J. gen. Virol. (1976), **32**, 249–259
Printed in Great Britain

Ultrastructure and Life Cycle of the Lipid-containing Bacteriophage $\phi 6$

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(*Accepted* 31 *March* 1976)

SUMMARY

An electron microscopic study of the lipid-containing bacteriophage $\phi 6$ revealed an electron dense compact inner core of 30 nm in diam., which apparently contains the nucleic acid of the virus. This inner particle is surrounded by a complex polyhedral capsid with an outer diam. of 50 nm. Outside this is the envelope, which gives the virus a total diam. of 65 to 75 nm. The envelope, which has a thickness of a unit membrane, could be removed by treating the phage with Triton X-100. A definite structure is seen inside the envelope of the phage tail.

In infection, phages are attached by their tails to the host cell pili. Occasional pili with a few attached phages were seen in a phage resistant mutant. In the course of the infection phages were also seen attached to the outer membrane of the cell. In a phage-tolerant mutant many normal-looking pili with adsorbed phages were visible, but we could never see phage-cell membrane associations. The membrane of the phage appears to fuse with the bacterial outer membrane and 50 nm virus particles could be seen in the periplasmic space of the bacterium, probably attached to the cytoplasmic membrane. Newly formed 50 nm particles appeared 45 min post infection (p.i.) centrally in the host cell. Assembly of the envelope also began at this time and by 80 minutes p.i. all the 50 nm particles were covered by the virus membrane. At no stage were phages seen in the periphery of the bacterium. Mature phages were finally released by a rupture of the host cell without spheroplast formation.

INTRODUCTION

Bacteriophage $\phi 6$ differs from all other known bacterial viruses in that it contains double-stranded RNA and is surrounded by a lipid envelope (Vidaver, Koski & Van Etten, 1973). The lipid content is high (25 %) and resembles that of many enveloped animal viruses (Vidaver *et al.* 1973). The lipid composition of the phage is different from the lipid composition of the host cell (Sands, 1973). However, the outer and inner membranes of the host cell envelope have not been studied separately. The envelope can be removed by organic solvents and sodium dodecyl sulphate (SDS) revealing a 50 nm particle (Ellis & Schlegel, 1974). If the envelope is removed, the infectivity of the virus is lost (Vivader *et al.* 1973; Ellis & Schlegel, 1974).

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The phages seem to have a blunt membranous tail with which they adsorb on to the pili of the host *Pseudomonas phaseolicola* (Vidaver *et al.* 1973). The development of the phage takes place in the nuclear region of the host cell (Ellis & Schlegel, 1974).

Recently the protein composition of the ϕ6 was shown to consist of two classes, the early and the late proteins. Some of the late proteins can be extracted from phages with Triton X-100. This suggests that they are membrane proteins (Sinclair *et al.* 1975).

Another lipid-containing bacteriophage PM2, described earlier (Espejo & Canelo, 1968), differs structurally and in its chemical composition from the ϕ6. PM2 is a double-stranded DNA phage (Espejo, Canelo & Sinsheimer, 1969) and its lipid is inside a protein layer (Brewer & Singer, 1974; Hinnen, Schäfer & Franklin, 1974). Two other lipid containing bacteriophages were recently described by Bradley & Rutherford (1975), but they seem to resemble PM2.

An electron-microscopic study of purified ϕ6 particles, using thin sectioning and negative staining techniques, is described. Different stages in the life cycle of the virus were examined and evidence is presented for a unique infection mechanism among bacterial viruses involving the fusion of virus and host membranes.

METHODS

Growth and purification of the phage. The ϕ6 bacteriophage and its host *Pseudomonas phaseolicola* HB 10 Y were kindly provided by Anne Vidaver, The semi-synthetic medium (SSM) described by Vidaver *et al.* (1973) was used for bacterial growth and phage production. The bacteria were grown at 26 °C until the extinction 0·1 at 620 nm was reached and phages were added, so that final phage-bacterium ratio was 4:1. (The culture used for studying the life cycle of the virus had a multiplicity of infection (m.o.i.) of about 60 for the thin section samples and an undetermined high m.o.i. for the negative staining.) The cell debris was removed by low-speed centrifugation after 3·5 h incubation. The resulting supernatant was concentrated with 9 % (w/v) polyethylene glycol 6000 (PEG) using the method of Yamamoto *et al.* (1970). The PEG-treated phage was allowed to stand at 4 °C overnight and was then pelleted by centrifugation at 12000 *g* for 30 min. The pellet was resuspended in buffer A (Vidaver *et al.* 1973) to one hundredth of the original lysate volume.

The phage was further purified by centrifugation in a 10 to 40 % (w/v) linear sucrose gradient in buffer A using a Spinco SW50.1 rotor at 27000 rev/min for 70 min. Fractions were collected from the bottom. Protein concentration (Lowry *et al.* 1951), infectious titre and extinction at 280 nm were determined for each fraction. Only the peak material was collected and dialysed overnight against buffer A at 4 °C, This material had a titre of about 5×10^{12} p.f.u./ml and was used in all electron microscopic studies.

