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Ultrastructure of Bacteriophages and Bacteriocins

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INTRODUCTION

Like many other organisms, bacteria are subject to infection or antibiosis by a range of viruses or viruslike particles which fall naturally into two physiologically separate groups, bacteriophages and bacteriocins. The members of the first group are true viruses, infecting their hosts and multiplying within them. The bacteriocins differ in that they do not multiply in the cell after infecting it, but only kill it.

Although the effects of bacteriophage infection were certainly encountered by many workers in the early part of this century and before, it was not until 1915 that Twort (212) observed lytic action on colonies of staphylococci and succeeded in isolating the agent responsible. Since Twort's objective was to study the nature of ultramicroscopic viruses, he naturally concluded that the lytic agent was such a particle, though at the time little was known about viruses in general. Two years later, d'Herelle (88) independently isolated a dysentery bacteriophage, characterizing it as an ultramicroscopic parasite of bacteria, and giving it the name "bacteriophage." As late as 1922 the true nature of bacteriophages was still a controversial subject (89), the virus theory not being fully accepted. Today, however, modern techniques have shown it to be correct, and Adams (2) defines bacteriophages as "autonomous microbes analogous to plant and animal viruses but obligately parasitic on bacteria." Until 1940, phage research mainly consisted of unsuccessful attempts to use the viruses for therapeutic purposes, and it was not until after World War II that more fundamental research was carried out.

Unlike bacteriophages, the discovery of bacteriocins cannot be pinpointed to a single date; the inhibition of one bacterial strain by another was frequently observed in the early part of this century. However, the first thorough investigation of the interaction of two strains of *Escherichia coli* was carried out by Gratia in 1925 (75) and subsequent years. Both Gratia and Fredericq, who continued this work, laid the foundation for our present knowledge of the subject which Fredericq has recently reviewed (72, 73). In his original investigation, Gratia succeeded in isolating an inhibitory substance from *E. coli* strain V which acted on *E. coli* strain ϕ and was called a "colicin." At first, this substance seemed very similar to a bacteriophage, but it possessed one essential difference: it was not reproduced by the sensitive bacterium as a bacteriophage would have been. Bacteriocins may thus be defined as a natural class of highly specific antibiotics.

Since 1945, bacteriophages have been intensely studied by many different techniques, but perhaps one of the most significant contributions to our knowledge of these and other viruses has been made by the use of one instrument, the electron microscope. The first feature electron microscopy revealed was the remarkable form of many phages; they resembled submicroscopic tadpoles with heads and with tails which could change shape. Other types had longer tails, sometimes with a knob on the end. In addition to this sort of information, the examination of infected cells by various procedures revealed much about the infective process. In contrast, bacteriocin research has been carried out by a comparatively small group of enthusiasts. The electron microscope has been little used to study bacteriocins, so that our knowledge of their morphology in general is almost nonexistent. Other aspects, such as their chemical nature (102), have been well studied. However, recent reviews, such as that of Reeves (175), indicate that our knowledge of these antibiotics is not nearly so detailed as is our understanding of bacteriophages, in spite of the fact that bacteriocins are of equal biological importance.

The electron microscope is particularly suit-

able for studying the morphology and physiology of viruses and similar particles; thus, the present review leans heavily on results obtained by electron microscopy in describing the structure and infective processes of bacteriolytic particles. Other aspects arising out of these studies, such as their classification and origin, will also be discussed.

CLASSES OF BACTERIOLYTIC PARTICLES

Since the discovery of bacteriophages and bacteriocins, a number of other related types of particles have been found associated with various species of bacteria. These have usually been recognized in the electron microscope by their resemblance to bacteriophages or bacteriophage components, but not all are biologically active. With respect to activity, bacteriophages come first. They have a conventional viral form: a nucleic acid core is surrounded by a protein coat, and most types have a tail by which they adsorb to the host cell. Bacteriophages can have two different replicative cycles. In the first, nucleic acid is injected into a sensitive cell which subsequently lyses. In the second type of cycle, nucleic acid is injected, but no intracellular virions are formed. Instead, the phage genome or a replica of it becomes inserted into the continuity of the bacterial chromosome and is thereafter replicated as a part of the bacterial chromosome. A bacterial strain infected in this way is called lysogenic and can be induced to produce phage particles by treatment with metabolic inhibitors; lysis and the release of phage progeny then occur as with lytic viruses. It is usual to call bacteriophages with the first type of infective cycle "virulent" and those with the second type "temperate." However, since many phages are capable of both replicative cycles, depending on the bacterial strain they infect, the two terms really apply to phage-host systems rather than to the bacteriophage alone. Obviously, it would be extremely difficult to prove that a so called "virulent" phage could never lysogenize any sensitive bacterium, or, conversely, that no sensitive strain existed towards which a particular "temperate" phage could behave in a virulent fashion. One cannot, therefore, separate bacteriophages into two distinct groups, though "virulent" and "temperate" can be used in a descriptive sense.

Recently, a number of phagelike particles with heads and tails have been found associated with *Bacillus subtilis* (18, 98, 189, 200). Like temperate phages, they are inducible, and, according to Seaman et al. (189), their genetic determinants are perpetuated in the carrier strains as episomes, replicating in synchrony with the bacterial chromosome. Unlike temperate phages, they cannot lysogenize a sensitive bacterium, nor can they multiply intracellularly in a virulent fashion. Their only biological activity is manifested by the ability to kill a sensitive cell. They are thus bactericidal like bacteriocins and not bacteriolytic like bacteriophages. These "protophages," or "killer particles" as they are called, are very similar to bacteriocins, being differentiated from them only by a rather uncertain genetic difference. The bacteriocins, whose determinants are believed to be plasmids, are biologically similar to killers, and are induced from carrier strains of bacteria defined as bacteriocinogenic; their release may not be accompanied by lysis. The structure of these agents is variable. As will be described below, some resemble bacteriophages; others appear to be phage components—headless tails, for example. A large group is entirely different, being in the form of very small molecules, e.g., colicin CA42-F with a sedimentation constant of only 3.6*S* (175). Clearly, the differences, if any, between large phagelike bacteriocins and killer particles are better discussed after they have both been described in detail.

All the particles described so far are biologically active against the bacteria with which they are associated. A class of particles exists, however, which appears to be a product of bacterial lysis and to have no obvious activity or function. While not strictly bacteriolytic, these particles can be grouped among the bacteriophages and bacteriocins because of a morphological resemblance. One of the most important is produced by the autolysis of the gliding organism *Saprospira grandis* (44) and resembles a phage tail. A second is also rod-shaped and is produced by *Proteus mirabilis* (101).

ISOLATION OF BACTERIOPHAGES AND BACTERIOCINS

Culturing Methods

In general, special growth media and culturing methods are not required for the isolation and production of bacteriophages and bacteriocins. All that is necessary is that optimal growth conditions for the particular species of bacterium prevail and that all the necessary nutrient requirements and trace elements be provided. There is, however, one procedure devised by Gratia (79) in 1936 which forms the basis of most of the isolation, growth, and titration procedures used in bacteriophage studies. It is the double agar layer method, which, together with many other experimental methods, is described in detail by Adams (2). Standard nutrient agar plates are prepared with 1.5 to 2% agar con-

taining the appropriate nutrients for the organisms being studied. The host bacterium, either with or without virus particles added, depending on the experiment, is suspended in about 1.0 ml of nutrient broth and mixed with an equal volume of 0.7 to 1.0% agar solution at 45 C. The mixture is immediately poured onto an agar plate which is rocked back and froth to spread it evenly. After allowing the soft agar layer to set on a level surface, the plate is incubated. An even opaque layer of bacterial growth is obtained in this way, and single virus particles form transparent plaques which are clearly visible in it.

Isolation of a Virulent Bacteriophage

Since one expects to find bacteriophages in the natural habitat of the host organism, it is from this source that new isolates are best obtained. One of the best sources for enteric bacteria is sewage. To isolate phages, a sample of the habitat material, or a broth extract of it if it is solid, is first cleaned by centrifugation (5,000 $\times$ *g* for 15 min) and sterilized with chloroform. About 0.1 ml is plated together with the host organism by the double agar layer method. After incubation, plaques should be found (if there are too many or too few, more or less habitat material may be required). The bacteriophages are removed from a plaque by stabbing with a sterile platinum needle, which is rinsed in 1.0 ml of broth. To ensure that phages from nearby plaques have not contaminated the suspension, a little is replated with host bacteria and a fresh plaque is stabbed. Two or three "plaque-picking" cycles will ensure a pure broth suspension.

With habitat material in which the phage concentration is very low, such as with seawater, enrichment procedures may be required similar to those used by Schmidt and Stanier for the isolation of *Caulobacter* phages (186).

Isolation of a Temperate Bacteriophage

Since a temperate phage is in effect reproduced by its host bacterium without lethal consequences, its isolation from a lysogenic strain requires rather different procedures. First, it is necessary to find a sensitive indicator strain; that is, one toward which the temperate phage will behave in a virulent fashion. Since small numbers of the phage are produced spontaneously by the host, all that is usually necessary is to prepare soft agar layer plates of suspected sensitive strains and to place a loopful of host bacterium on them. If, after incubation, the colony of the host strain is surrounded by a clear area, then the suspected indicator is sensitive. Next, serial dilutions of bacteria-free fluids from a broth culture of the host organism are plated by the soft agar layer method with the indicator; the plaques obtained can then be picked in the usual way. If an indicator cannot be obtained, the host organism can be induced, by methods to be described below, but under these circumstances one cannot be certain that one has a bacteriophage and not a bacteriocin or other intermediate bacteriolytic particle. This situation arises only when a phage-like particle is identified other than by its biological activity, such as in the electron microscope.

Detection of a Bacteriocin

Both bacteriocins and temperate phages are detected in the same way; the producing organism causes lysis of an indicator strain. Thus, to detect lysogeny or bacteriocinogeny, the plating procedure outlined above is used with numerous different combinations of bacterial strains of the same species. Other detailed procedures have been described by Fredericq (71, 73). Once an interacting pair of strains has been found, the lytic agent must be identified. Bacteria-free fluids from a culture or an induced culture (*see below*) are diluted serially. Loopfuls of each dilution are spotted on a soft agar plate of the indicator. After incubation, the presence of a temperate phage is indicated by the breaking up of the clear spots into discrete plaques as the dilution is increased. With a bacteriocin, no plaques are formed, the area of lysis becoming less transparent with greater dilution.

INDUCTION OF A TEMPERATE BACTERIOPHAGE OR BACTERIOCIN

The production of a large quantity of a temperate phage or a bacteriocin can be conveniently achieved by causing a logarithmic culture of the host bacterium to lyse by means of an inducing agent. There are many of these available, such as hydrogen peroxide, mustard gas, ultraviolet light, and mitomycin C. The last two are probably the best, ultraviolet light having first been applied to *B. megaterium* (138) in log phase at a concentration of 3.4×10^6 cells per ml in a 2-mm layer. The radiation time could be varied with little effect from 20 to 120 sec and an intensity of 2,000 ergs per mm^2 at the surface of the broth. Complete lysis ensued after incubation for about 80 min. Mitomycin C is very simple to use, a suitable quantity (between 0.1 and 1.0 μg/ml final concentration according to the species of bacterium) being added to a dilute log culture (10^8 to 5×10^8 cells/ml). Lysis may or may not occur, but bacteriocin or temperate phage production generally reaches a maximum after 5

to 7 hr. A study of mitomycin induction on various *Bacillus* species has been carried out by Altenbern and Stull (3). However, it should be pointed out that a few phages are not inducible.

PURIFICATION PROCEDURES

For the examination of a bacteriophage in an electron microscope, a more or less pure concentrated suspension of particles must be prepared; such a preparation is most easily attained by use of plates. About 1 ml of a suspension made by picking a plaque is plated with host bacteria by the double agar layer method. The resulting plate, which should contain many plaques, is extracted with a few milliliters of broth for several hours. After removing the bacteria by centrifugation, this suspension can be used to lyse a larger number of plates. These, in turn, are extracted with neutral 0.1 M ammonium acetate solution. Bacteria are again removed by centrifugation (5,000 $\times$ *g* for 15 min) and then the phages themselves are sedimented at about 30,000 $\times$ *g* for 2 hr (adequate for most types). Resuspension in ammonium acetate solution followed by two further cycles of low- and high-speed centrifugation will produce a clean enough preparation for electron microscopy. Other more sophisticated methods of purification include the use of density gradients in the ultracentrifuge, ion-exchange columns (193), and precipitation with ammonium sulfate or zinc hydroxide (162). These methods are more suitable for large volume lysates of broth cultures.

