0	0	32	35
	Os	0	0	41	48	0	0	26	32
	Glu-Os/UAc	0	0	19	46	0	0	17	31
49⁻	FoOs	99	–	22	–	–	–	–	–
	Os	–	–	–	–	–	–	–	–
	Glu-Os/UAc	67	–	21	–	–	–	–	–

*The bacteria were grown, infected and superinfected as described. At 30 min after infection aliquots were taken for the 3 fixation methods listed. Counting of phage-related particles was done independently by 2 different persons (I and II) as described in Materials and Methods 4.5.
bp) Black particles; gr) grizzled particles; ep) empty particles. For definition of the classes of phage-related particles see Results, section 2. If an experiment was not done, it is indicated by "–"

larly detectable when the sections have been stained with lead (44). In this case, serial sections of a grizzled particle always remain distinct from sections of full particles (34). This aspect is therefore not due to the level at which a particle has been sectioned. Again, as for the black particles, only rarely is the orientation of the particle relative to the sectioning plane adequate to render a surrounding shell visible.

Grizzled particles have in general a less pronounced polyhedral shape than black particles. The dimensions are the same as for black particles. From their appearance we can obviously not decide whether the grizzled particles originate from initially full heads by an artificial loss of DNA induced by fixation, or if they are in the process of being packed with DNA. Mutants 49⁻ are characterized by large amounts of grizzled particles showing variable packing densities, ranging from almost full to almost empty. This strongly suggests variable contents of DNA.

c) Empty large particles (Fig. 2c). Their shape is polyhedral; they are formed by a thin membrane (60–70Å) with "dots" of electron-scattering material inside in each corner. They have the same dimensions as the black and grizzled particles. On serial sections which are not poststained with lead, elps appear rarely on more than one section. The aspects of serial sections of elps are all different from those of serial sections of esps (34).

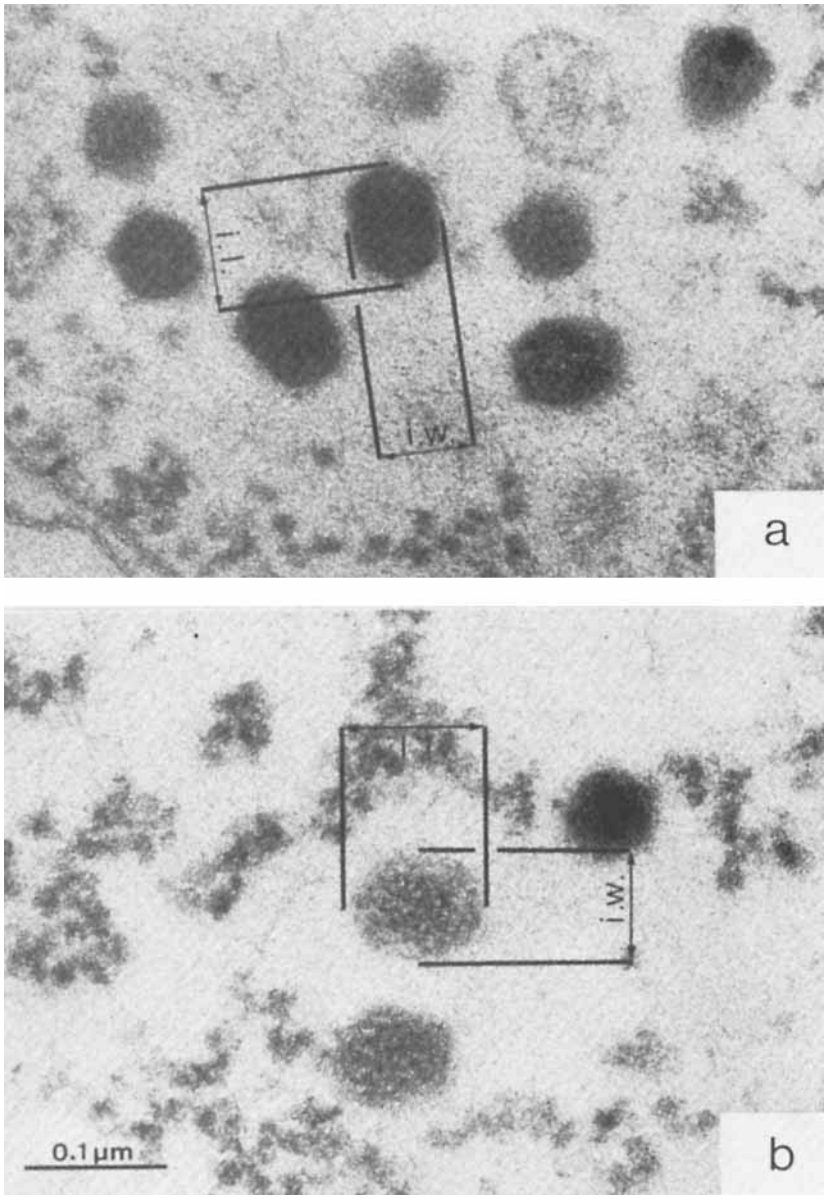
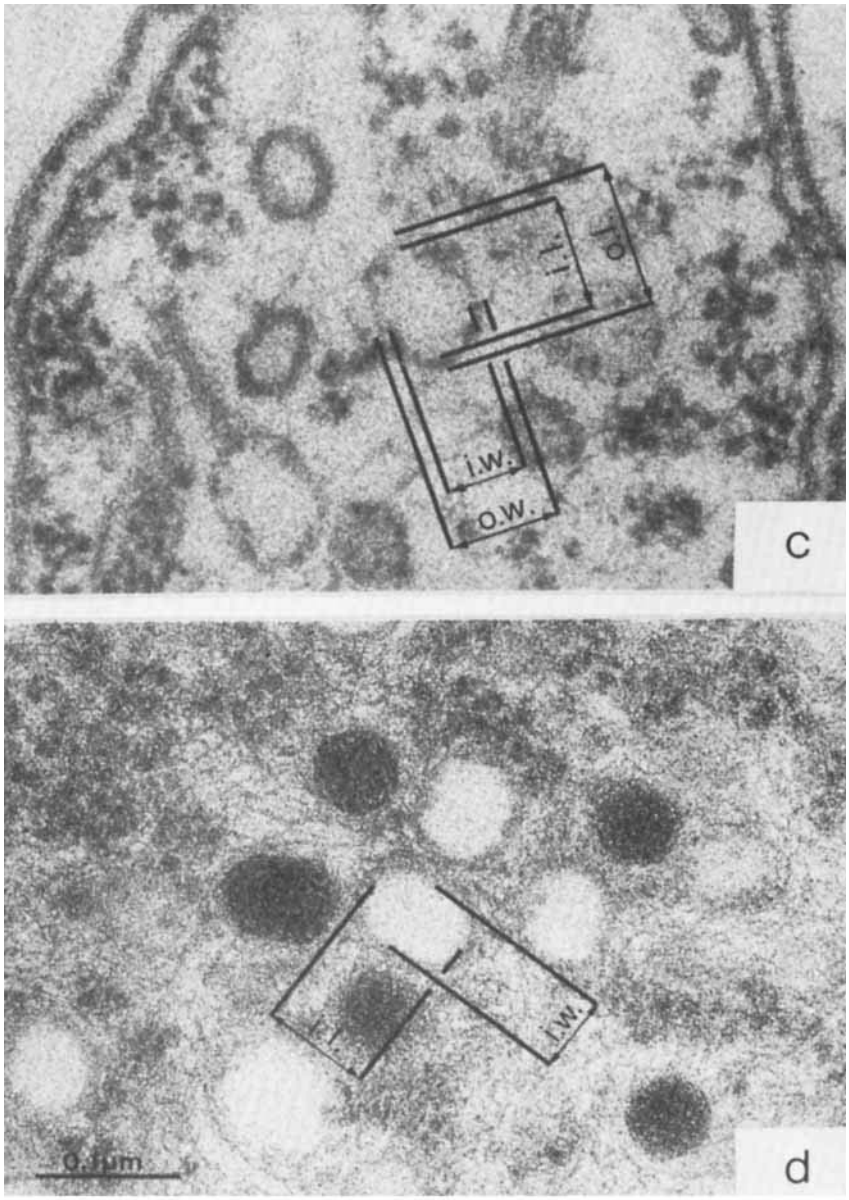