Detergent treatment. Triton X-100 was added to the dialysed phage to achieve 0·1 % (w/v) concentration. The preparation was allowed to stand 4 min at room temperature and then was negatively stained as described below. For thin sectioning (see also below) 200 μg of Triton X-100/100 μg of phage protein was added. This preparation was centrifuged at 35000 rev/min for 40 min using a SW 50.1 rotor to pellet the phage. The pellet was resuspended in equal amount of Triton X-100, centrifuged again, washed with buffer A and then prepared for thin sectioning.

Isolation of ϕ6 resistant mutants of Pseudomonas phaseolicola. About 10000 p.f.u. of the phage and 0·2 ml of the overnight broth culture of the host bacterium, were added to tubes with 3 ml molten soft agar (0·6 % [w/v] agar in H_2O). The tubes were whirled and poured on to nutrient broth yeast extract (NBY) plates (Vidaver, 1967). The plates were incubated at

26 °C for 48 h. After incubation, phage resistant colonies were isolated and purified by two successive single colony isolations on NBY plates. Their resistance to φ6 was tested. Two mutants, no. 11 and no. 21, were used in electron microscopic study of the phage infection

Electron microscopy. The phage was negatively stained with 2 % (w/v) phosphotungstic acid (PTA) in 0·1 M-sodium phosphate (pH 7·5). Thin sections were cut from the pure phage pellet that had been fixed for 1·5 h with 3 % (v/v) glutaraldehyde in 0·1 M-sodium cacodylate buffer (pH 7·2). Samples of different stages of the life cycle were fixed in suspension, in 3 % (v/v) glutaraldehyde. After 1·5 h these cells were pelleted at 5000 *g* for 20 min. Centrifuged pellets were postfixed with 1 % (w/v) osmium tetroxide in 0·1 M-sodium phosphate buffer (pH 7·2). The samples were embedded in Epon 812 and cut with a diamond knife in an LKB Ultramicrotome 1. The thin sections were stained with uranyl acetate and lead citrate. All the micrographs were taken with JEOL 100B electron microscope operating at 80 kV.

RESULTS

Ultrastructure of the φ6 bacteriophage

The diameter of the head of an intact PTA negatively stained φ6 particle is about 65 to 75 nm. All the negatively stained particles have a tail-like structure which varies in length (Fig. 1 *a*). The shape of the tail may vary from one particle to another, as also noted by Vidaver *et al.* (1973).

In thin sections the dimensions of the φ6 particles are identical with those of the negatively stained ones (Fig. 1 *b*). The particles have an inner electron dense core of about 30 nm (Fig. 1 *b*). Outside the core is an electron dense circle, approx. 50 nm in diam. (arrow in Fig. 1 *b*) and outside of this, a membranous layer approx. 8 nm thick. This could well correspond to a unit membrane that has been reported to have a thickness of 7·5 nm (Salton, 1964). The bilayer structure of the membrane is occasionally seen in sections – see arrow in Fig. 1 (*c*). Several 50 nm particles are now and then seen inside the same membrane (Fig. 1 *c*).

The length of the well-sectioned tail of the phage is approx. 40 nm (arrow in Fig. 1 *d*). The tail is about 20 nm wide and has a definite inner structure.

Detergents and organic solvents are known to inactivate the phage (Vidaver *et al.* 1973) and cause stripped particles in negatively stained preparates (Ellis & Schlegel, 1974). The purified and dialysed φ6 suspension was treated with 0·1 % (w/v) Triton X-100 and then negatively stained. Stripped particles were seen (Fig. 2 *a*). These particles were 45 to 50 nm in diam. and the shape indicated a more complex structure than a single icosahedral capsid. When the Triton X-100 treated and pelleted phage had been sectioned, particles with a diam. of approx. 50 nm were seen, with an inner core of about 30 nm (Fig. 2 *b*) corresponding to the 50 nm particle already seen in thin sections of the intact phage (Fig. 1 *b*).

The life cycle of the φ6 bacteriophage

Samples were collected during the life cycle of the phage at the times indicated by arrows in the one step growth curve (Fig. 3), which is identical to that of Vidaver *et al.* (1973). In the firts preparations (4 min), the phages are attached to the pili with their tails (Fig. 4 *a*, *b*) as also described by Vidaver *et al.* (1973). The pili are covered by numerous phages; the pilus itself is not visible in the thin section (Fig. 4 *b*), but the distribution of the phages suggests them to be attached to the pilus. Some phages are in close contact with the bacterial surface.