In some cases, it may be desirable to break down the bacteriophage into its individual components, e.g., purified heads or tails. This can be achieved to some extent by chemical and physical separation procedures (31, 162, 218).

LYSIS OF A BROTH CULTURE FOR STUDYING INTRACELLULAR MULTIPLICATION

In addition to producing larger quantities of phage than can be achieved by plating methods, the infection and lysis of a broth culture of bacteria by a bacteriophage permits the study of sections of infected cells in an electron microscope. The general procedure is to inoculate a suitable volume of nutrient broth with host bacteria and allow them to grow into log phase to a concentration of about 5 $\times$ 10^8 cells per ml, though this will, of course, vary according to the phage-host system under study. Phages are then added to a concentration of anything between 1 and 100 viable particles (plaque-forming units) per bacterium. Lysis will ensue between 0.5 and 4 or 5 hr according to the system. Samples can be taken for electron microscope preparation before and during lysis, and the condition of the inside of the host cells can be studied.

ELECTRON MICROSCOPY

Methods of preparation in electron microscopy have been the subject of many text books, a useful recent one being edited by Kay (111); therefore, only very brief outlines of methods need be included here.

Negative Staining

Negative staining (80, 97) consists of embedding the phage particle in an electron-dense matrix; the virus shows up white against a dark background in a micrograph. Various procedures and chemicals can be used (14, 30), the easiest method being to mix equal volumes of phage suspension (in 0.1 M ammonium acetate solution) and neutral 2% potassium phosphotungstate solution (PTA). A drop of mixture is allowed to dry onto an electron microscope specimen grid covered with a carbon support film. The resolution obtainable with this process is very high, morphological subunits of only 10 A or so being revealed.

Shadow-Casting

The process of shadow-casting was superseded around 1959 by negative staining for the study of fine structure, but it is nevertheless superior for showing up fine fibrous elements such as are found on the tails of some bacteriophages. [Details are given by Kay (111).] The method consists of drying a suspension of phage particles in water or ammonium acetate solution onto a support film and coating it with a thin film of heavy metal deposited at an angle by vacuum evaporation. Objects form transparent "shadows" in the metal layer providing high contrast but low resolution, the limitation being the granular structure of the shadowing film.

Sectioning

The preparation of ultrathin sections is necessary for studying the intracellular multiplication of bacteriophages. It has been the subject of a great deal of research and is summarized in detail in appropriate books (111, 169). For the examination of infected cells, bacteria from a phage-infected broth culture may be fixed in glutaraldehyde (180) and then osmium tetroxide. After dehydration in acetone containing uranyl acetate, they are embedded in Vestopal or other suitable plastics (111). Sections are cut on an ultramicrotome and mounted unsupported on

specimen grids (for serial sections, a support film may be required); additional staining with lead citrate (177) and other metal complexes, singly or in combination, is usually carried out before examination in the electron microscope. Although this procedure shows up virus particles particularly well, changes in the nucleoplasm are not so clear, and for this purpose it may be more desirable to follow the procedures of Kellenberger, Séchaud, and Ryter (119), who used only buffered osmium tetroxide in the presence of Ca^{++} and amino acids for fixation.

Rotational and Linear Integration Printing

When a repeating structure is obtained on an electron micrograph, its contrast and resolution can often be enhanced by special printing procedures (144). This applies whether the repeat is rotational or linear. If, for example, a hexagonal disc is to be printed, a normal print is first prepared and fixed with its center accurately positioned on the axis of rotation of a turntable, which is placed under an enlarger containing the original negative. After mating the projected negative with the print, a piece of printing paper is placed on top of the latter, the enlarger being first switched off. Six exposures are then made, the turntable being rotated by one-sixth of a revolution (because the rotational symmetry of the hexagon is sixfold) between each. It can be seen that the final print will contain information represented by the superimposition of each 60° segment of the hexagon on the rest; contrast and resolution will be thus improved. In the case of linear objects, the principle is the same but is rather more difficult to put into practice (145). Although the above procedures are effective, it has been found that both types of repeat structure can be printed more easily (22). For rotation, the electron micrograph is spun on a variable-speed centrifuge under stroboscopic illumination and photographed. For linear structures, the centrifuge is replaced by a Mickle disintegrator; since this vibrates at a fixed frequency, that of the stroboscope must be variable. The disintegrator does, however, provide the necessary variability in amplitude. Results obtained in this way are, of course, of a qualitative kind, permitting an improvement in visual appearance rather than especially accurate measurements. The interpretation of micrographs printed in this way must be carried out with great care, but a great deal of additional information can often be gleaned from a single picture, particularly from negatively stained specimens.

A more complicated but extremely efficient method of evaluating micrographs of repeating structures (125), based on the optical diffraction techniques of Taylor and Lipson (210), provides accurate quantitative measurements of periodicities, etc. This has been applied to the structure of phage tails (7).

EXAMINATION OF BACTERIOPHAGE NUCLEIC ACIDS

Spreading Nucleic Acids for Electron Microscopy

The length of a phage nucleic acid molecule and whether or not it is a closed ring structure can be ascertained in the electron microscope by a spreading technique (124). After osmotically shocking the phage or extracting its nucleic acid chemically, a solution of the viral nucleic acid is spread on the surface of a liquid in a Langmuir trough. This is achieved by mixing a protein (e.g., cytochrome *c*) with the nucleic acid solution and applying it to the surface of the liquid substrate. The spreading of the protein into a monolayer untangles the nucleic acid, which can be picked up on grids and shadowed to provide contrast.

Identification of Nucleic Acid Type

It is, of course, extremely important to know the type of nucleic acid in a virus particle, especially if it has been newly isolated. Fluorescent staining with acridine orange (23, 150) provides a simple method of determining this. A pure phage suspension with no free nucleic acids present at a concentration of about 5×10^{11} particles per ml is prepared in phosphate-buffered saline. Droplets are dried onto glass slides, fixed in Carnoy's fluid, and stained in 1% acridine orange in McIlvaine's buffer at *p*H 3.8. After soaking in 0.15 M Na_2HPO_4 for 15 min, the slides are examined under 2,570 A ultraviolet (UV), a green fluorescence indicating double-stranded deoxyribonucleic acid (2-DNA) or ribonucleic acid (2-RNA) and a red color single-stranded DNA (1-DNA) or RNA (1-RNA). To determine whether the particles contain DNA or RNA, the slide is treated for a few seconds with molybdic acid solution (Table 1). Confirmation of these results can be achieved by treating the slides with ribonuclease and deoxyribonuclease before staining (Table 1).

BASIC STRUCTURE OF BACTERIOPHAGES

Architecture of a Virus

Fundamentally, most if not all viruses are built on the same principles: they are in the form of a nucleic acid core surrounded by a protein coat. However, the shape and size of the protein coat varies greatly. The nucleic acid is in the form of a long filamentous molecule and may be of a double- or single-stranded type.

TABLE 1. *Colors obtained from fluorescent staining of nucleic acids*

Nucleic acid	Treatment			
	Na_2HPO_4	Molybdic acid	Ribonuclease[a]	Deoxyribonuclease[a]
2-DNA	Green	Green	−	+
2-RNA	Green	Green fades	−	−
1-DNA	Red	Paler green	−	+
1-RNA	Red	Paler red	+	−

[a] Resistance to an enzyme is shown by − and susceptibility by +.

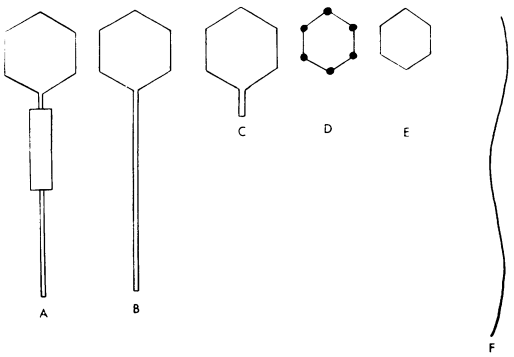


FIG. 1. *Basic morphological types of bacteriophages.*

The protein coat is an assembly of identical morphological subunits, which may themselves be composed of a number of smaller units, e.g., single molecules or assemblies of molecules. The morphological subunits may or may not be visible in the electron microscope. The particle may have appendages attached to it or it may be surrounded by an envelope. To clarify morphological descriptions, a special terminology, developed by Lwoff et al. (136), has been adopted to describe these parts. As has been mentioned, the nucleic acid is called the *core*; the protein coat as a whole is known as the *capsid*, and the morphological subunits which form it as the *capsomeres*. The complete infective virus particle is known as the *virion*.

As will be discussed in detail with reference to bacteriophages, all viruses are built on strict geometrical lines and obey rules of symmetry; Horne and Wildy have reviewed this aspect in detail (94). The fundamental principles underlying virus structure were predicted by Crick and Watson (49, 50), who argued from general principles applied in particular to small RNA-containing virions. They pointed out that the RNA was insufficient to code for more than a small number of low molecular weight proteins, so that the only way to build a capsid was to employ repeatedly the same sort of molecule. These are the chemical subunits already mentioned above. They then argued that the best way to pack similar subunits was to provide them each with a similar environment, i.e., to arrange them symmetrically. Since these predictions were made, the electron microscope has shown that they are valid, not only for small virions but for many large and complex viruses, including bacteriophages.

Basic Morphological Types of Bacteriophages

The bacterial viruses exhibit a greater diversity of form than any other group, but what at first glance appears to be a jumble of unrelated shapes and sizes falls conveniently into six basic morphological types. The most complex of these (Fig. 1A) has a head with a hexagonal outline which may or may not be elongated; a tail with a contractile sheath is attached to it. This is usually rigid and may have various appendages, such as fibers or terminal structures. It would be possible to subdivide this group according to their form. The second group in order of structural complexity (Fig. 1B) also has a six-sided head and a tail. Here, however, the tail is relatively flexible; it may or may not have terminal appendages; it is longer than the head diameter and has no contractile apparatus. The third type also has a tail and a six-sided head, but the tail is shorter than the head diameter or maximal dimension, and may also have appendages attached to it, but, again, it is noncontractile (Fig. 1C). The fourth group has no tail (Fig. 1D), but is still six-sided in outline though of symmetrical appearance. Each apex of the hexagon has a knob or large capsomere on it. In the next group (Fig. 1E), the large capsomeres are absent and the virion presents a simple regular hexagonal outline. The final group (Fig. 1F) is quite unlike the others, the virion being in the form of a long flexible filament; there are no additional structures of any kind attached to

TABLE 2. *Basic morphological groups of bacteriophages with types of nucleic acid*

Group	Description	Nucleic acid type
A	Contractile tail	2-DNA
B	Long noncontractile tail	2-DNA
C	Short noncontractile tail	2-DNA
D	No tail, large capsomeres	1-DNA
E	No tail, small capsomeres	1-RNA
F	No head, flexible filament	1-DNA

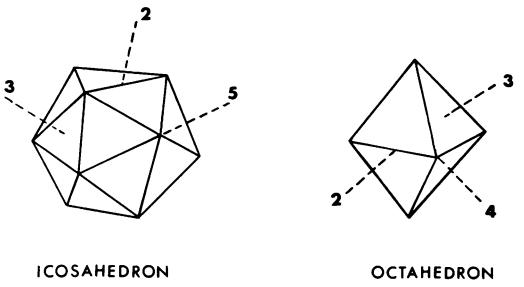


FIG. 2. *Basic geometrical forms of phage heads showing axes of symmetry.*

it. As might be expected, a fundamental difference in morphology reflects a difference in nucleic acid type. The kind of nucleic acid found in the members of each group has proved to be the same regardless of the genus of host bacterium. A suggested nomenclature, which will be adhered to in the ensuing material, together with the various nucleic acid types is given in Table 2.

It is of some importance to compare these structures with those found in other groups of viruses. The first three groups (with tails) are, as far as is known, unique to bacteria or very closely allied organisms such as algae. Group D could, with a stretch of the imagination, be said to resemble some of the small mammalian and plant viruses with large capsomeres, but, again, there is nothing exactly like it outside the bacteriophages. Groups E and F are different. There are many plant, animal, and insect viruses which exhibit a regular hexagonal outline and, moreover, are of more or less similar dimensions to their bacteriophage counterparts. The flexible filamentous form is also common, particularly among plant viruses. Although the morphological dissimilarity of the tailed bacteriophages compared with other viruses does not necessarily imply a complete lack of physiological similarity, in the cases of the filamentous and tail-less "spherical" bacteriophages, a comparison would be much more justifiable. Since Groups A to D are unique to the bacterial viruses, they would be expected to possess unique modes of action unlikely to be encountered elsewhere in the virus kingdom.