Fig. 2. Thin sections of phage-related particles found in the maturation mutants. Fixation: FoOs; poststaining with 5% uranyl acetate. The measured distances are indicated: i.w.) Inner width; o.w.) outer width; i.l.) inner length; 0.l.) outer length; c.w.) core width; c.l.) core length. The values are given in Table IV. a) Black particle (bp); b) grizzled particle (gr); c) empty large particle (elp); d) empty small particle (esp) in a well-preserved cell; e) empty small particle (esp) in a partially lysed cell; f) τ particle.



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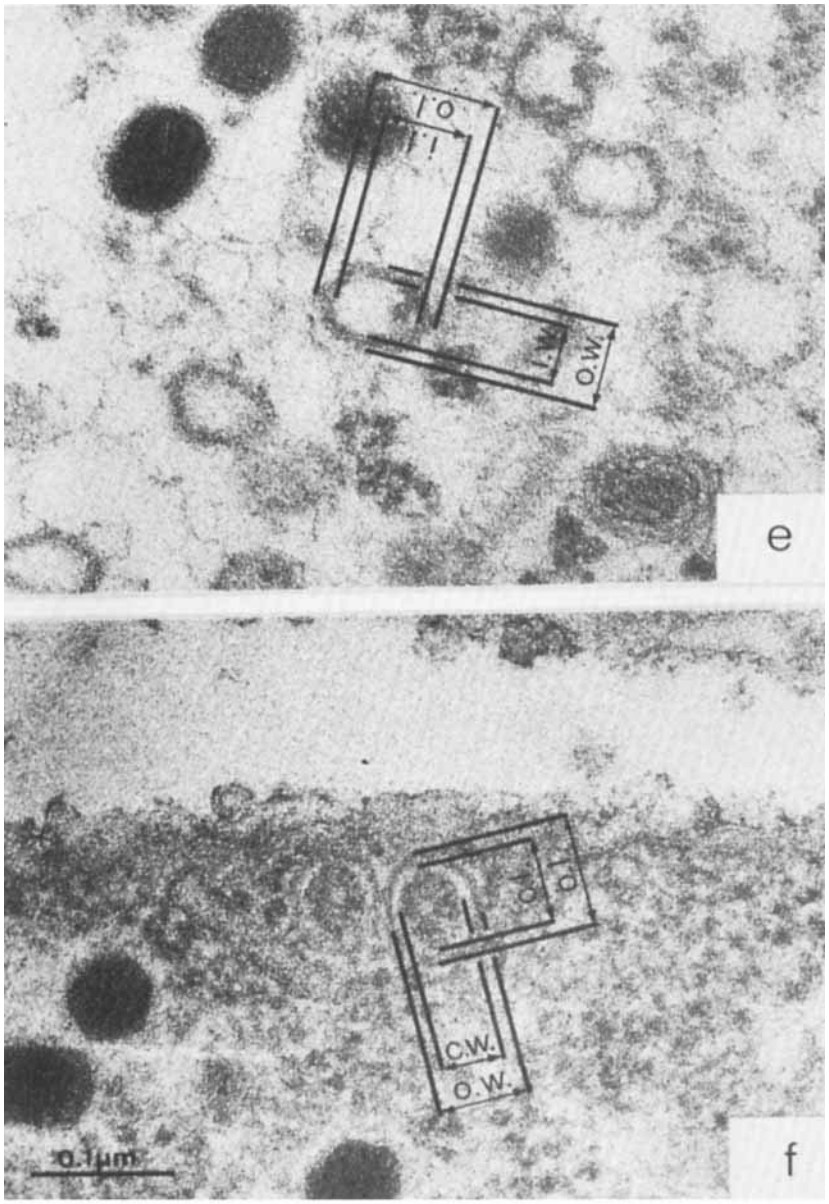

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TABLE IV., Dimensions of Phage-Related Particles*

Particle	No. of particles measured	State of bacterium: partial lysis	Length outside[a]	Width outside[a]	Length inside[a]	Width inside[a]
bp	44	no	1051 ± 32	823 ± 48	921 ± 31	666 ± 23
	20	yes			921 ± 26	665 ± 28
gr	41	no	c	c	940 ± 37	694 ± 28
	30	yes			968 ± 58	722 ± 46
elp	0	no				
	9	yes	1132[d]	894[d]	907 ± 57	676 ± 26
esp	64	no			653 ± 23	472 ± 38
	99	yes	797 ± 42	608 ± 41	603 ± 41	428 ± 18
τ	33	no	856 ± 46	699 ± 42	639 ± 46	472 ± 39
	25	yes	884 ± 46	731 ± 44	630 ± 38[b]	481 ± 37[b]

*The particles were measured as described in Materials and Methods 4.5., including more than 40 experiments which had been done over a period of 2 years. The variation was determined by calculation of the standard deviation.

[a]The dimensions measured for every particle are inserted in Fig. 2.

[b]Dimensions of the core.

[c]The inside measurements are more reliable because the membranes are only rarely seen distinct from the surrounding cytoplasm.