To investigate the role of the pili in phage infection, we isolated phage resistant mutants of the host strain and studied the adsorption of the phages to these mutants. In the case of mutant No. 11 the pilus structure was either changed or the pili were absent, and only few

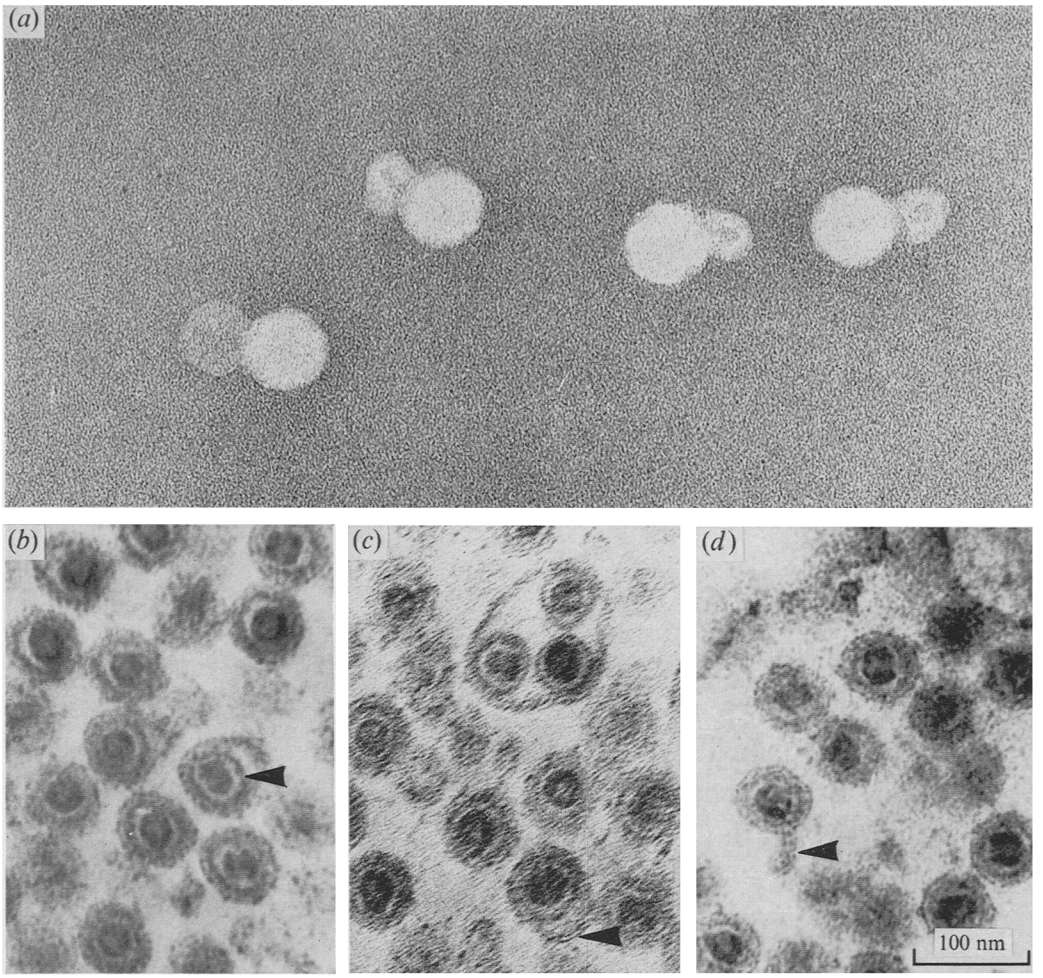

Fig. 1. Purified bacteriophage ϕ6. (*a*) Negatively stained preparation revealing the general structure of the phage. (*b*) The phage seen in thin section, where the different parts of the head of the phage are clearly seen. The arrow indicates the 50 nm particle, surrounding the 30 nm core. The outermost dark circle is the phage membrane. (*c*) A section with several 50 nm particles inside one membrane. The bilayer structure of the phage membrane is clearly seen (arrow). (*d*) A section of the phage where a well-sectioned tail is seen (arrow).

phages were seen in contact with the bacterial projections (Fig. 5*a*). The other mutant, no. 21, was apparently a phage tolerant one. The high affinity of phages to pili was not diminished (Fig. 5*b*). These mutant strains showed neither phage particles inside the cells nor lysed cells in thin sections fixed 80 min p.i. At this time after infection lysed cells are normally seen (as described below).

At 4 min p.i. different stages of phage-bacterium membrane fusions are seen (Fig. 6*a* to *e*). Fig. 6(*a*, *b*) show phages attached to the outer membrane of the cell. Fig. 6(*c*, *d*) show the phage envelope and the cell membrane fused. In some bacteria the 50 nm particles are seen in the periplasmic space and probably attached to the cytoplasmic membrane of the bulging bacterium (Fig. 6*e*). Similar structures are also seen in preparations at 8 min p.i. but they are less frequent suggesting that penetration normally takes place in a short time.