SYMMETRY IN BACTERIOPHAGES

The electron microscope has shown that bacteriophages obey the predictions of Crick and Watson (49, 50) and are built on strictly geometrical lines. This applies to all the morphological groups described above. Horne and Wildy (94) have detailed the principles of symmetry as applied to viruses in general, and here it is only necessary to mention how some of them apply to bacteriophages in particular. There are two basic forms of symmetry found in viruses, cubic and helical. The first occurs in regular polyhedra and the second in rod-shaped structures. In bacteriophages, therefore, heads will have cubic and tails helical symmetries. Five Platonic bodies exhibit cubic symmetry: the regular tetrahedron, the cube, the octahedron, the regular dodecahedron, and the icosahedron. Of these, only the octahedron and icosahedron have been found by electron microscopy (see Fig. 2), together with their derivatives which are often asymmetrical and extremely difficult to identify. A regular body has axes of symmetry, that is, lines (for three-dimensional objects) or points (for two-dimensional objects), about which it can be rotated to give a number of similar appearances. For example, a six-pointed star appears identical in any one of six positions if rotated about its center; thus, it has sixfold symmetry. The icosahedron and octahedron have three different symmetry axes each: 5:3:2 (fivefold, threefold, and twofold) and 4:3:2, respectively; they are shown in Fig. 2. Rod-shaped bodies, including phage tails, have helical symmetry which can be seen to have two components: when viewed end-on and when viewed from the side. An end-on view presents a radial or rotational symmetry like that of the symmetry axes of the Platonic bodies. A side view shows the symmetry perpendicular to the long axis. The three-dimensional helical symmetry is provided by a combination of the two. It is important to bear this in mind when interpreting electron micrographs in terms of three dimensions.

With the tailed phage particles it is clear that a combination of cubic and helical symmetry exists, and by the rules of geometry the one must fit the other at the point of contact. This does not necessarily mean that the symmetry axes must be identical: it is probably reasonable to fit a sixfold tail onto a threefold head axis. It has been argued further by Moody with reference to the T-even phages (155) that two unlike symmetries could be adapted by an intermediate structure. For example, a sixfold tail could be joined to a fivefold head axis by a 15- or 30-fold adaptor. While this suggestion would be applicable to all tailed phages, it seems on the whole that it is stretching nature's capabilities and is not compatible with those few convincing observations available, all of which show identical symmetries between tail and head [e.g., phages E1 (15) and SP50 (61)]. It is important to note that the predictions of Caspar and Klug (38) have been borne out, particularly among the small bacteriophages with their preferred icosa-

TABLE 3. *Distribution of morphological groups of bacteriophages*

Host genus	Morphological groups[a]
Escherichia	A, B, C, D, E, F
Pseudomonas	A, B, C, E, F
Bacillus	A, B, C, E
Staphylococcus	A, B

[a] See Fig. 1.

hedral head shape. The larger ones often have octahedral heads, though the well-known T-even phages exhibit a more complex shape which is still a controversial matter. A knowledge of the shape of the capsid is of practical value for the calculation of such things as the volume or the number and size of the capsomeres.

GENERAL MORPHOLOGY OF BACTERIOPHAGES

Occurrence of the Basic Morphological Types in the Bacterial Kingdom

Bacteriophages have been isolated for a large proportion of bacterial genera, but the most work has been carried out on those whose hosts have the greatest effect on mankind. As a result, the present overall picture of the distribution of the six basic morphological forms may be a distorted one. One or two fundamental facts are, however, apparent. First, a basic morphological type of phage is not restricted to any one bacterial genus or species; that is to say, contractile phages have been isolated for both gram-positive and gram-negative bacteria of widely separated genera. These phages may have a close resemblance to one another, though they almost always differ in some detail, such as dimensions or the nature of an appendage. Furthermore, a single phage is highly specific, its activity being usually restricted to closely allied species of a single genus.

Some morphological forms are more common than others. The two forms most frequently encountered over a wide range of organisms are the contractile types and those with long noncontractile tails. It is hard to say which is the most common, but in my experience the latter is found over a wider range of host organisms.

Some idea of the distribution of morphological types within various host genera can be obtained from Table 3. The genera chosen have received more or less equal attention, except for *Escherichia*, whose phages have been particularly well studied. In *Escherichia* each group is represented, and with *Pseudomonas* only tail-less phages with large capsomeres are missing. *Bacillus* has four forms, but *Staphylococcus* only two. Distribution is therefore uneven, and it is impossible to say what affects it. One point which does emerge, however, is that despite a fairly intense search for bacteriophages over the past few years no new morphological type has been found since the isolation of the first filamentous form by Marvin and Hoffmann-Berling (147) in 1963.

The exceptions to the specificity observed in bacteriophages of most bacterial genera are those of the *Enterobacteriaceae* family. Many can infect more than one host genus; the most closely related are probably those of *E. coli* and *Salmonella typhi*, which are sometimes called coli-typhoid phages. Likewise, some phages isolated on *Serratia marcescens* also grow on *E. coli*. This situation is possibly due to the classification of the *Enterobacteriaceae*, which describes as separate genera organisms which are extremely closely related. In the ensuing descriptions, phages will be associated with their best-known hosts.

Bacteriophages of Escherichia coli

Bacteriophages of *E. coli*, or "coliphages," have been studied in greater detail than any others. The best-known group, the "T" phages of Demerec and Fano (57), have been studied since their isolation. Though there are only seven isolates, the group contains three different morphological types. Phages T2, T4, and T6, better known as the "T-even" phages, are contractile, with a complex tail assembly and a head with an elongated six-sided outline. Because of their importance, they will be described in detail below. Phages T1 and T5 represent morphological group B (with long noncontractile tails). T3 and T7 have short noncontractile tails. Many isolates morphologically similar to the "T" phages have since been found in natural habitats, such as sewage and manure. Extensive searches by many workers have provided examples of the remaining morphological groups (Fig. 1).

Coliphages of the contractile group contain two different forms: the large T-even phages with 1,000 A heads and tails, and a smaller type with an 800 A head and a 1,000 A tail. The second form, a typical isolate being E1, has, according to Bradley (15), an octahedral head (Fig. 3a). Its tail has a number of cross-striations when extended and shows axial striations when contracted (Fig. 3b). The tail tip has four fibers attached to an obscure base-plate (Fig. 3a), these being more obvious in a shadowed preparation (Fig. 3c). This suggests a fourfold radial symmetry for the tail which is presumably attached to a fourfold axis of the octahedral head (Fig. 3a), thus obeying the rules of symmetry mentioned above.

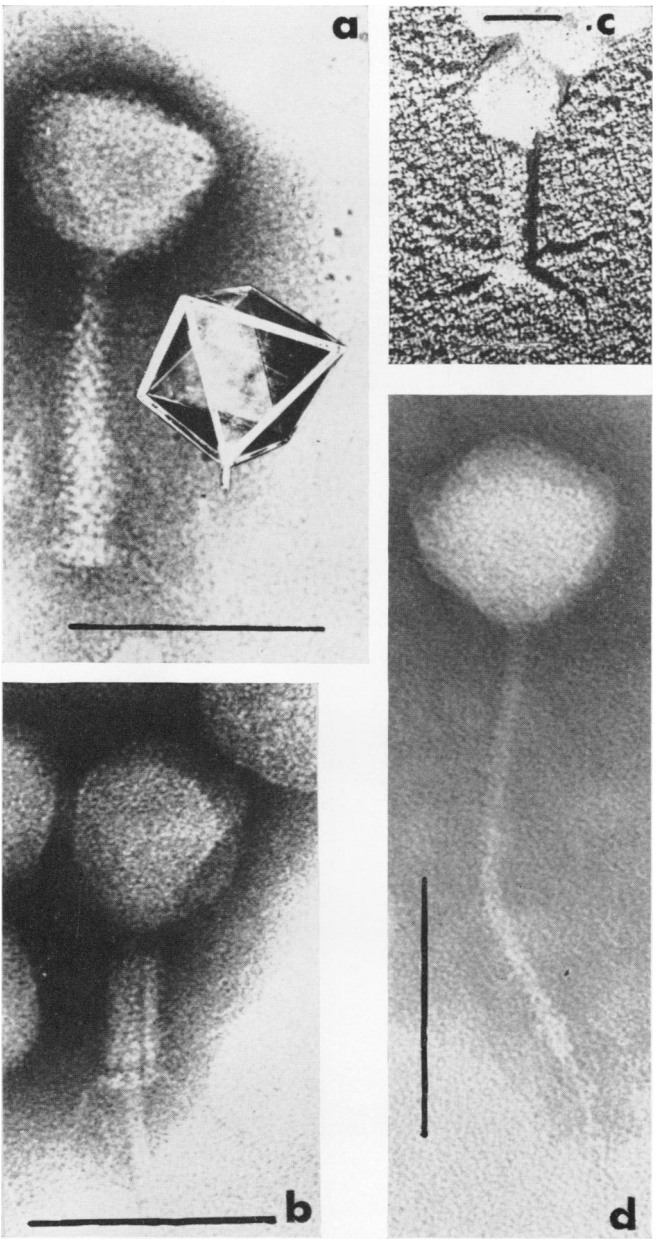


FIG. 3. *(a) Coliphage E1 showing octahedral form (15), PTA, × 333,000, scale 1,000 A. (b) Coliphage E1 with contracted tail (15), PTA, × 333,000, scale 1,000 A. (c) Shadowed coliphage E1 showing tail fibers (15), × 100,000, scale 1,000 A. (d) Coliphage T5, PTA, × 333,000, scale 1,000 A.*

Coliphages with long noncontractile tails also vary in appearance both in the shape of the head and in the nature of the tail tip. Phages T1 and T5 are more or less similar (Fig. 3d). The well-known phage λ is one of the few which exhibits head capsomeres (61), as shown in Fig. 4. The head (600 A) has a more or less regular hexagonal outline and a mottled appearance (Fig. 4a). On the intact phage, the tail (1,600 A) appears solid with fine cross-striations. It has a single fine fiber at the tip about 240 A long. The empty virion with its transparent head

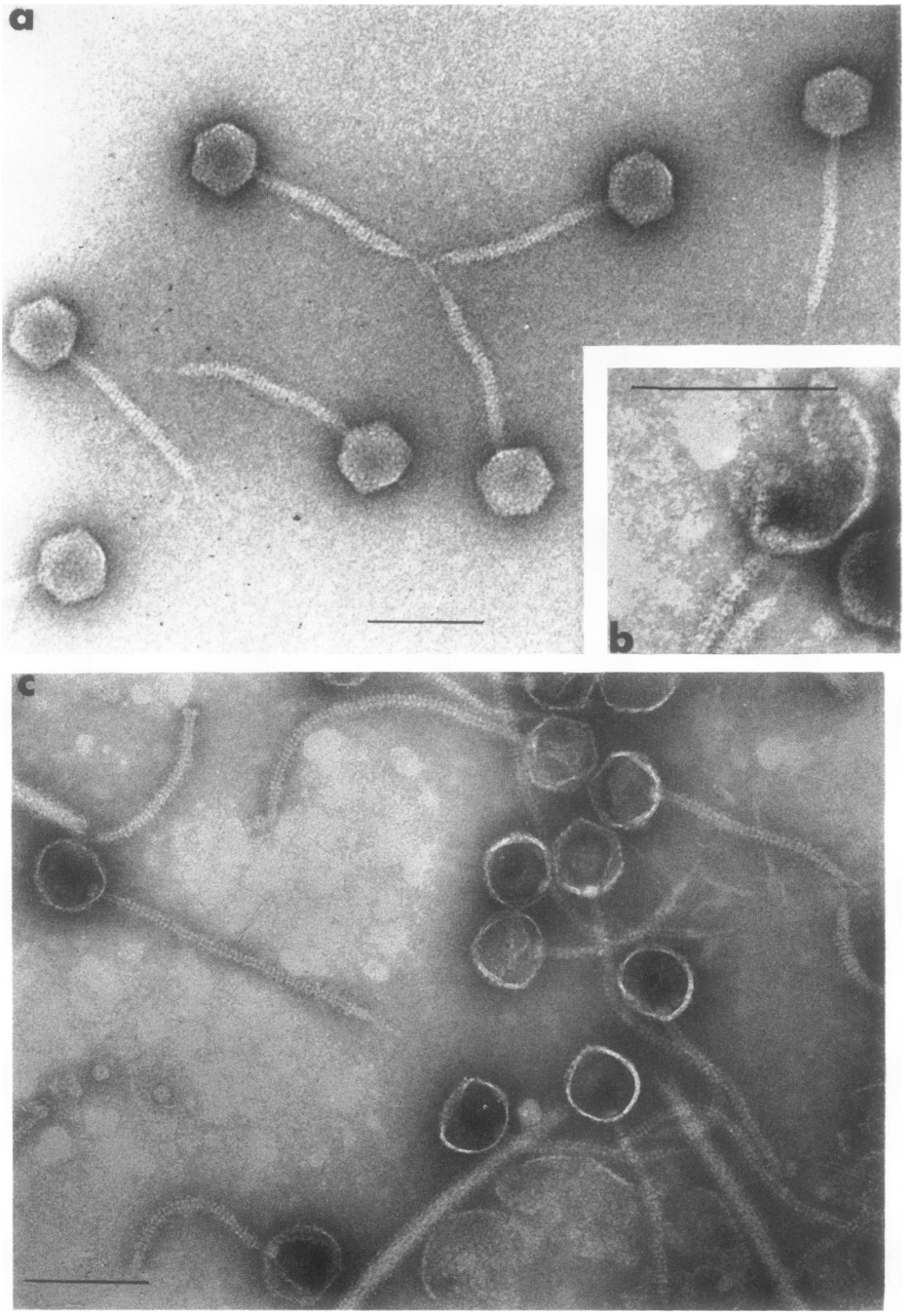


FIG. 4. *After Eiserling and Boy de la Tour (61), with permission of Karger, Basel/New York. (a) Coliphage λ, PTA, × 150,000, scale 1,000 A. (b) Disrupted head of phage λ showing head capsomeres, PTA, × 275,000, scale 1,000 A. (c) Phage λ with empty heads, PTA, × 150,000, scale 1,000 A.*