[d]Only 2 values.

bp) Black particles; gr) grizzled particles; elp) empty large particles; esp) empty small particles.

d) Empty small particles (Fig. 2d and 2e). They are seen clearly in partially lysed cells and show a rather thick membrane (90–100 Å) without any visible content. They have the same outer shape and size as τ particles and likewise have much less pronounced vertices than black particles. Only exceptionally do they approach a polyhedral shape; very often deformed particles are found. The same species of particles are clearly visible on photomicrographs of sections made by Okinaka (37); in fact his observations made us aware of these particles which are clearly different from the elps described above. None of the sections of a series of esps have aspects which are similar to elps (34).

In well-preserved bacteria most of the shells cannot be distinguished and the particles appear as "holes." Then only inside diameters can be measured. Inside these "holes" the density is so low that they appear very similar to the polysaccharide granules (glycogen) which are observed in uninfected E. coli grown in certain media (5).

In order to eliminate this possibility J. Robertson and P. Lyttleton kindly applied their procedure of polysaccharide staining (39) to our sections. The "holes" reacted negatively. In some sections they found a few positively reacting glycogen granules, as well as many unstained, phage-related holes, thus demonstrating the specificity of the reaction (results not shown).

e) τ Particles (Fig. 2f). Our observations agree with previously published photomicrographs (21, 2): τ particles are always membrane-attached, prolate, and even when they approach a polyhedral shape, the vertices are rounded and but little pronounced. The core is inhomogeneous in density and its morphology depends strongly on the fixation procedure used (21).

We summarize by distinguishing 2 groups according to particle size; on the one hand, we have the "large" particles including bp, gr, and elp, while on the other hand we find the "small" particles esp and τ.

Except for τ and grizzled particles, we occasionally found particles of all species with tails attached.

3. Comparison of the Kinetics of Head-Related Particles Produced by Mutants in the Maturation Genes and by Wild Type

For mutants in the maturation genes we followed the formation of the different classes of head-related particles which are characterized in the previous section. Using the procedure described in Materials and Methods, we infected E. coli BE grown on M9a with the different mutants. At 20, 30, 40, 50, 60, and 90 min after infection samples were taken for fixation with formaldehyde-OsO_4. The different kinetics are presented in Fig. 3. As we have used standard conditions of media and growth, the numbers can be compared directly, taking into account an accuracy of 10–20% for quantitative electron microscopy (46). We find that all mutants except 17^- have yields of head-related particles of which the total is comparable to that of wild type. However 17^- has only about half the yield of that of wild type. This observation agrees with determinations of total phage-related proteins (46) by which we found that 17^- also synthesizes only about half of the amount of wild type. This result was obtained with a backcrossed mutant, which makes the influence of an additional mutation other than 17^-, unlikely.

The numbers of plaque forming units (pfu) are also plotted in Fig. 3. The kinetics of formation of black particles are the same for wild type and the mutants 64^-, 50^-, 2^-, and 4^-. For wild type the pfu follow the curve of black particles while for the others the number of pfu is less than 1 per bacterium.

The 2 classes of empty particles (elp and esp) have not been distinguished quantitatively. Indeed, elps rarely appear on more than one section of a series, while esps, due to their thick shell are visible on 2–3 sections. This obviously influences the multiplication factor F by more than twofold (34). Hence, we only determined and plotted the total number of empty particles. By the morphologically defined particles present, by their relative amounts, and by their kinetics of appearance, we can distinguish only 4 different classes of phenotypes:

a) Black particle producers (Fig. 4). Cells infected by bp producers are morphologically indistinguishable from wild type-infected cells, the majority of the particles being black. Among all the head-related particles only the grizzled ones reach a constant amount, 30–60 per bacterium. Practically no empty particles are found. The experiments were not designed to get significant estimates of τ particles (see Materials and Methods 4.5.); "bp producers" are: 64^-, 50^-, 2^-, and 4^-.

b) 49^- (Fig. 5). About half of the particles are black and the other half are grizzled particles at 30 as well as at 60 min after infection. The proportion of the 2 types can vary by some 10–20% from experiment to experiment. Less than 20% of empty particles are present. From the general aspect of the protoplasm we tend to believe that the DNA pool is enlarged. This phenotype is represented by the backcrossed T4·49(*am*E727 × 1) as well as by the original strain containing an additional mutation in the *wac* gene (7).

c) 17^- (Fig. 6). Throughout the time after infection we find, besides the τ particles, the number of which is slightly increased at later times, only empty particles (elp and esp). This phenotype is represented by 17(*am*N56) and 3 of the 5 tested *ts* mutants in this gene (Table V).

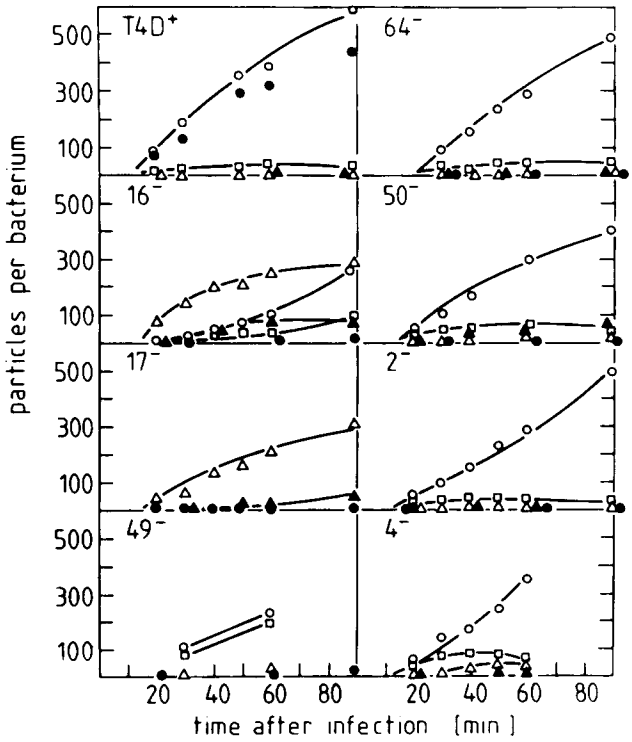

Fig. 3. Kinetics of head-related particles produced in the maturation mutants and in wild type at 37°C. E.coli BE grown on M9a were infected and superinfected with the mutants indicated. Aliquots were taken at different times after infection and fixed with FoOs. Phage-related particles were counted directly on the microscope screen as described (Materials and Methods 4.5.). ○—○) Black particles (bp); □—□) grizzled particles (gr); △—△) empty particles (ep); ▲—▲) τ particles; ●—●) pfu.

d) 16⁻ (Fig. 7). At early times after infection (up to 30 min) this phenotype is indistinguishable from 17⁻. Later we find increasing numbers of black and grizzled particles, reaching together more than 50% of the total number of particles present at 90 min after infection. τ Particles reach a constant number of about 70 per bacterium at later times. This phenotype is represented by 16(*am*N66) as well as by a double mutant 16(*am*N66·*am*N87). This indicates that the late filling is not due to a translational leakiness, because the probability of a read-through in a double mutant is expected to be much lower than in each of the single mutants. Unfortunately, no *ts* mutants are available in gene 16.