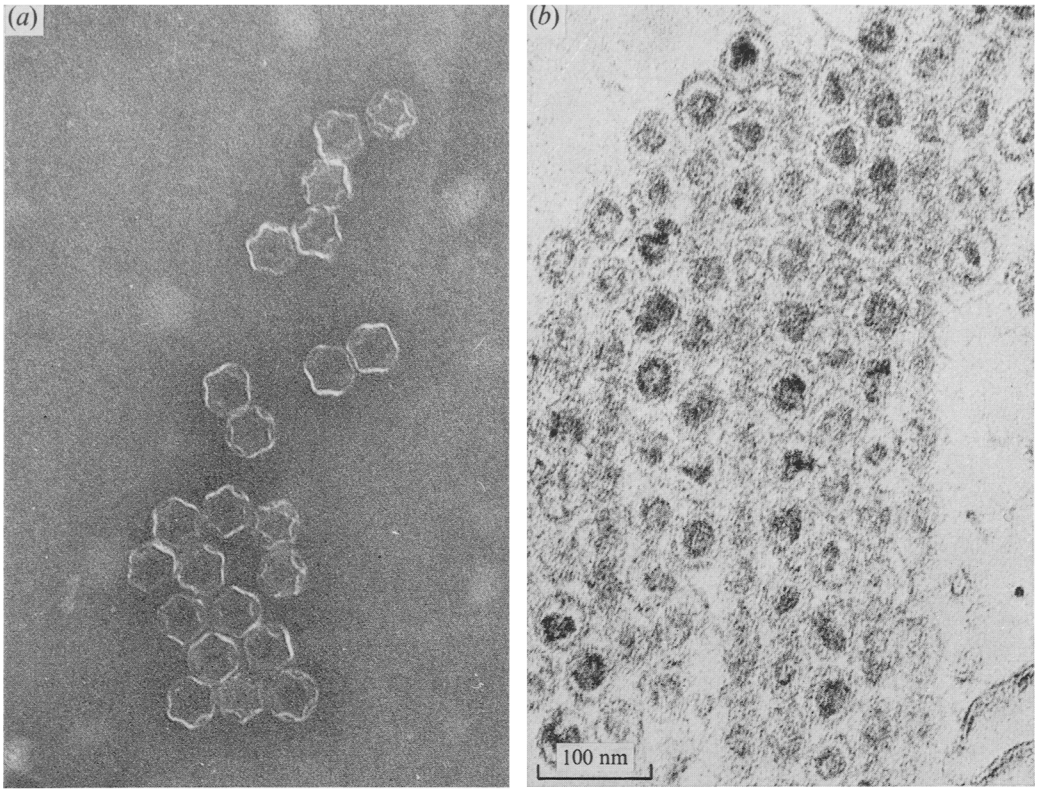

Fig. 2. Triton X-100 treated phage. (*a*) A negatively stained preparation where the 45 to 50 nm large rather complex capsid structure is seen. (*b*) Sectioned pellet of the phage, where the 30 nm core is seen inside the 50 nm particle.

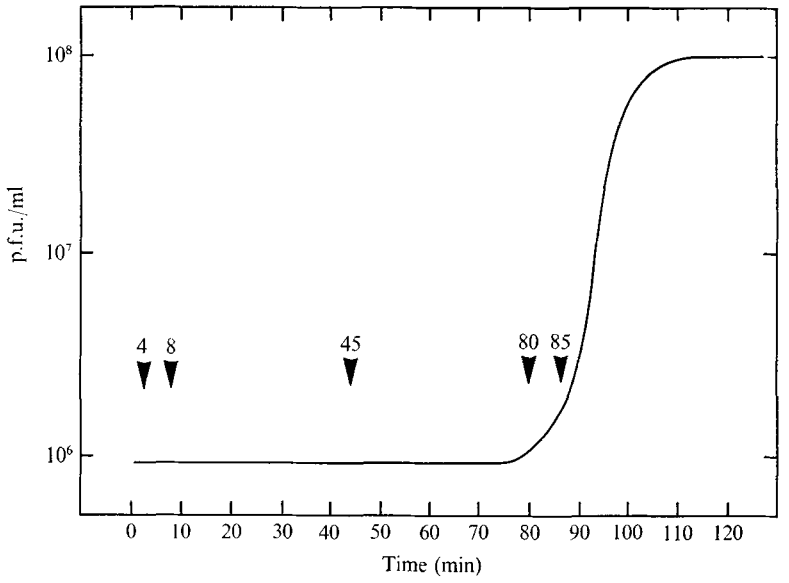

Fig. 3 Schematic one step growth curve with arrows indicating the times when the samples were collected for the study of the life cycle.