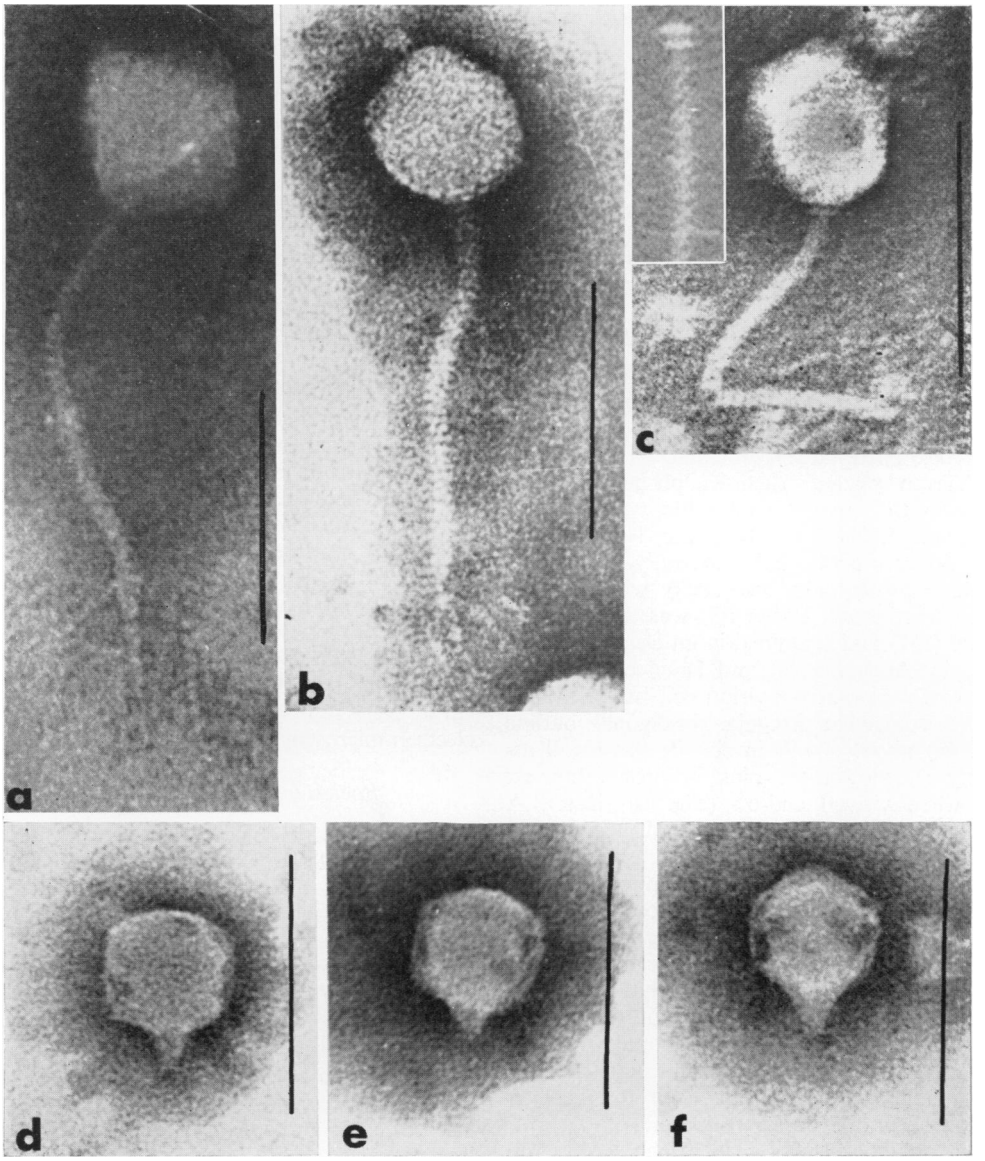


FIG. 5. (*a*) *Coliphage WAK/2 (17), PTA, × 333,000, scale 1,000 A.* (*b*) *Coliphage γ 2 (17), PTA, × 333,000, scale 1,000 A.* (*c*) *Coliphage ZG3A (17); inset, headless tail; PTA, × 333,000, scale 1,000 A.* (*d*), (*e*), (*f*) *Coliphage T3, PTA, × 333,000, scales 1,000 A.*

(Fig. 4c) has a hollow tail exhibiting the capsomere structure more clearly. The head capsomeres (Fig. 4b) are about 90 A in size; unfortunately, it is not possible to see their arrangement clearly and so determine the geometrical form of the head. On the other hand, Bradley (17) has shown that phage WAK/2 has a forked tip and probably an octahedral head (Fig. 5a). Smaller phages in this group have flexible striated tails with a few fine fibers at the tip (Fig. 5b). Another distinctive type has an elongated head, the isolate ZG/3A (17) being shown in Fig. 5c. This is of particular structural interest, since a double disc or collar (Fig. 5c, inset) is found on headless tails. This, presumably, is for attaching the head firmly; the bottom disc of the assembly is visible on the intact virion.

The best known of the coliphages with short noncontractile tails is T3; T7 is similar. They

both have a head with a hexagonal outline often appearing irregular and a wedge-shaped tail (Fig. 5d, e, f). The head shape is not known.

The remaining three forms of coliphages will be described in greater detail below. They are: the tail-less phages with large capsomeres, such as ϕX174 (193) which is in the form of an icosahedron with 12 apical capsomeres; those containing ribonucleic acid (1-RNA), such as f2 (134), which are icosahedra with small capsomeres; and the filamentous types (147).

Bacteriophages of Salmonella

S. typhi bacteriophages are active against other species of *Salmonella* and also against *E. coli*, *S. marcescens*, and other *Enterobacteriaceae* There are representatives similar to the T-even phages, such as phage 66t (15), and also the smaller contractile type ϕ2 (27) which resembles E1. There are isolates with long noncontractile tails generally similar to coliphages, but only one (P22) with a short noncontractile tail. Phage P22 was described by Levine (131) and examined in an electron microscope by Anderson (6) and Israel et al. (100). It can be seen that the virion consists of a head with a somewhat irregular hexagonal outline (Fig. 6a) about 600 A in size. It has a tail assembly showing three pins in profile but six pins when viewed end-on (Fig. 6a, inset). A short tube attaches it to the head. The sixfold radial symmetry of the tail plate suggests the presence of a sixfold axis of symmetry in the head, which rules out either an icosahedron or an octahedron for the head shape unless the tail is attached to the center of a triangular face, which is unlikely. Some other shape, perhaps resembling a shortened version of the T-even phage head, is thus likely, assuming that the rules of symmetry are obeyed and that the short tube joining the sixfold plate to the head is not some sort of symmetry adaptor.

The only tail-less phage which grows on *Salmonella* is the ϕX174 type with large capsomeres, phage ϕR (112); it also grows on strains of *E. coli*.

Bacteriophages of Serratia and Aerobacter

The phages of these two genera are generally similar to those of *Escherichia* and *Salmonella* with one or two exceptions. *S. marcescens* has representatives of all the tailed forms, including one identical to the T-even bacteriophages, and most notably a massive type, probably the largest bacteriophage so far isolated. The two isolates described (19), numbered SMP and SM2, have heads some 1,350 A in size with an irregular six-sided outline (Fig. 6b). The massive tails (2,350 A $\times$ 275 A) are contractile. In the case of SM2, the tail is surrounded by many curly filaments (Fig. 7) believed to be unwinding strings of tail capsomeres, rather than functional tail fibers as with the T-even phages. The tail of SMP is striated when extended (Fig. 6c) but, as would be expected, changes its appearance when contracted (Fig. 6d). The size of a phage head is best illustrated by considering its volume. In the case of SMP and SM2, it is estimated at about 6×10^8 A^3 (assuming octahedral form), compared with 3.6×10^8 A^3 for T-even phages and 7.4×10^6 A^3 for RNA phages. This evidently highly complex virion contains 2-DNA (by acridine orange staining).

Other isolates described (19) resemble T5 and ZG3A with its elongated head and double adaptor assembly. There is also a form not unlike the *Salmonella* phage P22 shown in Fig. 8a; it has a more complex tail assembly than phage P22.

I have found a similar range of phages for *Aerobacter aerogenes*, except for the ZG3A and P22 types. The isolates seem to differ only in dimensions, though the T-even forms are identical in every respect, as far as can be seen in the electron microscope.

Bacteriophages of Pseudomonas

All the morphological types except tail-less phages with large capsomeres (group D) are represented in this genus. The contractile ones are similar to the smaller coliphages and appear to have octahedral heads (16, 24, 64, 195). A particularly good example is shown in Fig. 8b, c. This isolate (12 S, growing on *P. syringae*) shows the facets of the octahedron remarkably clearly, and there is no doubt that the tail is attached to an apex. There are the usual cross-striations and a few fine fibers at the tail tip. The structure and infective process of a typical isolate specific to *P. aeruginosa* will be described in detail below.

Pseudomonas phages with long noncontractile tails exhibit a fairly varied morphology. It is usually the tail tip which is distinctive, having anything from a few fine fibers to a knob (Fig. 8e) or something resembling a cross (Fig. 8d). The isolate shown in Fig. 8d (24) also has an elongated head and is not unlike its coliphage counterpart 2G3A (17). Phages with short noncontractile tails are particularly common and are identical in appearance to the coliphage T3. A few isolates with a complex fibrous tail have been found (16), a typical one, numbered 12B, being illustrated here (Fig. 9a). It is to be noted