Wild type, bp producers, and 49⁻ are closely related in having few empty particles from the beginning. Only the proportion of bp to gr varies between them. Types 17⁻ and 16⁻ can clearly be distinguished from the above mentioned and from one another in that bacteria infected with 17⁻ only produced empty particles, whereas those infected with 16⁻ have black, grizzled, and τ particles at times later than 30 min after infection. Gel electrophoretic studies by Vanderslice and Yegian (45) suggested that gene 16 might be polar on gene 17. We could not confirm these results (Kellenberger and Kellenberger, unpublished results) and found equally good evidence for the alternative explanation that the band thought to be gp17 is rather the uncleaved, native form of protein B_1 (6), which, after

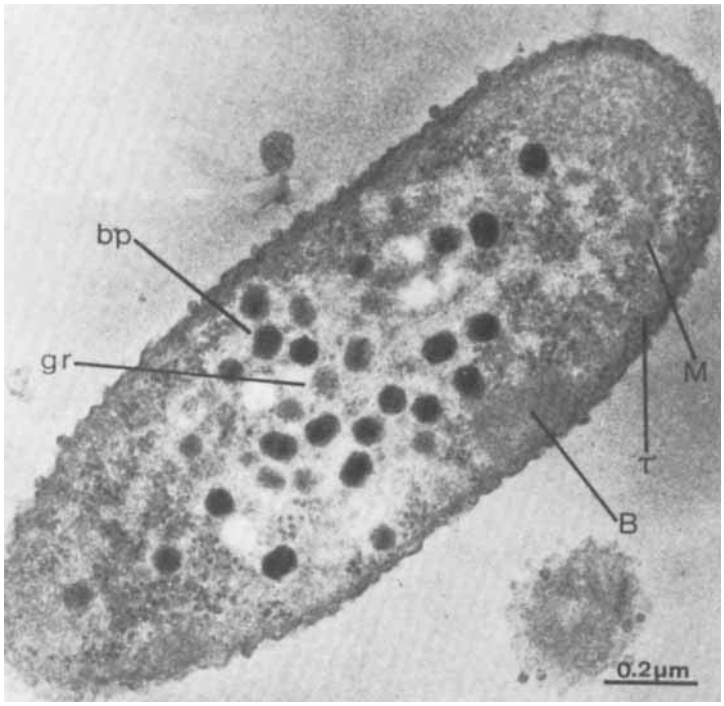

Fig. 4. Thin section of E. coli BE 60 min after infection and superinfection with T4•4(*am*N112) (m.o.i. 5; 37°C). Black particles (bp), grizzled particles (gr) and τ particles are found. Note the "lump-like body" (B) and the membranous structure (M). The appearance is very similar to wild type-infected bacteria. Fixation: FoOs.

cleavage, gives the adenylribosylation enzyme of the polymerase which can be isolated from phage (40) and which would be the product of a gene (alt) which is not yet genetically identified.

We have studied the phenotypes of 5 different *ts* mutants in gene 17, 60 min after infection and growth at nonpermissive temperature (40.5°C); the results are summarized in Table V. Three of these *ts* mutants (*ts*L2, *ts*N20, and *ts*N21) have approximately the same phenotype as 17(*am*N56), whereas the remaining two (*ts*CB80 and *ts*L51) have many grizzled particles. The yields are about the same as for the amber mutant. The 5 *ts* mutants in gene 17 were also tested for "reversibility" of gp17. We measured the number of pfu after a shift to permissive temperature (30°C) in the presence or absence of 25 μg/ml of chloramphenicol, which was added 5 min before shift. In none of the 5 mutants did more than 5 pfu per bacterium mature in the presence of chloramphenicol, independent of the time of shift to permissive temperature (30, 60, and 90 min after infection). Without the addition of chloramphenicol infective phages started to appear linearly at a rate of 1–3 pfu per min about 20 min after shift to permissive temperature. These results indicate that in all 5 mutants the particles synthesized at nonpermissive temperature have entered an abortive pathway. They obviously do not rule out the possibility that empty particles might be competent for DNA-filling. Experiments on this will be reported later.

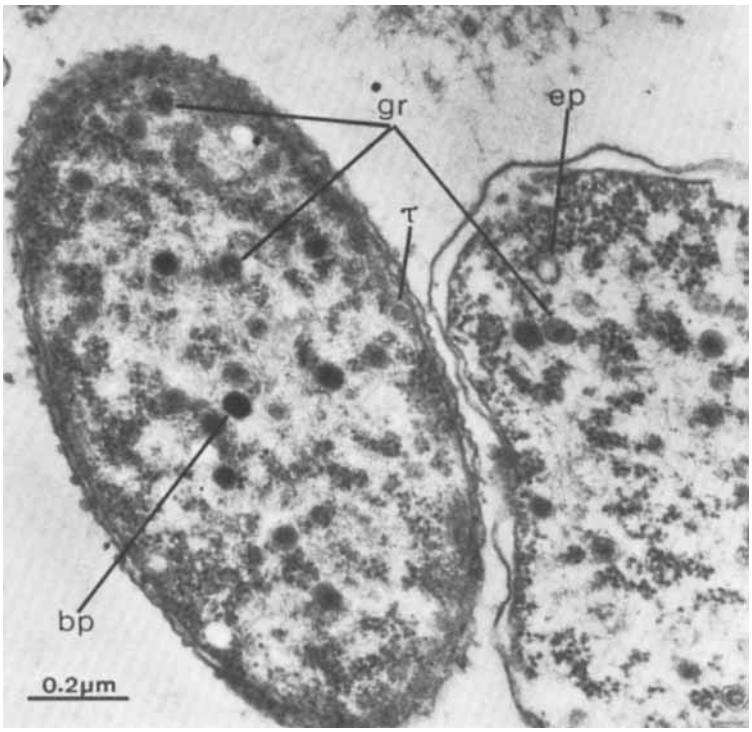

Fig. 5. Thin section of E. coli BE 60 min after infection and superinfection with T4•49(*am*E727)•64(*am*C86) (m.o.i. 5; 37°C). All types of particles are found. Grizzled particles (gr) with different degrees of full- or emptiness are present. The general aspect is the same as for 49^- single mutant-infected cells. Note the enlarged DNA pool, which is characteristic for 49^-. At the right a partially lysed cell is present in which the membranes of the particles are more clearly visible than in well preserved cells. Fixation: FoOs.