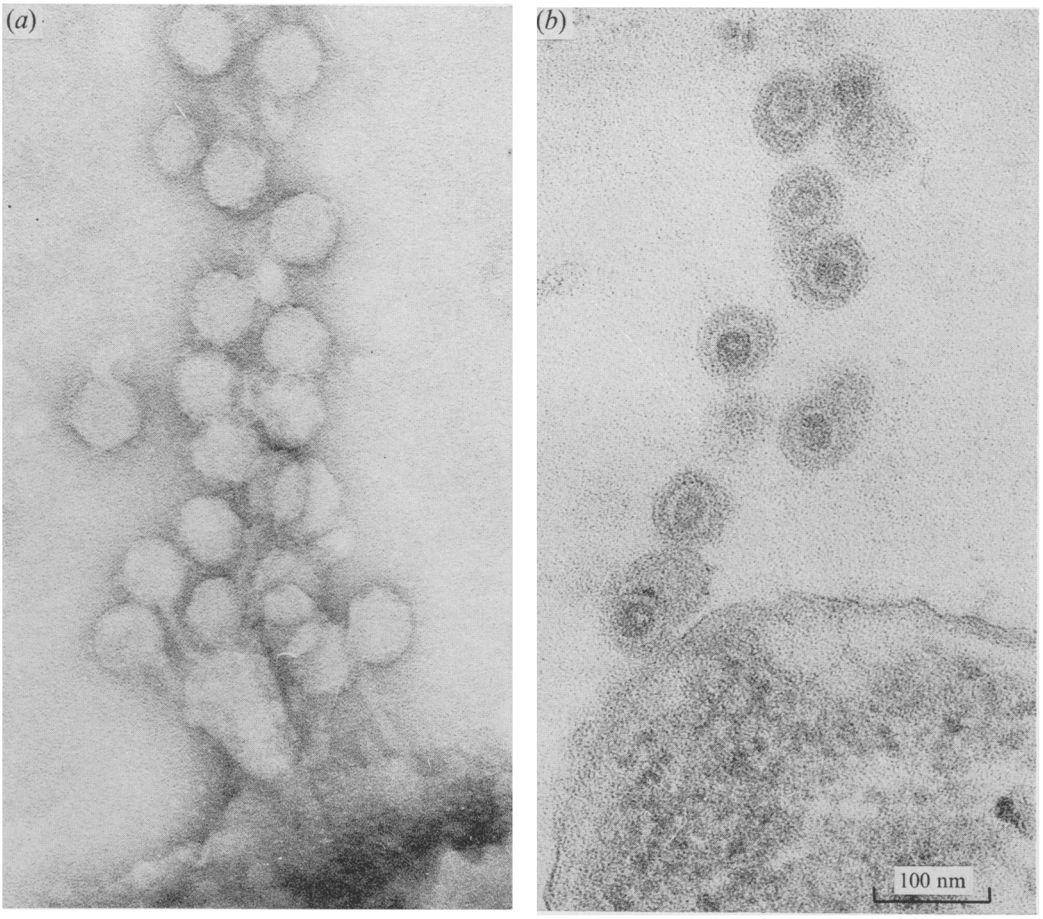

Fig. 4. The attachment of the phages to the pilus of the *Pseudomonas phaseolicola* HB 10 Y (*a*) negatively stained. (*b*) thin sectioned.

Visible $\phi 6$ particles can again be seen 45 min p.i. (Fig. 7*a*). At this time 50 nm large particles with 30 nm cores, but without the membrane, are frequently seen centrally in the cells. The first sign of membrane formation around a 50 nm particle is also evident (arrow in Fig. 7*a*). This takes place in the central part of the cell. No association of the phage particles to the bacterial membrane is seen. In fact the periphery of the cell is conspicuously free of phages.

Eighty min after infection, all the particles inside the cells have a membrane, their diam. is about 65 to 75 nm (Fig. 7*b*) and they resemble intact purified phages. These probably correspond to the particles Ellis & Schlegel have described (1974). At this time many of the cells are already lysed.

Phage liberation is supposed to be the result of a burst and lysis of the cell (Ellis & Schlegel, 1974). This is evident in Fig. 8, taken 85 min p.i., in which a bursting cell liberates phage particles. Even at this stage the peripheral area of the cell is free of phages. Remnants of numerous lysed cells are also seen.