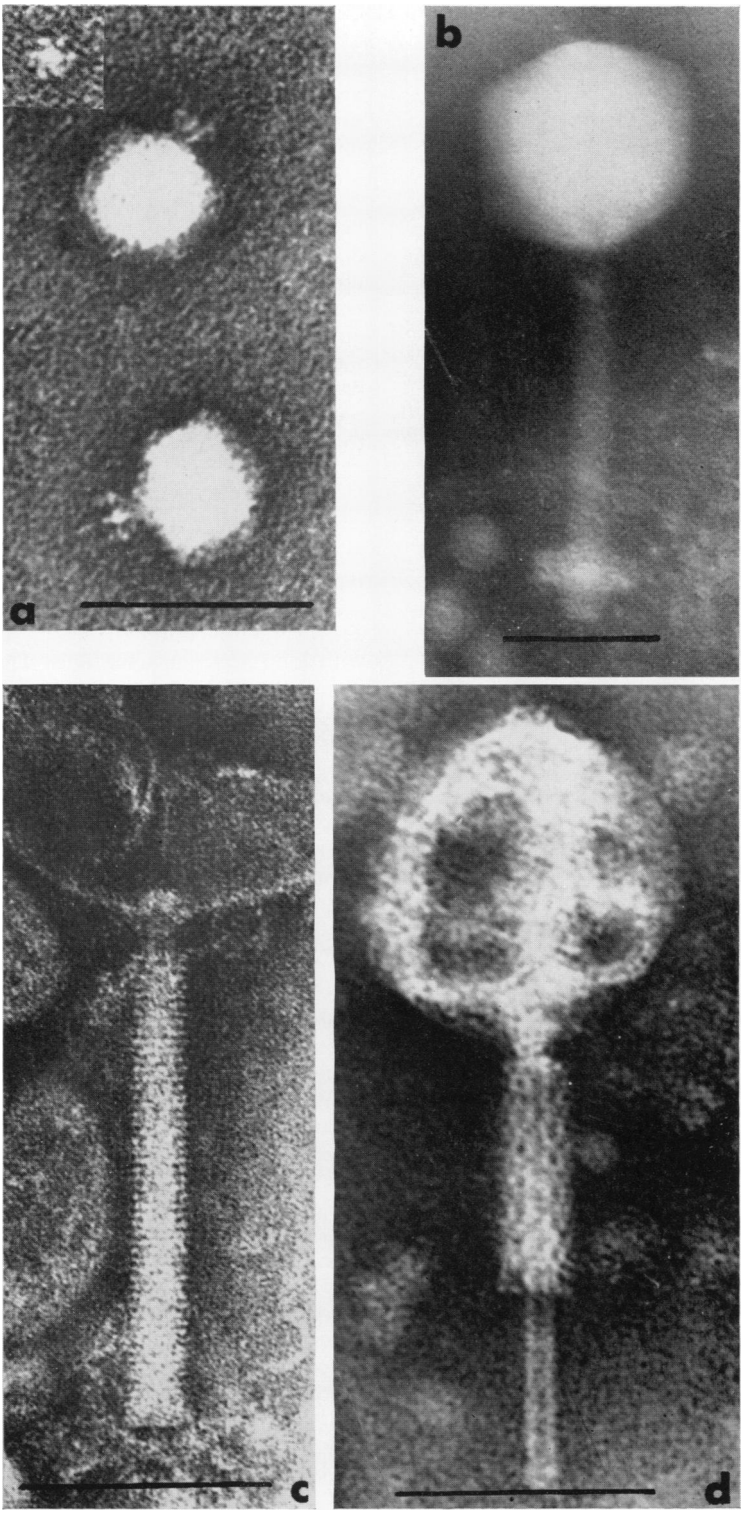


FIG. 6. *(a) Temperate Salmonella phage P22, inset end-on base-plate, PTA, × 300,000, scale 1,000 A, after T. F. Anderson (6), with permission of De Nederlandse vereniging voor Electronenmicroscopie. (b) Serratia marcescens phage SMP (19), PTA, × 200,000, scale 1,000 A. (c) Phage SMP showing tail striations (19), PTA, × 333,000, scale 1,000 A. (d) Phage SMP with contracted sheath (19), PTA, × 333,000, scale 1,000 A. (b), (c), (d) With permission of J. Appl. Bacteriol.*

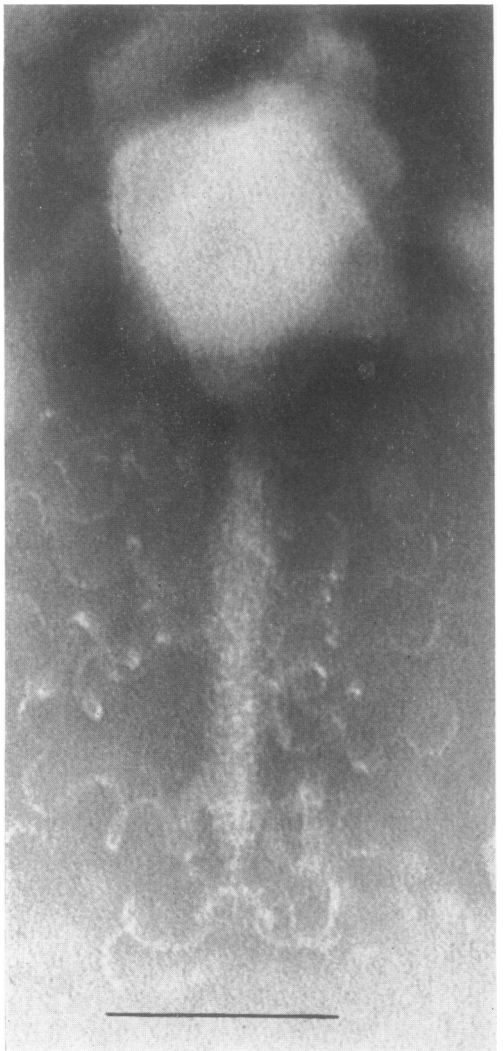


FIG. 7. *Serratia marcescens phage SM2 (19), PTA, × 300,000, scale 1,000 A; with permission of J. Appl. Bacteriol.*

that the two morphologically similar phages in the micrograph are of different sizes. This unexplained phenomenon has been encountered in some *Salmonella* phages (6) and in a *Streptococcus* phage to be described below. In Fig. 9b, two virions of this type (12B) can be seen to have absorbed to a piece of debris and ejected their nucleic acid. The empty particles now have hollow tails, as is the case with all tailed phages. Empty and full virions are shown at higher magnification in Fig. 9c and d.

So far, only two phages with no tails and small capsomeres (containing 1-RNA) have been isolated for *P. aeruginosa*, and neither attacks other species in the genus (24, 64). They are generally similar in appearance to other RNA phages, but their infective process has some unusual features which will be described.

Pseudomonas is the only genus other than *Escherichia* for which filamentous phages have been isolated (207). Their length is 16,000 A and diameter about 75 A; the ends of the filaments taper to a point (Fig. 9e). These phages tend to wind round one another to form ropes. The virion consists of about 12% of 1-DNA. As yet, there is no information about the infective process of this phage, but it is likely to be as remarkable as that of the filamentous coliphages to be described below.

Bacteriophages of Stalked Bacteria

Electron microscopic studies by Schmidt and Stanier (186) have revealed two morphological types of phages active against species of the genera *Caulobacter* and *Asticcacaulis* (173). There are no contractile types, but two forms with long noncontractile tails. The *Asticcacaulis* phage ϕAc20 is conventional with a hexagonal head and a simple tail with no terminal appendage, but the *Caulobacter* phage ϕCb13 has an elongated head and a short tail (Fig. 10a). Other isolates are tail-less with small capsomeres and contain RNA (Fig. 10b). They are similar in appearance to the RNA coliphages and *Pseudomonas* phages. Little is known about their infective process save that they adsorb to polar pili (185).

Staphylococcus and Streptococcus Bacteriophages

Staphylococcus phages are of particular interest because they have been widely used for typing strains of *S. aureus* and other species (219). Contractile and noncontractile phages with long tails are represented. The former are large complex virions (129). The noncontractile types are often conventional in form (like coliphage T5) but with knobs on the tips of their tails (16), however, many isolates have been found which have elongated heads and very long tails (Fig. 10e). The tail tips have a sixfold star-shaped assembly (Fig. 10c, d). In the empty virion (Fig. 10e) the tail is hollow, and in the full ones (Fig. 10d) it is filled as with other tailed phages.

The *Staphylococcus* phages studied by Bradley (16) are of taxonomic interest, since their morphological and serological groupings correspond (see Table 4); a small morphological difference such as tail length is reflected in a different serological grouping.

A *Streptococcus lactis* phage is included here because it shows a feature of particular interest:

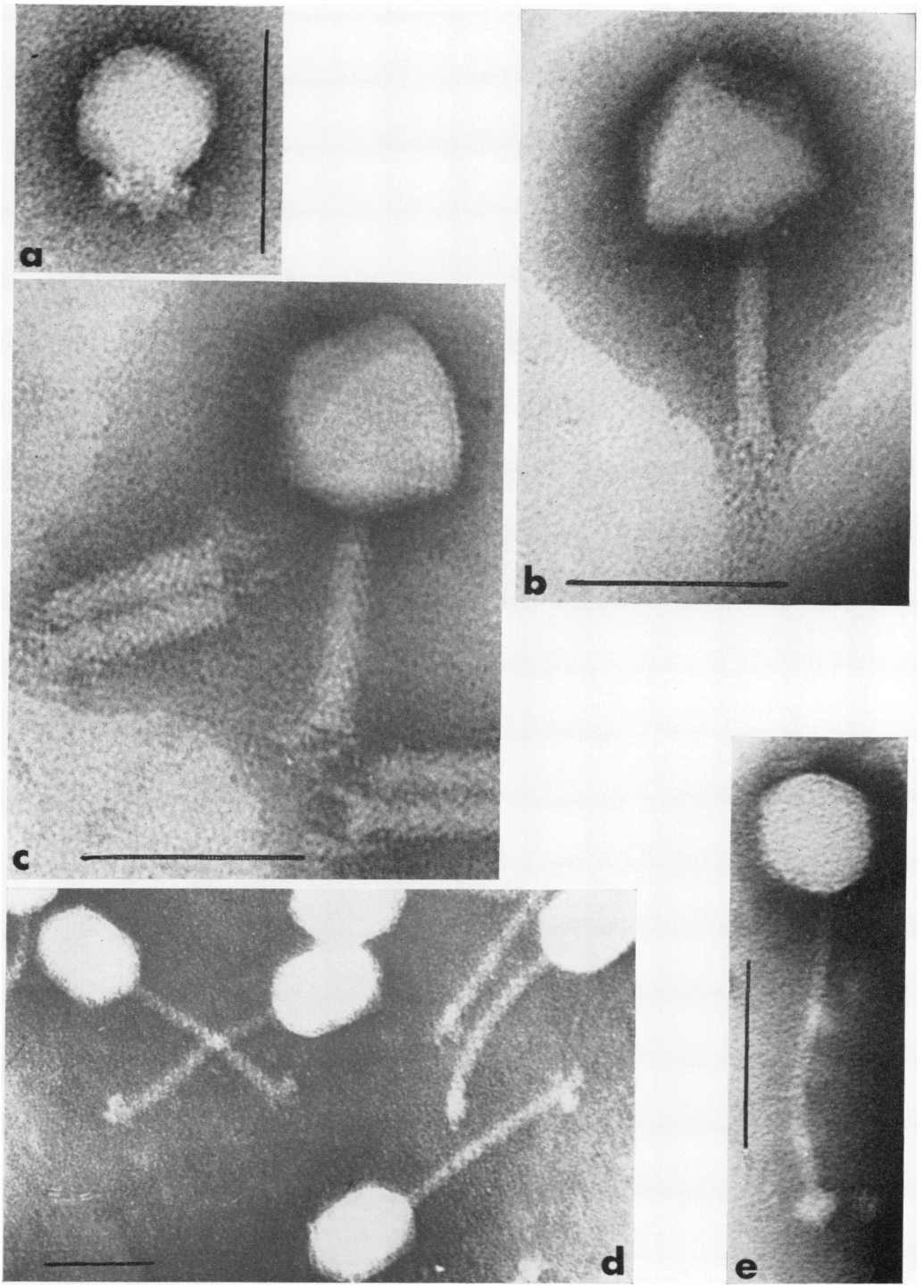


FIG. 8. *(a) Serratia marcescens phage SM4, PTA, × 333,000, scale 1,000 A; with permission of J. Appl. Bacteriol. (19). (b), (c) Pseudomonas syringae phage 12S (16), PTA, × 333,000, scales 1,000 A; with permission of J. Ultrastruct. Res. (d) P. aeruginosa phage PB-2 (24), PTA, × 165,000, scale 1,000 A. (e) P. aeruginosa phage Pc (24), PTA, × 275,000, scale 1,000 A.*