4. Epistatic Behavior of Mutants in the Maturation Genes and in Gene 24

In the previous section we established the morphological phenotypes of mutants in the maturation genes. We now establish the epistasy of these mutants by studying the phenotypes of the double mutants thereof and with 24^-. E. coli BE grown on M9a was infected and superinfected as before. Samples for the electron microscopy of thin sections were taken from the cultures at 30 and 60 min after infection; the results are summarized in Table VI. Mutants 17^- are eptistatic on those of all other maturation genes, all double mutants with 17^- having the same phenotypes as 17^- alone. For mutants in the remaining genes no clear-cut statement can be made about the epistasy. Most of the double mutants among 49^-, 50^-, 2^-, 4^-, and 64^- have similar phenotypes, comparable to either "bp producers" or type "49^-." The observed proportions of black to grizzled particles range from 9:1 to 1:9. These differences are far greater than the experimental variation of ± 20%. Many double mutants with 49^- contain grizzled particles which are less dense than those found in 49^- alone, suggesting a lower DNA content.

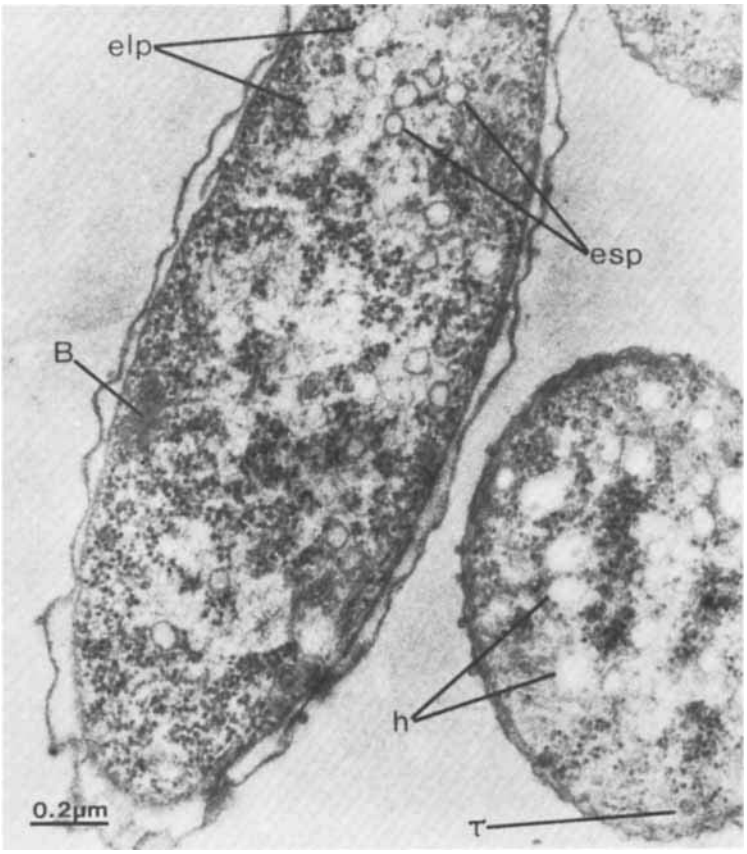

Fig. 6. Thin section of E. coli BE 60 min after infection and superinfection with T4•17(*am*N56)•64(*am*C86) (m.o.i. 5; 37°C). Same aspect as bacteria infected with the 17(*am*N56) single mutant. Note the 2 types of cells which always occur in 17^--infected cells. In the bacterium on the right the empty particles appear as "holes" (h), whereas in the bacterium on left empty small (esp) and empty large (elp) particles can be distinguished. B) "Lump-like body." Fixation: FoOs.

All double mutants with 16^- show the characteristic production of variable amounts of DNA-containing particles (bp and gr) at later times after infection.

The results obtained with the double mutants with 24^- are not included in Table VI; all of them had the same phenotype as 24^- alone, namely τ particles and polyheads. This suggests that gp24 acts before all maturation gene products.

We attempted to correlate these morphological phenotypes with a specific cleavage pattern of gp23. Cleavage was measured in continuously labeled cultures and expressed as the ratio of gp23* to (gp23 + gp23*). The experiments were carried out under standard conditions details of which have been previously described (46). In Fig. 8 the results for mutants in the maturation genes are summarized. We can see that, with the exception of 16^-, in which we have a gp23* ratio of as little as 0.6 at later times, we always have ratios between 0.7 and 0.85 (wild type: 0.85). Hence we cannot attribute a certain cleavage pat-

TABLE V. Head-Related Particles in 17⁻ *ts* Mutants*

Mutant	Particles per bacterium				
	bp	gr	ep	τ	pfu
*ts*CB80	3	59	74	2	1
*ts*L51	1	84	35	1	< 1
*ts*L2	< 1	< 1	130	2	1
*ts*N20	7	3	143	4	10
*ts*N21	16	< 1	132	< 1	10
*am*N56	0	0	153	5	< 1

*The bacteria were grown at 37°C up to 1 × 10^8 per ml, and then shifted to 40.5°C. At 2.2 × 10^8 bacteria per ml they were infected and superinfected as described in Materials and Methods. At 60 min after infection aliquots were taken for fixation and embedding and counting of pfu. FoOs fixation was used. Counting of head-related particles was done as described in Materials and Methods 4.5. For comparison the results with 17(*am*N56) are included in the table.
bp) black particles; gr) grizzled particles; ep) empty particles.

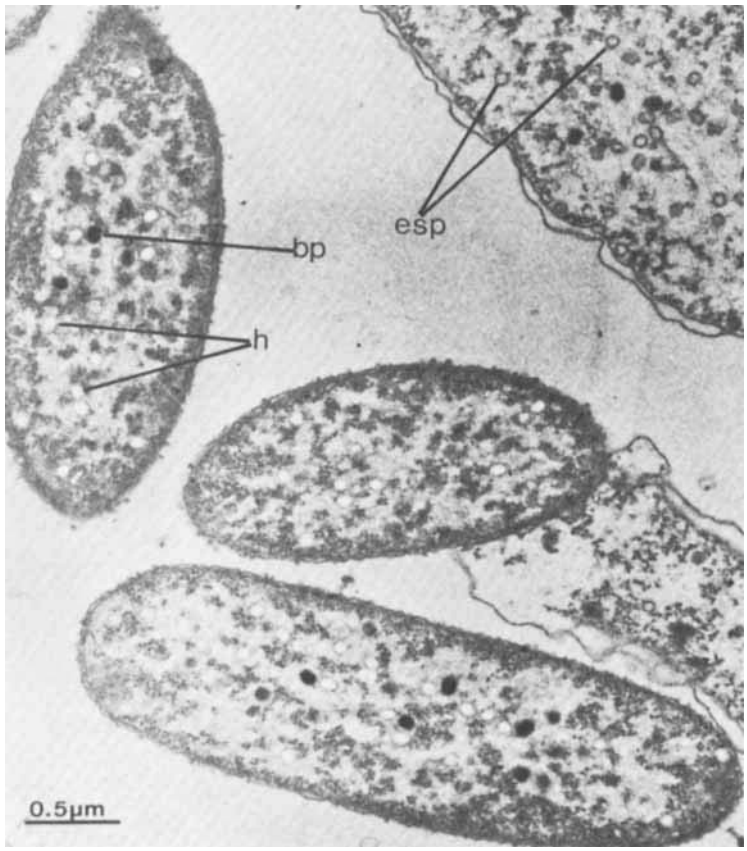

Fig. 7. Thin section of E. coli BE 30 min after infection and superinfection with T4·(*am*N66·*am*N87) (m.o.i. 5; 37°C). The cells were fixed by the partial lysis method (21). Besides 3 well-preserved cells, there are 2 partially lysed bacteria, in which many empty small particles (esp) are visible. In the well-preserved bacteria the empty particles appear as "holes" (h). At 30 min after infection full particles (bp) start to be formed. Fixation: FoOs.