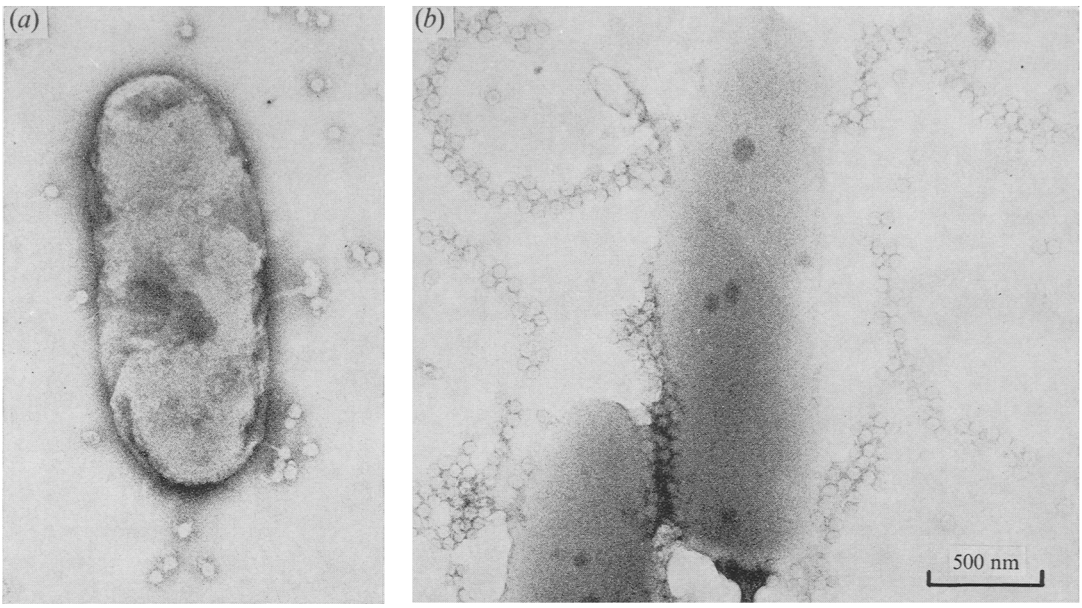

Fig. 5. Phage φ6 resistant *Pseudomonas phaseolicola* HB 10 Y mutants. (*a*) A mutant with very few phages attached to the bacterial projections. (*b*) A phage tolerant mutant strain with pili crowded by phages.

DISCUSSION

We could show by electron microscopy that the bacteriophage φ6 is of uniform size – in contrast to a previous claim (Ellis & Schlegel, 1974) – having a diameter of 65 to 75 nm. The membrane surrounding the phage has the thickness corresponding to that of a unit membrane. Solubilization of the membrane with organic solvents and detergents suggests that it has the same basic structure as any biological membrane.

The up to 100 nm negatively stained particles previously described (Ellis & Schlegel, 1974) may result from the fusion of the membranes of several individual phages as seen in Fig. 1 (*c*) or from loosening of the phage membrane. In fresh preparations we have never seen individual particles larger than about 75 nm.

The phage has a blunt membranous tail of variable morphology (Vidaver *et al.* 1973). It has been suggested that the tail structure may be an artefact of streaming of the loose lipid envelope (Ellis & Schlegel, 1974). Our pictures (arrow in Fig. 1 *d*) show a definite inner structure in the tail. Our studies on disrupted phages (to be published) suggest that this structure may contain specific tail protein. The tail is most probably the attachment organelle to the pilus (Vidaver *et al.* 1973, and Fig. 4).

The 50 nm capsid structure described before in negatively stained preparations (Vidaver *et al.* 1973; Ellis & Schlegel, 1974) can be clearly visualized also in thin sections of the intact phage (Fig. 1 *b*). After solubilization of the phage membrane with Triton X-100 this 50 nm particle (Fig. 2 *a*, *b*) remained visible after both negative staining and thin sectioning.

In thin sections a compact inner core particle of about 30 nm in diam. is clearly visible. It is probable that the core contains the nucleic acid of the phage. The compact form of the 30 nm particle suggests that it may also contain an inner protein capsid analogous to several double-stranded RNA viruses of eucaryotic organisms (Wood, 1973).