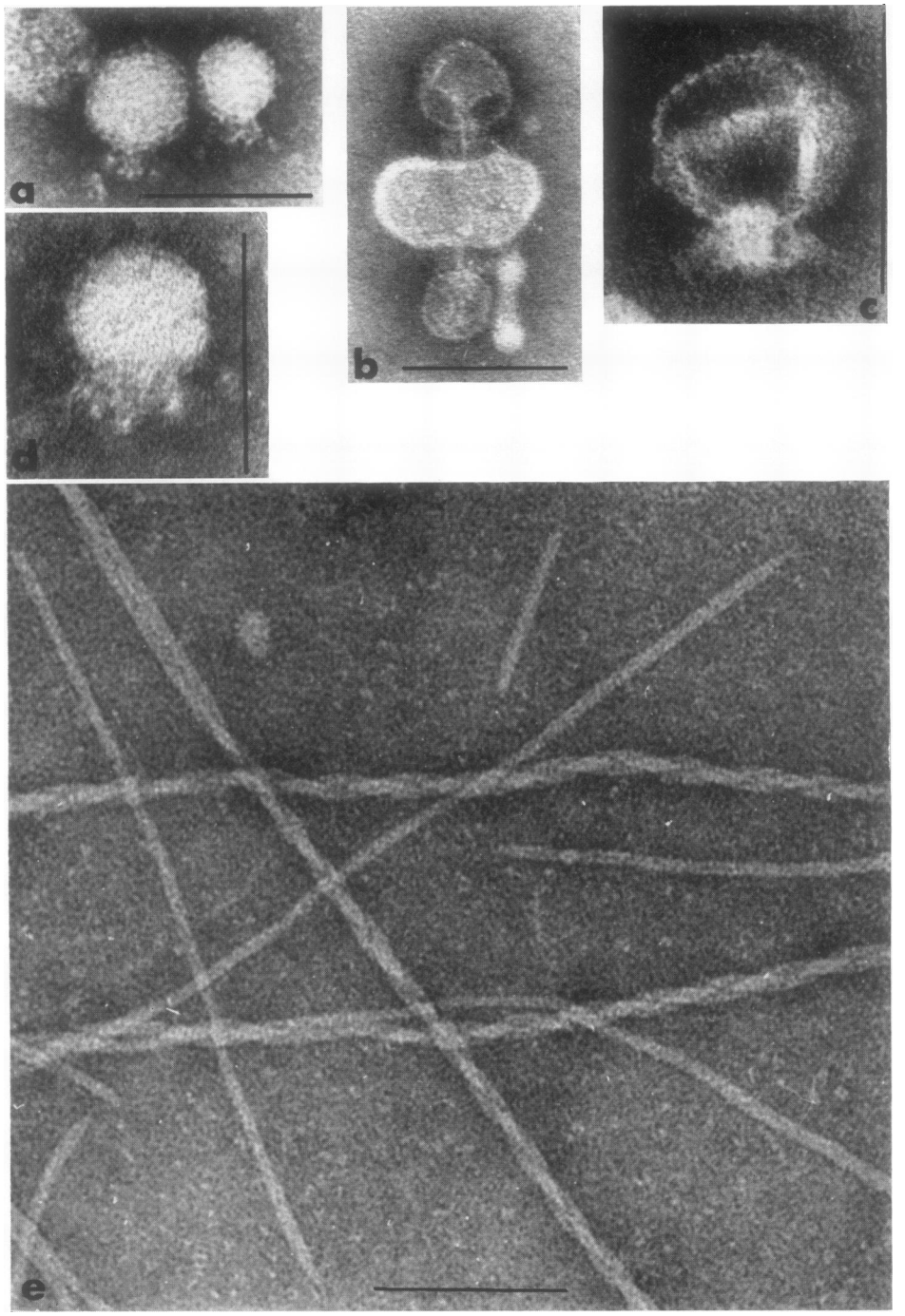


FIG. 9. *(a), (b), (c), (d) Pseudomonas syringae phage 12B (16), PTA, scales 1,000 A. (a), (b) Head dimorphism, × 230,000. (c) Empty virion, × 380,000. (d) Full virion, × 333,000. (e) The filamentous P. aeruginosa phage, PTA, × 265,000, scale 1,000 A; after Takeya and Amako (207), with permission of Virology, Academic Press, Inc. (a), (b) With permission of J. Ultrastruct. Res.*

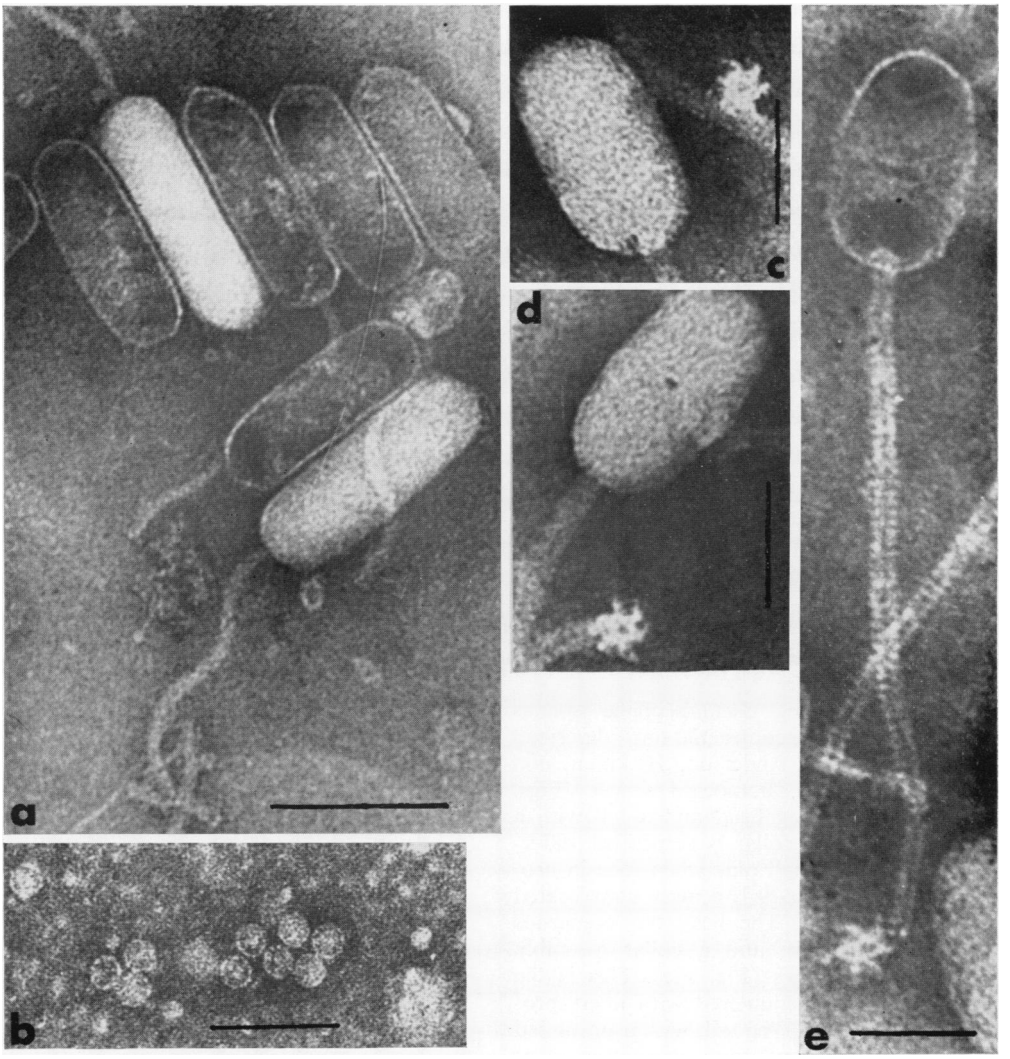


FIG. 10. *(a) Caulobacter phage φCb13, PTA, × 230,000, scale 1,000 A, after Schmidt and Stanier (186) (b) RNA Caulobacter phage φCb8r, PTA, × 175,000, scale 1,000 A; after Schmidt and Stanier (186). (c), (d), (e) Staphylococcus phage 594n, PTA, × 333,000, scale 500 A; with permission of J. Ultrastruct. Res. (16).*

head dimorphism, already encountered in *Pseudomonas* phages. In Fig. 11a, it can be seen that the head of one virion is twice as long as the other. The tails are long and noncontractile, without any appendage.

Bacteriophages of Bacillus

Species of the genus *Bacillus* are attacked by the three morphological types of tailed phage. The contractile forms are large and provide some of the few examples where head capsomeres are visible (61). In Fig. 11b, which illustrates a *B. subtilis* phage (SP50), they show up as a more or less regular mottling on the capsid, and they are also visible on the head of the intact virion in Fig. 11c. In neither case are they clear enough to allow a positive identification of the head shape by their arrangement, though Eiserling and Boy de la Tour (61) favor an icosahedron. This is supported to some extent by an apparently five-pronged base plate (Fig. 11c) which would correspond to the fivefold axis of the head. On the other hand, the large triangular faces of the virion tend to suggest an octahedron. Similar

TABLE 4. *Morphological and serological groups of Staphylococcus phages*[a]

Basic morphology	No.	Head dimensions	Tail length	Serological group
		A	A	
Long noncontractile tail, symmetrical head	77	550	2,200	F
	P42D	550	2,300	F
	581	550	2,400	F
	187	600	1,700	L
	71	500	1,500	B
	P52A	500	1,500	B
	52	500	1,500	B
Long noncontractile tail, elongated head	3B	800 × 600	3,000	A
	6	920 × 400	3,000	A
	594n	960 × 550	3,000	A
	70	980 × 530	3,000	A

[a] The serological grouping is that of the *Staphylococcus* and *Streptococcus* Reference Laboratory, London, England. Measurements were made from phosphotungstate preparations.

phages have been described by Davison (53) and Bradley (18).

The most interesting *Bacillus* phage isolates are two with short noncontractile tails, described by Bradley (18) and Anderson, Hickman, and Reilly (4). The first, numbered GA/1 (Figs. 12a, b), has an oblong head and a tail with a collar around it; the collar is sometimes differentiated into three small pins when seen in profile (Fig. 12b). Phage ϕ29 is a more complicated version (Fig. 12c, d). The head has numerous fine projections on it, and a collar assembly with twelve appendages (Fig. 12c). This is the only phage so far encountered with fibers projecting from the head. The tail, with a presumed 12-fold radial symmetry, could well reflect a 6- or 12-fold symmetry on the long axis of the head.

The only known tail-less *Bacillus* phage, numbered $\phi\mu$-4, has been described by Shafia and Thompson (190). It is only about 100 A in size and exhibits no obvious structure in shadowed preparations (Fig. 13). Although there appears to be little doubt that it is a true bacteriophage, little is known about it.

Bacteriophages of Other Genera

The species of many other bacterial genera have phages active against them, but they are too numerous to describe in detail. Some of the more important have been selected in Table 5, which gives their morphological types and references.

INFECTIVE PROCESS OF BACTERIOPHAGES

The infective process of a virulent phage can be conveniently divided into four stages: adsorption, injection, intracellular multiplication, and lysis. All but the injection stage can be studied in an electron microscope. Adsorption consists of the attachment of the virion to the surface of the cell or to some appendage on it; most phages adsorb to the cell wall, but the electron microscope has shown that some infect via pili (33, 48), and a noncontractile *Salmonella* phage has been observed to attach to bacterial flagella (154). The *B. subtilis* phage PBS1 forms plaques only on motile strains of the host organism (108), the inference being that it also uses flagella as receptor sites. Phage PBS1 is a contractile form with very thick tail fibers (60). There are thus two kinds of phage receptors, those which are an integral part of the cell wall, and those which are not. Weidel and Kellenberger (216) were able to isolate and purify the receptors for coliphage T5 from cell walls of *E. coli* B and to study their reaction with T5 virions in the electron microscope. The receptors consisted of small spherical molecules which were found attached to the tips of the long noncontractile tails of the phage particles. This prevented the phages from adsorbing to cell walls, and in many cases caused ejection of the nucleic acid. The receptors from the cell wall of the T5-resistant mutant *E. coli* B/1,5 looked the same in the electron microscope but did not attach to the tail tips, nor did they inactivate the phage. It can also be inferred that the site of attachment of the phage lies at the tail tip. This has also been observed directly in the electron microscope for a number of tailed phages.

The injection stage cannot usefully be studied in the electron microscope. All that can be seen is empty or half-empty phage heads and nothing can be learned about the mechanism which causes the nucleic acid to be ejected. Nevertheless, Weidel and Kellenberger's experiment does suggest that the energy for ejection lies within the virion itself rather than in the bacterium, since the adsorption of single receptor molecules initiates it.