TABLE VI. Phage-Related Particles and Cleavage of gp23 in Double Mutants of the Maturation Genes*

	16⁻			17⁻			49⁻			50⁻			2⁻			4⁻			64⁻					
	bp	gr	ep	bp	gr	ep	bp	gr	ep	bp	gr	ep	bp	gr	ep	bp	gr	ep	bp	gr	ep	bp	gr	ep
	6	6	77[a]																					
16⁻	23	8	49[b]																					
		0.58[c]																						
	< 1	< 1	99	< 1	4	92																		
17⁻	< 1	< 1	99	< 1	2	92																		
		0.67			0.74																			
	< 1	< 1	99	< 1	< 1	99	54	43	2															
49⁻	< 1	10	88	< 1	< 1	99	52	43	4															
		0.56			0.70			0.83																
	13	12	75	< 1	< 1	98		–		65	30	3												
50⁻	35	15	47	< 1	< 1	96	3	40	56	72	17	5												
		0.51			0.70			0.75			0.82													
	13	12	72	< 1	< 1	98		–			–		72	21	4									
22⁻	29	14	54	< 1	< 1	98	9	81	9	66	16	18	87	10	1									
		0.50			0.60			0.60			0.65			0.84										
	< 1	1	99	< 1	1	98	< 1	77	21		–		50	40	10	64	33	2						
4⁻	4	14	82	< 1	6	94	1	90	9		–		50	44	6	79	12	7						
		0.46			0.57			0.64			–			0.50			0.82					T4D⁺		
	15	21	62	< 1	< 1	95	91	8	< 1		–		90	8	2	60	40	< 1	70	23	4	90	10	< 1
64⁻	34	21	44	< 1	2	93	90	6	3	63	16	19	97	2	1	70	30	< 1	83	11	3	90	10	< 1
		0.44			0.65			0.61			0.62			0.50			0.48			0.76			0.86	

E. coli BE grown on M9a was infected and superinfected with the different mutants as described in Materials and Methods and Table I. For fixation (FoOs) and embedding aliquots were taken at 30 min ([a]) and 60 min ([b]) after infection. Counting of phage-related particles was done as described in Materials and Methods 4.5. The numbers of particles are given as percentage of the total phage-related particles present. The differences between 100% and the sum of the values listed is accounted for by τ particles, which are not included in the table. bp) Black particles; gr) grizzled particles; ep) empty particles. Cleavage of gp23 ([c]): The cultures were continuously labeled with ^{14}C-labeled amino acids (see Materials and Methods) starting at 30 sec after infection. At 60 min after infection aliquots were taken for gel electrophoresis. Autoradiograms of the gels were used to determine the amounts of gp23 and gp23. The extent of cleavage is expressed as the ratio of gp23* to (gp23 + gp23*). Details on this procedure are given in (46).

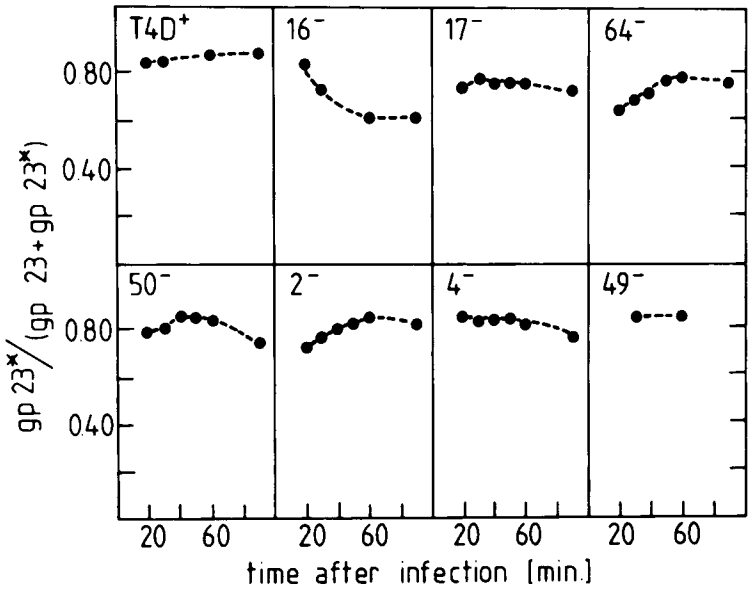

Fig. 8. Cleavage of gp23 in the maturation mutants as measured by continuous label. E. coli BE was grown on M9a at 37°C and infected and superinfected with the mutants indicated (m.o.i. 5). At 30 sec after infection ^{14}C-labeled amino acids were added (specific radioactivity 2.7 μCi per mg). At different times after infection aliquots (1 ml) were precipitated with ethanol (1:2). After centrifugation, the precipitations were dissolved in "sample buffer" (27) and run on a 10% polyacrylamide gel. The dried gel was exposed to Kodirex film and the density of the gp23 and gp23* bands measured with a densitometer. The peak areas were calculated by multiplication of peak height times peak width at half height. For details on the procedure see (46). The extent of cleavage is expressed as the ratio gp23* to (gp23 + gp23*).

tern to a certain phenotype. The following can be concluded: 1) Most of the bp (in wild type, 49$^-$, 50$^-$ 2$^-$, 4$^-$, and 64$^-$) contain gp23*; 2) the grizzled particles in 49$^-$ contain gp23*; 3) most of the empty particles in 17$^-$ at all times and in 16$^-$ at early times after infection contain gp23*.

From among all the single maturation mutants, 16$^-$ is the only one in which the data suggest an accumulation of particles with uncleaved gp23.

For the double mutants we find larger differences in the cleavage of gp23 (Table VI). The mutants in the matrix can be arranged such that, with some exceptions, we find an increasing or decreasing gp23* ratio along a horizontal or vertical line. These data could tentatively be interpreted as indicating that some of the particles of the mutants with low gp23* ratio might be made with uncleaved gp23. To test this hypothesis we correlated quantitatively the amount of phage-related particles per bacterium counted on thin sections with the amount of gp23* equivalents per bacterium using standard conditions. The results are reported elsewhere (46). For the most likely candidate, 64$^-$·2$^-$ this hypothesis turned out not to be verified as correct.