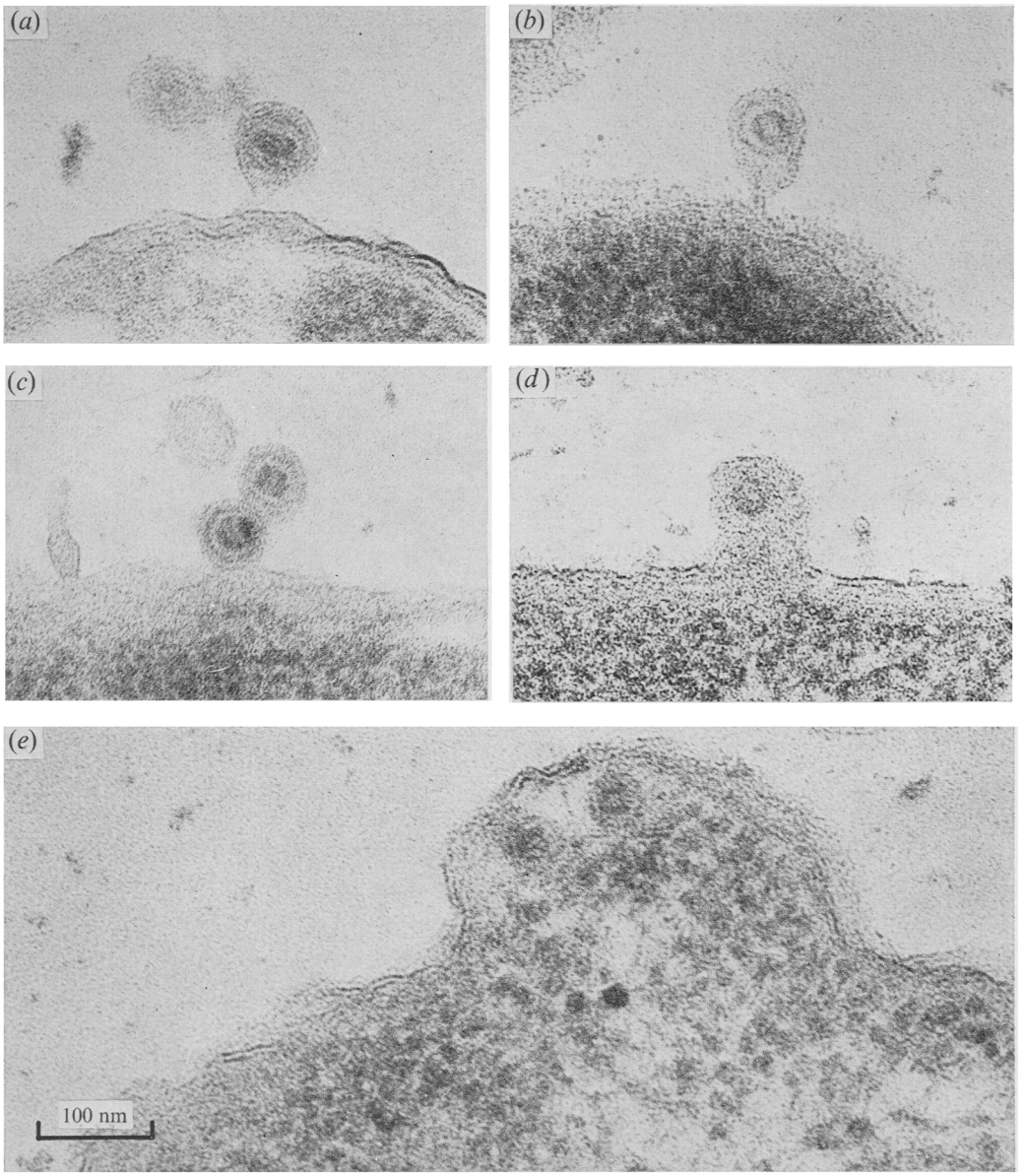

Fig. 6. The process of entry of the phage into the bacterium in samples prepared 4 min p.i. (*a* to *d*) Different stages of phage-bacterium membrane association. (*e*) 50 nm particles in the periplasmic space of the bacterium.

One of the earliest steps in the infection is the adsorption of the phages by their tails to the host cell pili (Fig. 4). This adsorption seems to be a necessary step in the infection, as shown by the fact that a phage resistant mutant which had either changed or absent pili had only few phages attached (Fig. 5*a*).

In the early stages of infection we found different stages of apparent phage-bacteria membrane fusions (Fig. 6*a* to *d*). In some pictures the 50 nm particles are seen in the periplasmic