More precise information has been obtained by methods other than electron microscopy. Lanni (126), using ^{32}P-labeled T5 bacteriophage, found that only about 10% of the phage DNA was transferred rapidly to the host cell; the remainder proceeded much more slowly. The first portion of the DNA, known as first-step-transfer material, consisted of a segment of the

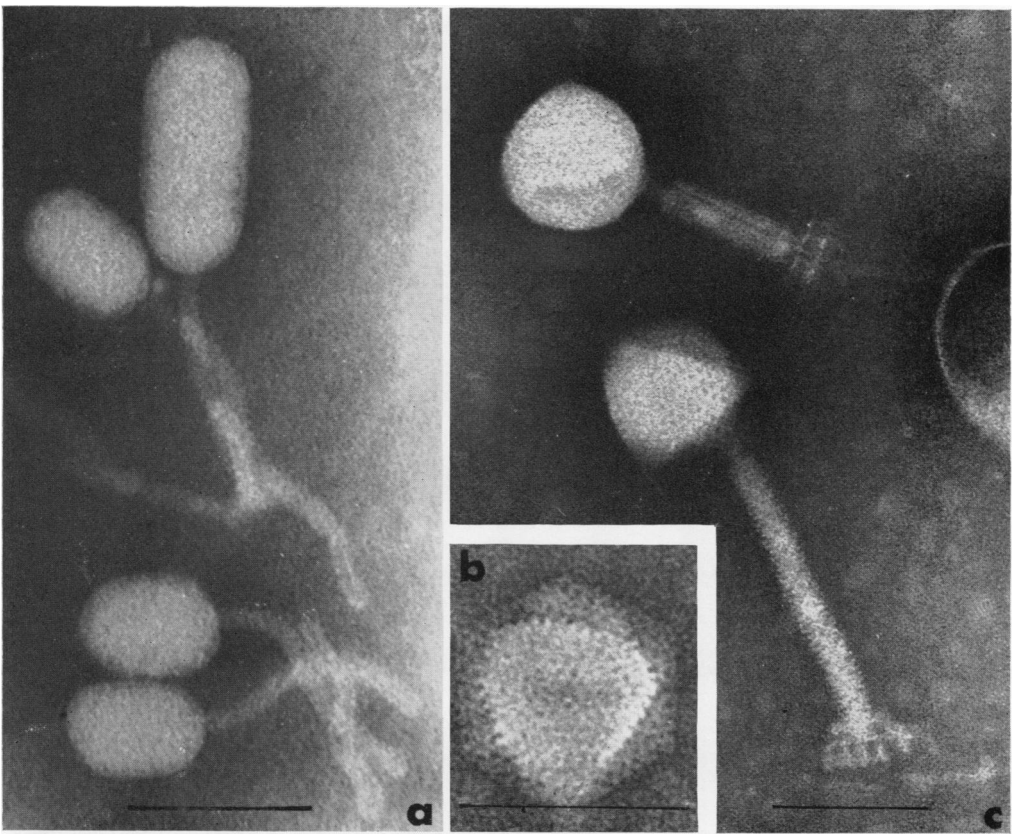


FIG. 11. (*a*) *Streptococcus phage 3ML, showing different head sizes (16), PTA, × 235,000, scale 1,000 A; with permission of J. Ultrastruct. Res.* (*b*), (*c*) *Bacillus subtilis phage SP50, PTA; after Eiserling and Boy de la Tour (61), with permission of Karger, Basel/New York.* (*b*) *× 300,000, scale 1,000 A.* (*c*) *× 200,000, scale 1,000 A.*

phage DNA molecule (128, 152), which passed into the host cell in the absence of protein synthesis. The remainder transferred only after fresh protein synthesis within the bacterium. Lanni (127) suggested that the first-step-transfer material contained the genetic information required for this protein synthesis. It is thus evident that nucleic acid injection, at least in the case of bacteriophage T5, is a complex process involving energy from both phage and bacterium and is much more than a single-step transfer of the phage nucleic acid molecule.

Intracellular multiplication has been examined in comparatively few cases by use of ultrathin sections. In the T-even phages, changes in the nucleoplasm have been observed (119) prior to the formation of mature intracellular phages. With RNA phages, crystals of virions are formed prior to lysis (188). With *P. aeruginosa* RNA phages, this is accompanied by spheroplast formation (24). With filamentous coliphages, multiple cell wall and cell membrane invaginations are produced (26). In the case of the tailed mycobacteriophage B-1, Takeya et al. (208) reported the presence of empty phage heads within the host cell prior to lysis, a feature not observed with T2 (119). Clearly, phage infection can initiate a variety of cytological changes in a bacterium, but the significance of many such observations is at present uncertain.

The release of vegetative phages is usually achieved by lysis of the host cells, an event which can be observed in thin sections as well as in negatively stained preparations. Usually, the bacterium merely ruptures, releasing virions into the surrounding medium together with the remains of its contents (Fig. 14, *S. aureus*). In *E. coli* cells infected with T2, lysis is caused by the cessation of cell wall synthesis accompanied by the synthesis of lysozyme (45). It is

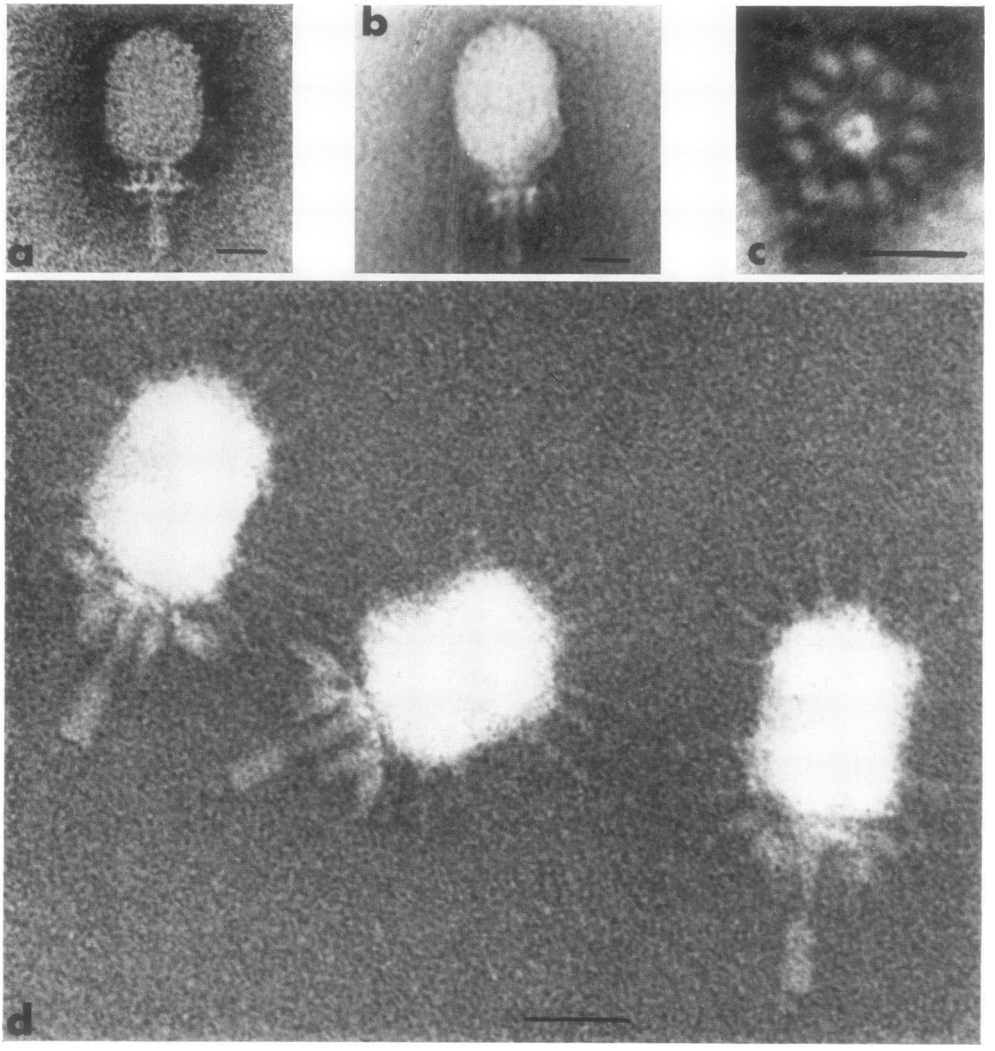


FIG. 12. *(a), (b) Bacillus subtilis phage GA-1 (18), PTA, × 333,000, scales 200 A. (c), (d) B. subtilis phage φ29, PTA, × 700,000, scale 200 A; after Anderson, Hickman, and Reilly (4), with permission of J. Bacteriol. (c) Tail end-on. (d) Intact virions.*

thought that *P. aeruginosa* cells burst by osmosis when lysed by their RNA phages (24).

Clearly, there is a great deal of variation in the infective processes of the different morphological types, and the following section describes the more important of these in detail.

MOLECULAR STRUCTURE AND INFECTIVE PROCESSES OF THE MORE IMPORTANT BACTERIOPHAGES

Structure of the T-even Phages

The three T-even phages, T2, T4, and T6, are morphologically similar and belong to the contractile group (Fig. 1A). Other morphologically identical phages are known for the principal species of the *Enterobacteriaceae*. Electron microscopy has provided a nearly complete picture of their structure.

The 2-DNA core of the head consists of a single molecule 49 μ long (Fig. 15) when spread on a Langmuir trough (124); the two free ends of the filament can be seen. It is not known for certain how it is coiled within the head, but the use of uranyl formate negative staining (67) shows a curious whorled appearance which could represent the arrangement of the DNA (Fig. 16a) since a similar structure is not visible on empty heads. A comparison of the volume

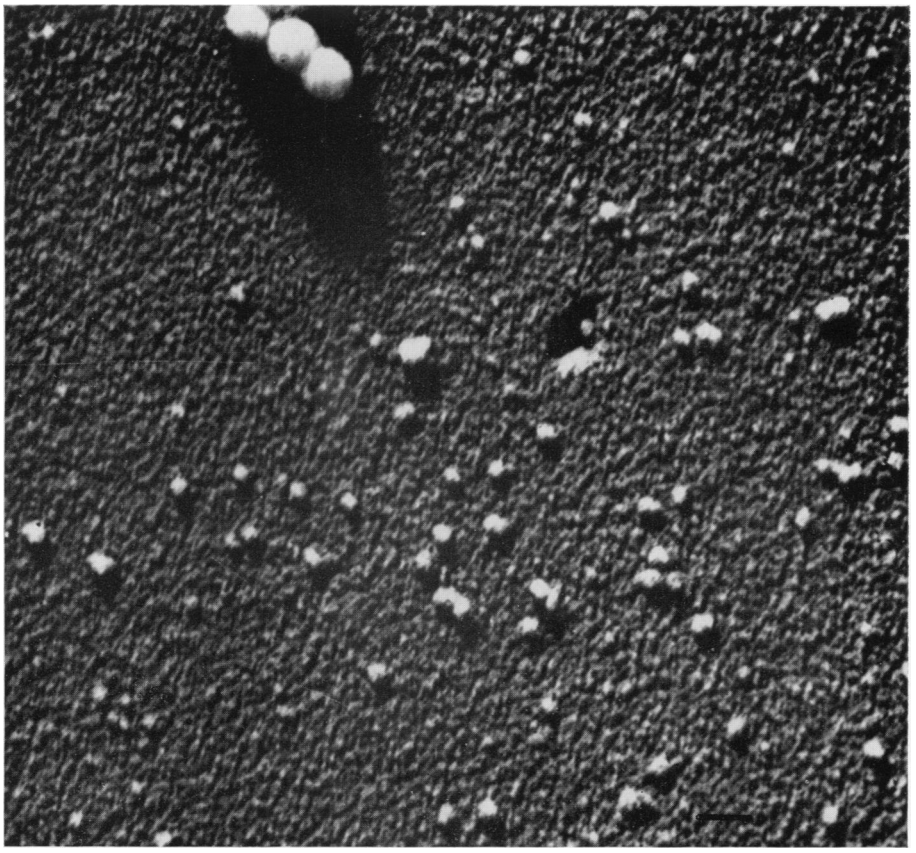


FIG. 13. *Bacillus phage $\phi\mu 4$ with 880 A latex particles, shadowed, × 60,000, scale 1,000 A, after Shafia (190), with permission of J. Bacteriol.*

TABLE 5. *Morphological types of miscellaneous bacteriophages*

Host species or genus	Phage no.	Tail type	Group (Fig. 1)	Reference
Agrobacterium radiobacter	PR-1001, PS-192, PR-590a, PsR-1012	Short, noncontractile	C	179
Alcaligenes faecalis	A64/A62, 8764	Long	B	140
A. faecalis	A11/A79, A6	Contractile	A	140
Brucella	About 25 isolates	Short, noncontractile	C	32, 167, 168
Chondrococcus columnaris	—	Contractile	A	122
Clostridium perfringens	80	Short, noncontractile	C	215
Corynebacterium diphtheriae	B, BL,	Long, noncontractile	B	149
Flavobacterium	NCMB 385	Long, noncontractile	B	20
Lactobacillus	—	Contractile and long, noncontractile	A	54
Listeria monocytogenes	—	Long, noncontractile	B	205
Mycobacterium	B1	Long, noncontractile	B	206
Myxococcus xanthus	MX-1	Contractile	A	35
Proteus	28 isolates	Contractile, long and short noncontractile	A B C	174
Providencia	PL25, PL26, PL27	Short, noncontractile	C	42
Saprospira grandis	—	Contractile	A	133
Streptomyces	K_1, R_1, R_2	Long noncontractile	B	47
Vibrio	Groups I & III	Short, noncontractile	C	52
	Group II	Contractile	A	
	Group IV	Long, noncontractile	B	