DISCUSSION

With a view toward preserving fragile particles which are expected to occur with mutants in the maturation genes, we compared different fixation methods. We found no

qualitative difference between them: Even without prefixation the types of particles found were identical by either method. However, the quantitative comparison revealed large differences between the fixation procedures used. Only for those in which the infected bacteria were well preserved and homogeneous were the numbers of phage-related particles per bacterium reproducible. This difference was not found for wild type-infected bacteria, but only in those infected with one of the maturation mutants, confirming the prediction that any intermediate or defective head related particle is likely to be unstable (11, 26) and hence its number will decrease when the sample is prepared under inadequate conditions.

Normal phage heads and τ particles define 2 size classes: 1051 ± 32 Å × 823 ± 48 Å and 856 ± 46 Å × 699 ± 42 Å, respectively. All head-related particles which were found in the maturation mutants can be attributed to either of these 2 size classes:

a) "Large" particles (Fig. 9). This class comprises: black particles (bp), grizzled particles (gr), and empty large particles (elp). All of them are found in the cytoplasm and are never membrane-attached. The class of black particles, although on thin sections morphologically not distinct, comprises at least 3 different types: the relatively stable, mature heads; the much less stable immature full heads, which break down into empty capsids upon lysis; and finally, as we will demonstrate in a later paper of this series, the condensed DNA-containing particles of which also the shell is fragile.The grizzled particles, which are about 50% of the population occurring in 49^-, correspond to what Luftig et al. (36) have called "structures in the size and shape of intracellualr filled heads, but considerably more diffuse in appearance," and Okinaka (37) "diffuse heads." We have found that lead staining unambiguously characterizes these particles as being clearly distinct from any polas section of a bp (34). The patchy appearance, which leads us to call these particles "grizzled," suggests either an incomplete filling or an unstably packed DNA complement which is partly lost from the phage heads during fixation. Partially filled or partially emptied particles are found in the lysates of 49^-, containing between 32% (36, 35) and 50% (28) DNA, according to the procedure of lysis. This strongly suggests a fragility of the particles produced by 49^-.

In (46) and in later papers of this series, we will report experiments designed to test that the grizzled particles in general are truly partially filled particles which are maturable as is demonstrated for 49^- (32, 46).

The empty large particles (elp) which likely are constituted of gp23* (see Table VI) are morphologically identical to heads of ghosts on thin sections, showing the same characteristic dots in every corner. We do not know if these dots are composed of DNA or protein.

b) "Small" particles (Fig. 9). Two types of particles belong to this class: τ particles and empty small particles (esp). τ Particles are mainly found in mutants 21^- and 24^-, but they occur in all maturation mutants and wild type in small numbers (1–10 per cell). They contain a core which is surrounded by the major head protein (gp23); they are always membrane-attached. Empty small particles (esp), without a distinct core, however are only found in the cytoplasm, and we have strong indications (Ref. 46 and Table 6) that they are composed of gp23*, although they have the appearance of empty τ particles. Empty small particles always occur together with elps. From the experiments presented here, we cannot decide whether elps and esps occur simultaneously in vivo or are generated artificially one from the other by either swelling or shrinkage. We only know that they occur with all fixation methods used, and that the esps can be detected morphologically as flattened particles with a rough appearance in lysates and are found to be unstable

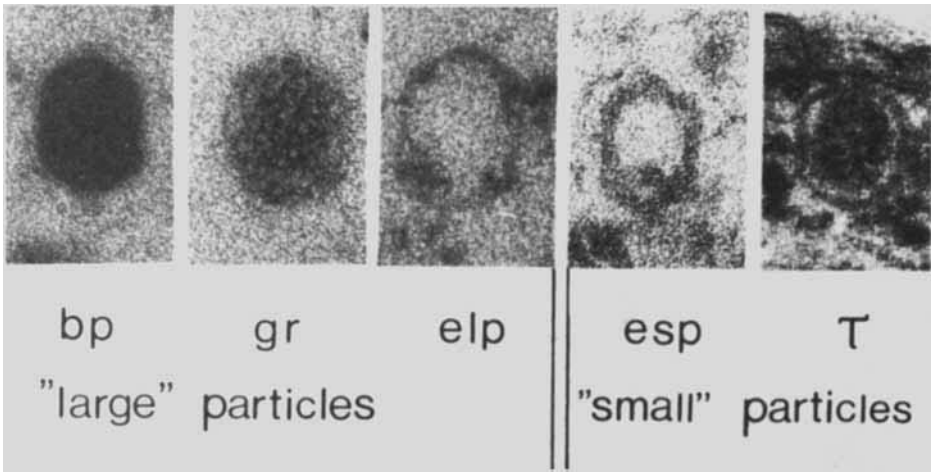

Fig. 9. Classes of head-related particles found in the T4 head maturation mutants. bp) Black particles; gr) grizzled particles; elp) empty large particles; esp) empty small particles. Fixation: FoOs.

when compared to the elps (46). The identification of these particles with those defined in the literature by their sedimentation constants in sucrose gradients is not yet unambiguously possible.

It is interesting to note, that a triple *am* mutant, 43^-(polymerase)·30^-(ligase)·46^- (exonuclease) only produces empty particles of both types of the same morphology (46, 47). This mutant is unable to replicate the viral genome and the formation of phage-related particles is a function of the multiplicity of infection. The implications of this finding are discussed in paper II of this series (47).

By comparing thin sections of cells infected with single mutants in the maturation genes and the double mutants between them, we can roughly distinguish 4 classes of phenotypes (Fig. 10). 24^- and the double mutants with it have not been included in the scheme, 24^- being epistatic on all head maturation genes.

Class I. This class is characterized by the production of empty particles at early times and the appearance of full particles at later times. It includes 16^- and the double mutants 16^-·50^- and 16^-·2^-. Note that 16^-·49^- and 16^-·17^- do not produce DNA-containing particles even at later times.

Class II. More than 90% of the phage-related particles are empty and many of them have tails attached. Independent of the morphology, cleavage of gp23 is decreased. This type includes 17^- and all double mutants between 17^- and mutants in the remaining maturation genes and 16^-·49^-. 17^- is epistatic on all other maturation gene mutants. This can be explained either by postulating that gp17 acts before all maturation genes or that gp17 acts on the DNA by rendering it competent for packaging (46). In 17^-, as in the mutants of the head assembly genes, the maturation of DNA to head-full pieces is blocked (14). Together with our results, this suggests that in the absence of DNA and packaging, cleavage of gp23 and processing of the core proteins occurs and leads to empty shells which are competent for tail attachment. The latter point is in favor of the 17^- particles being rather abortive than true precursors (46).