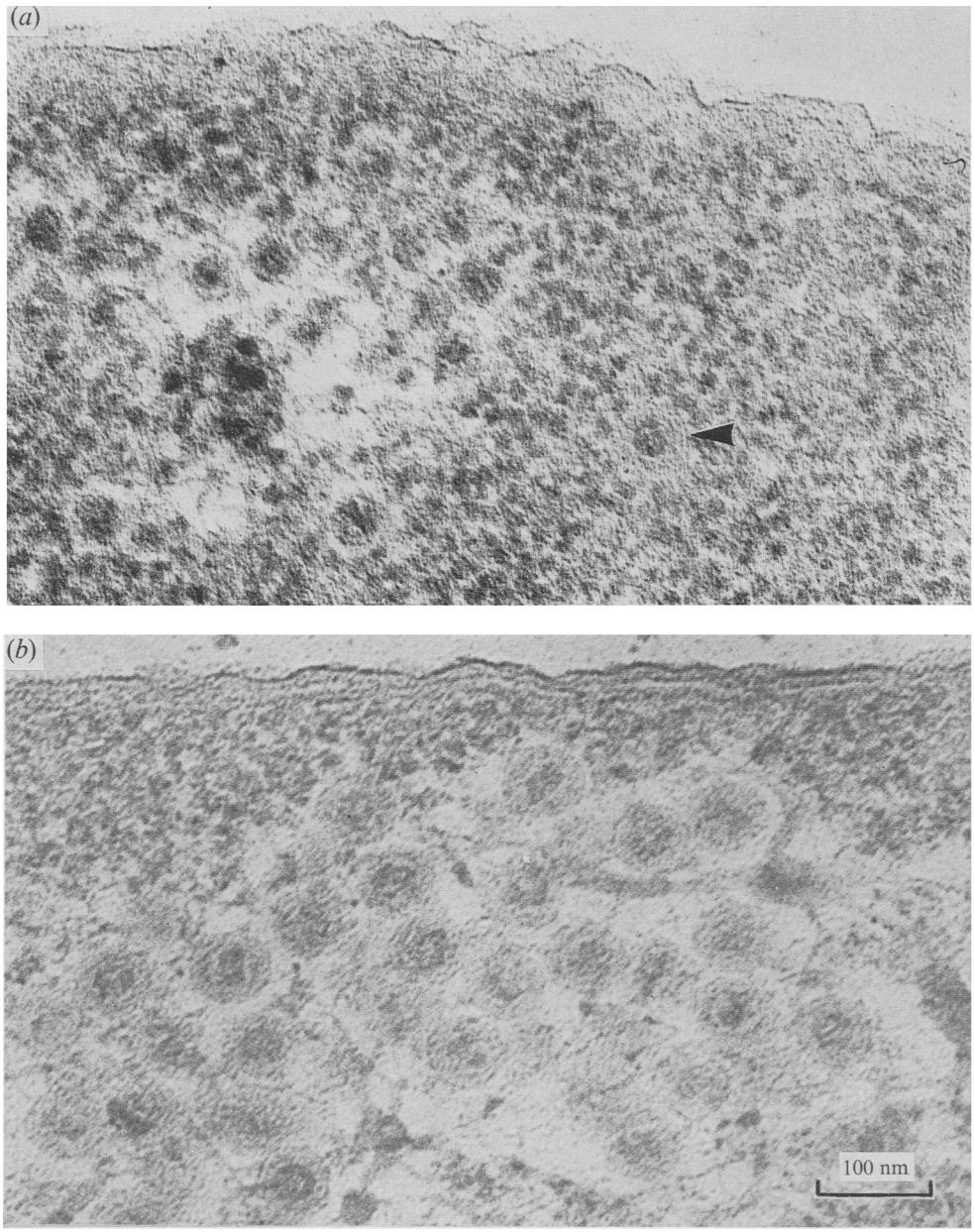

Fig. 7. Phage maturation. (*a*) 45 min p.i. 50 nm particles with 30 nm cores centrally located in the bacterium; first sign of the membrane formation is also seen (arrow). (*b*) 80 min p.i., nuclear region is filled with 65 to 75 nm phage particles.

space of the bacterium (Fig. 6*e*). It is tentatively proposed, that the phage membrane first fuses with the outer membrane of the cell releasing the 50 nm particle into the space in between the outer membrane and plasma membrane. The 50 nm particle in the periplasmic space seems to be associated with the cytoplasmic membrane. The next step in the infection could be the injection of the nucleic acid into the cytoplasm. In the phage tolerant mutant,

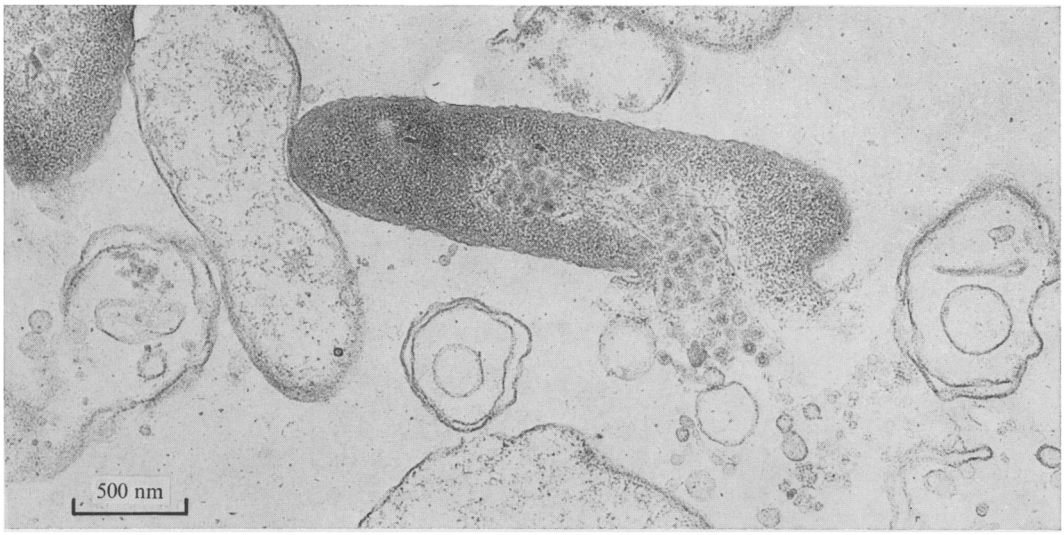

Fig. 8. 85 min p.i.: the burst releasing the phages from the bacterium.

no. 21, in which the adsorption to the pili seemed to be normal, no attachment of the virus to the bacterial membrane was seen. This observation suggests that the penetration of the phage through the outer membrane is also a necessary step in infection.

Later in the life cycle 50 nm particles were first detected in central parts of the cell. The 30 nm particles were never seen alone and it is possible that they are not compact enough to be distinguished from other bacterial components.

The first signs of the phage envelope, around the 50 nm particles, are seen 45 min p.i. It is known that there is a temperature sensitive step in phage assembly about 50 min after infection (Sands *et al.* 1974). The late phage proteins appear at about this time (Sinclair *et al.* 1975). These correspond with the time when the phage envelope begins to appear morphologically. The phage particles are always seen in the central regions of the cells and never in contact with cell membranes, as also described by Ellis & Schlegel (1974), contrary to PM2 maturation which occurs in cell periphery (Dahlberg & Franklin, 1970).

ϕ6 is the first bacteriophage described with features in common with enveloped animal viruses. This makes the ϕ6 an interesting and important bacteriophage to be used as a possible model system for studies on some animal viruses.

The authors are very grateful to Dr Anne K. Vidaver for ϕ6 bacteriophage and its host, and to Professor P. Helena Mäkelä for the critical reading of the manuscript.

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(*Received* 5 *January* 1976)