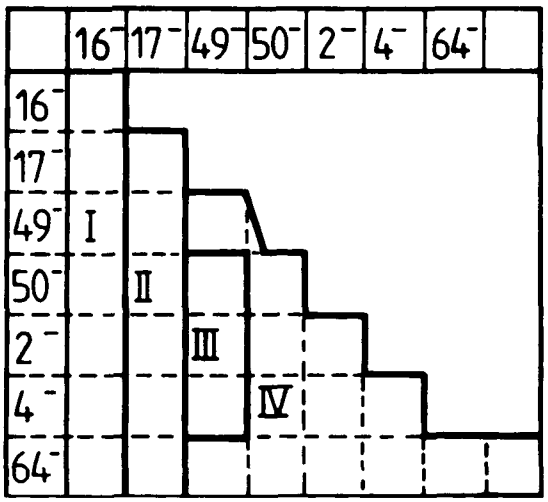

Fig. 10. Schematic representation of the main classes of epistatic behavior of the T4 head maturation mutants (for explanation see text).

Class III. This class includes $49^{-}\cdot 50^{-}$, $49^{-}\cdot 2^{-}$, and $49^{-}\cdot 4^{-}$. All of them show a full range between dark and pale grizzled and empty particles suggesting variable DNA contents. In contrast to 49^{-}, practically no bps are found. On thin sections we did not find a single particle with a tail attached. Cleavage of gp23 is decreased. From the aspect of the protoplasm in thin sections, we tend to believe that the mutants of this class have an enlarged DNA pool as in the case of the 49^{-} single mutant (Fig. 5). We found the same for $49^{-}\cdot 64^{-}$ but not for $49^{-}\cdot 17^{-}$. This morphologically detectable, large DNA pool is likely to correspond to the $>$ 1,000S form of DNA which has been described for 49^{-} (13, 24). At the same time, it was found that in 49^{-} lysates a nucleolytic activity is lacking which is present in all other maturation gene mutants and in wild type (13). The function of gp49 in head maturation could thus be indirect by the bias of an action on the vegetative phage DNA. Cross-links due to arrested recombination events prevent the DNA from being completely packaged into a head precursor (23).

Class IV. The single and double mutants of this type (49^{-}, 50^{-}, 2^{-}, 4^{-}, 64^{-}, $49^{-}\cdot 64^{-}$, $50^{-}\cdot 2^{-}$, $50^{-}\cdot 4^{-}$, $50^{-}\cdot 64^{-}$, $2^{-}\cdot 4^{-}$, $4^{-}\cdot 64^{-}$) contain more than 50% of black particles and less than 20% of empty particles. The overall aspect of sections of bacteria infected with one of these mutants or double mutants is very similar to wild type-infected cells, but less than 1 pfu per bacterium is found. Hence, most of these black particles are noninfectious and fragile as indicated by the study of lysates (Ref. 15 and unpublished results of our laboratory). In view of DNA maturation and tail attachment, 49^{-} and $49^{-}\cdot 64^{-}$ belong rather to class III than to this class as discussed before; both of them show the enlarged DNA pool on thin sections, and in both of them we never found attached tails on thin sections.

According to Fujisawa and Minagawa (14) cutting of the DNA is normal in 2^{-}, 4^{-}, 50^{-}, and 64^{-}. In sections we find some particles with tails. We have extensively studied in situ lysed cells infected with these mutants. Confirming previous results (15), we observe that heads produced by the latter mutants frequently have tails attached. By comparing

with lysates we find that the junction of tail to head is very fragile and breaks easily upon lysis.

In this group large differences in the extent of cleavage of gp23 occur. In some cases only about 50% of gp23* is found whereas about 90% of phage-related particles are bps. We have shown (46) that the accumulation of uncleaved gp23 in $64^{-}\cdot 2^{-}$ is not due to the accumulation of DNA-filled particles made of uncleaved gp23, as might be expected, but is rather due to an unbalance in another step of regulation of phage morphogenesis.

The above reported results are obviously not compatible with a linear pathway in which a given maturation step is simply halted when the involved gene product is not active: Mutants in none of the maturation genes lead to the accumulation of a single species of particles. In all the cases we observe mixed populations composed of particles of at least 2 species from among empty small, empty large, grizzled, or black particles. Gene 17 provides the only mutants which produce only empty particles (empty small and empty large) and which are epistatic on mutants in all other maturation genes. Two *ts* mutants in this gene, however, also produce grizzled particles.

The phenotypes of single and double mutants of the maturation genes can only be described by the proportions of different particles present and the amount of cleavage. The observed epistatic behavior, with the possible exception of mutants in gene 17, cannot be understood on the basis of obligatory sequential actions of the gene products considered. The following hypothesis provides an explanation of the observations.

The actions of the products of most of the maturation genes are independent from each other. In order to act on a precursor particle a given gene product does not obligatorily depend on the previous action of another gene product involved. Such independent actions necessarily lead then either a) to bypasses of the "normal" pathway or b) to abortive side tracks ending with particles which are no longer maturable to active phage.
For the latter alternative, we have examples from the head assembly pathway with its side tracks of abortive polyheads. Particularly relevant is the example of τ particles: Without active gp21, abortive τ particles are produced; without active gp24 both maturable τ particles and abortive polyheads are produced. The latter is also observed when both gp21 and gp24 are lacking (31). Everything happens as if an action of gp24 without the previous action of gp21 is possible but leads to the abortiveness.

Above we have discussed the observations which suggest for gp49 an action on the vegetative DNA of the pool and which prevents complete packaging. Other maturation gene products possibly also act via the vegetative phage DNA. On the one hand, the double mutants $49^{-}\cdot 4^{-}$ and $49^{-}\cdot 2^{-}$ of class III phenotypes produce grizzled particles of all degrees of filling. On the other hand, the preponderance of grizzled particles produced in 49^{-} can be counteracted by a combination with 64^{-} or 16^{-}. In both cases we do not deal simply with an epistatic behavior of 64^{-} or 16^{-} over 49^{-} since the double mutants show more black and less grizzled particles than 64^{-} or 16^{-} alone.

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E. Kellenberger and F. Traub have further investigated the heterogeneous response of a population of infected cells observed on thin sections by electron microscopy and which reflects in different degrees of preservation of leakage (in preparation). It is present with all FoOs fixed cells, but particularly obvious with amber mutants in 17^{-}, which in general have an abnormal lysis behavior. When prefixed with 0.01% OsO_4, the heterogeneity is further enhanced and results in leakage and partial lysis of many cells. We observed that a prefixation with glutaraldehyde at room temperature, which, after a wash in buffer, is followed

by the main fixation in OsO_4 under RK conditions, leads to a minimal heterogeneity in all cases. These and other preliminary observations with differently fixed 17⁻ infected cells suggest however that the number of recognizable, empty particles is decreased in well prepared cells, and suggests the existence of a form of particles which is difficult to visualize because they contain internal material. This will be described in a note added to the following paper of this series (47).

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