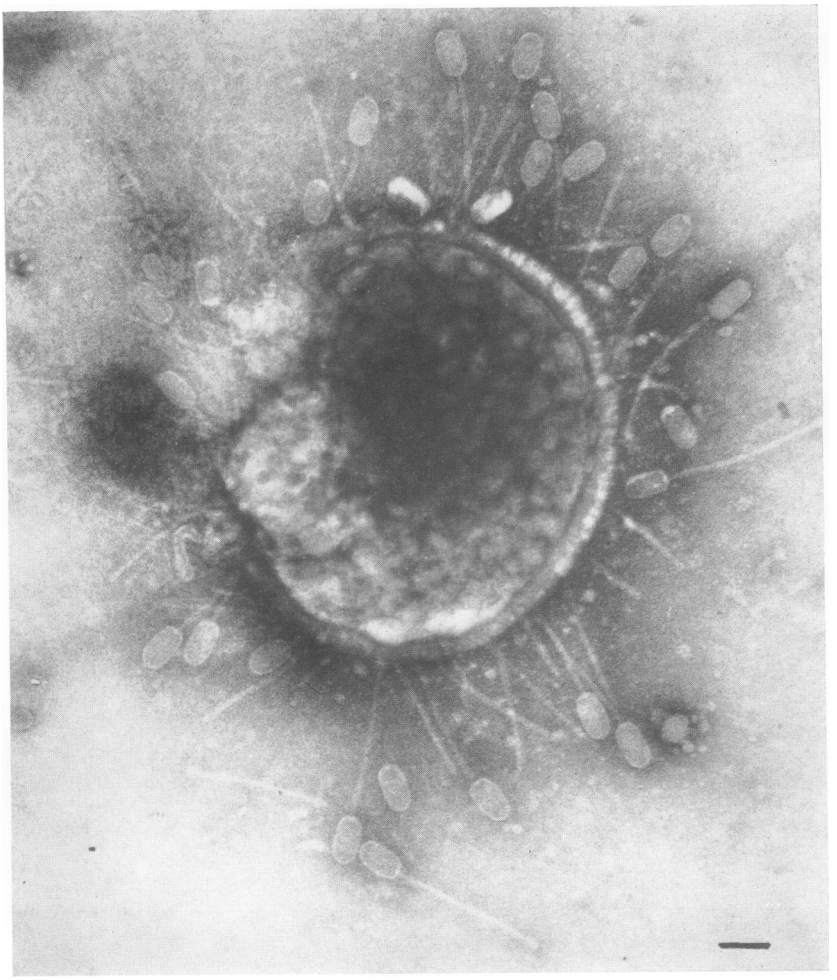


FIG. 14. *Bacteriophage 594n lysing Staphylococcus aureus (20), PTA. × 68,000, scale 1,000 A. With permission of the Royal Microscopical Society.*

of the head with that of the DNA within it is relevant to this observation. Although the figure of 3.58×10^8 A^3 for the head volume, based on a bipyramidal hexagonal prism, is only approximate because the exact head shape is not known, the various other forms to be mentioned below will not depart greatly from this volume. That of the DNA is about 1.55×10^8 A^3, based on a 20 A diameter filament. Thus, only about 43% is occupied, a low percentage, suggesting a loose coil. The head capsomeres have only been resolved after the degradation of the head (171). They are about 50 A in size and have a sedimentation coefficient of 6.2*S*. Since they have not yet been resolved on the intact head, it is difficult to be certain of the geometrical form of the capsid. Williams and Fraser (217) suggested, on the evidence of shadowed virions, that it was in the form of a bipyramidal hexagonal prism. More recently, negative staining has provided two possible alternatives. In the first, Bradley (21) suggested that a more likely shape was a distortion of Williams and Fraser's model formed by rotating the two pyramids by 30° with respect to one another (Fig. 16b, c). It can be seen that the edges visible in the electron micrographs correspond with those shown as continuous lines in the superimposed wire model. A cardboard model is shown in Fig. 16d and e, the name of the figure being a bipyramidal hexagonal antiprism. Moody (155) and Boy de la Tour and Kellenberger (12) favor a prolate icosahedron, which is an icosahedron with its middle section extended. Moody's observations are based on theoretical arguments on electron micrographs of aberrant forms of T-even heads. The electron

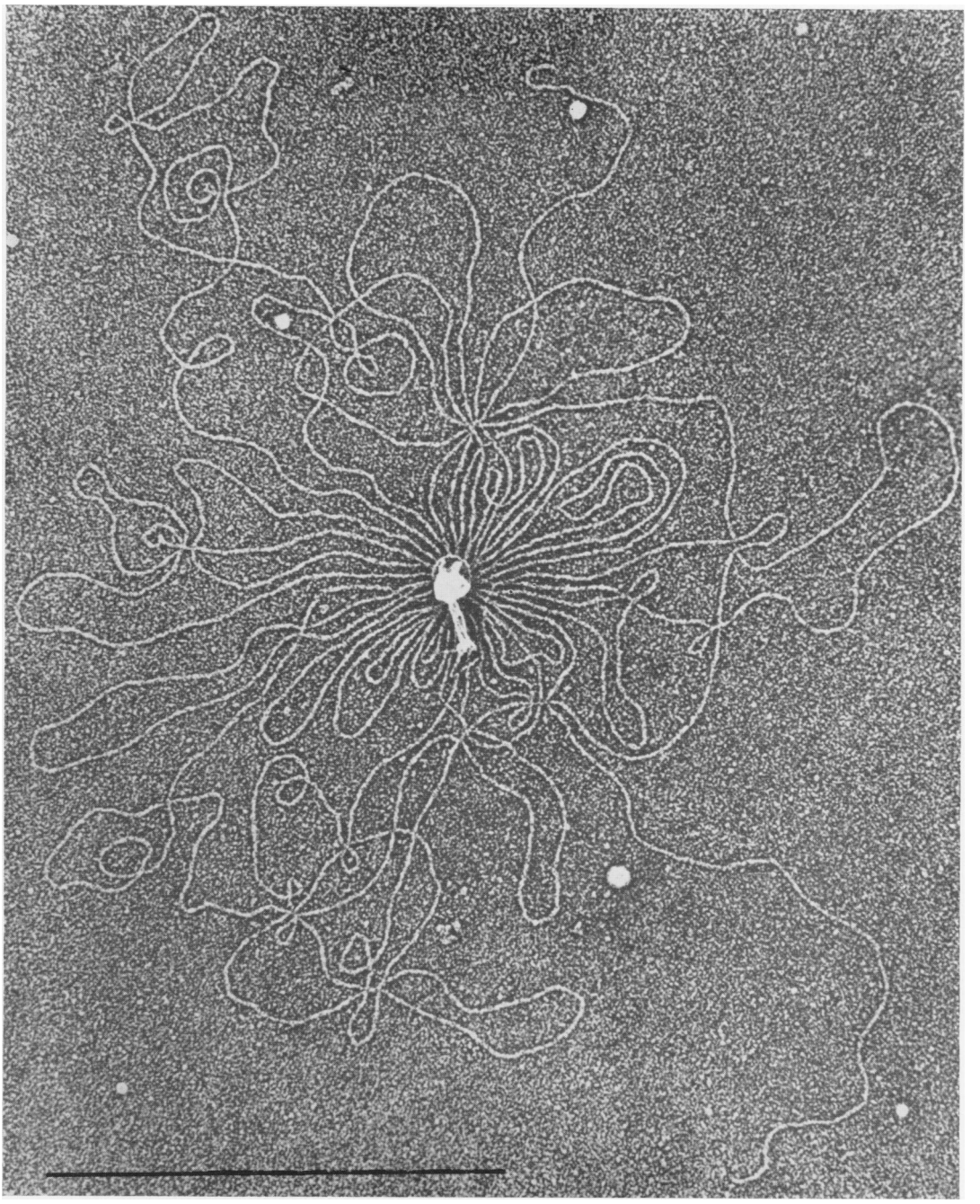


FIG. 15. *Coliphage T2 DNA spread on a Langmuir trough, × 66,000, scale 1 μ, after Kleinschmidt et al. (124); with permission of Biochim. Biophys. Acta.*

micrographs of Boy de la Tour and Kellenberger (Fig. 17a, b) which also show aberrant heads with two tails, support Moody's observations. The main objection to a prolate icosahedron is that a tail having sixfold rotational symmetry would have to be fitted to the fivefold axis of the head. While not impossible, assuming the presence of an adaptor plate, this seems rather

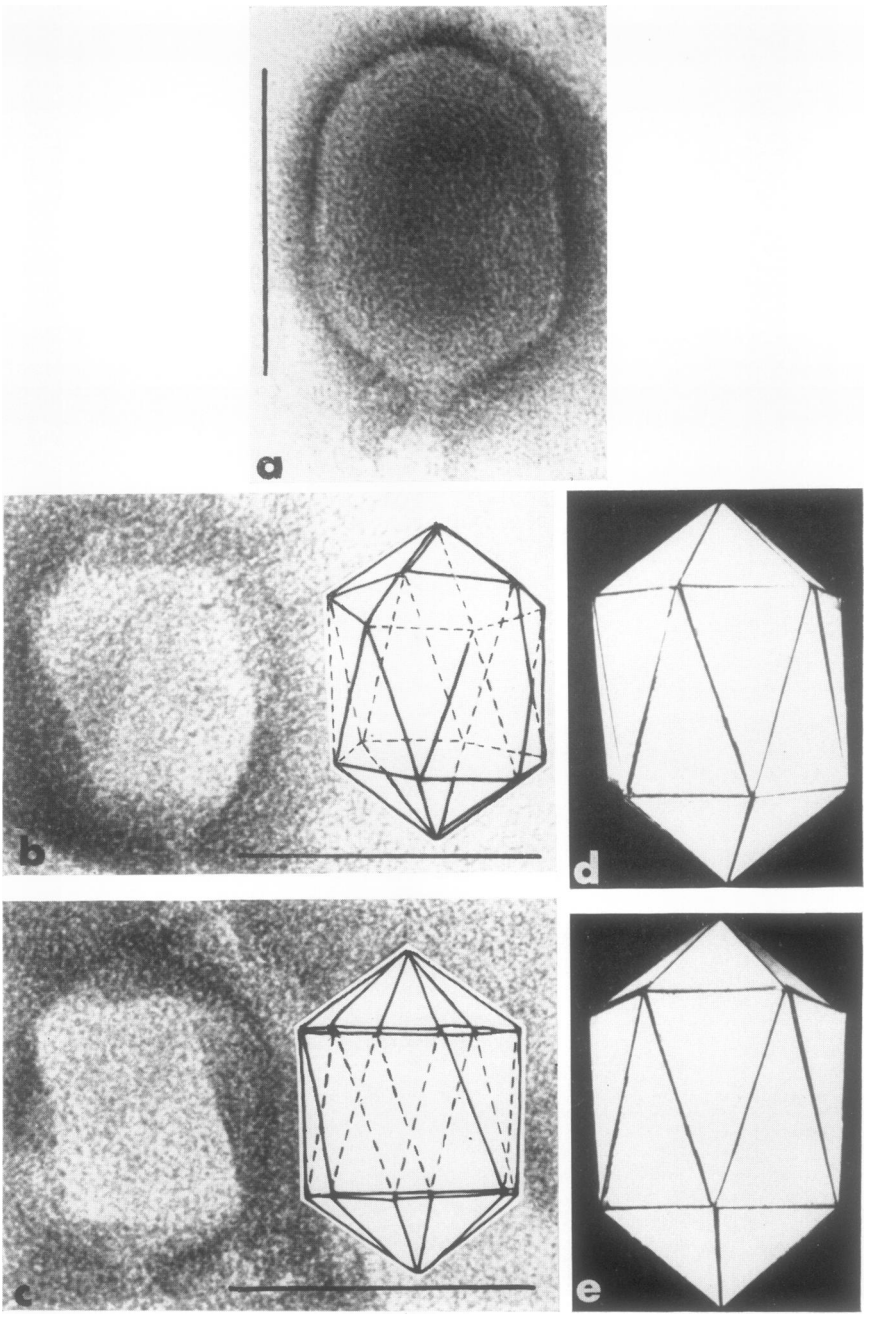


FIG. 16. *(a) Coliphage T4 head negative stained in uranyl formate, × 400,000, scale 1,000 A (21). (b), (c) Phage T2 heads in uranyl acetate, × 380,000, scales 1,000 A (21). (d), (e) cardboard models of T2 heads (21).*

less favorable than having two matching symmetries. There are two schools of thought, one favoring an adaptor and one not. Since both figures proposed differ only in the rotational symmetry of the long axis, its determination would clear up the matter. Sections of phage heads in infected cells tend to produce a hexagonal outline, but this will be the case with both figures. Only an end-on picture of a vegetative particle would be of value. This is difficult to

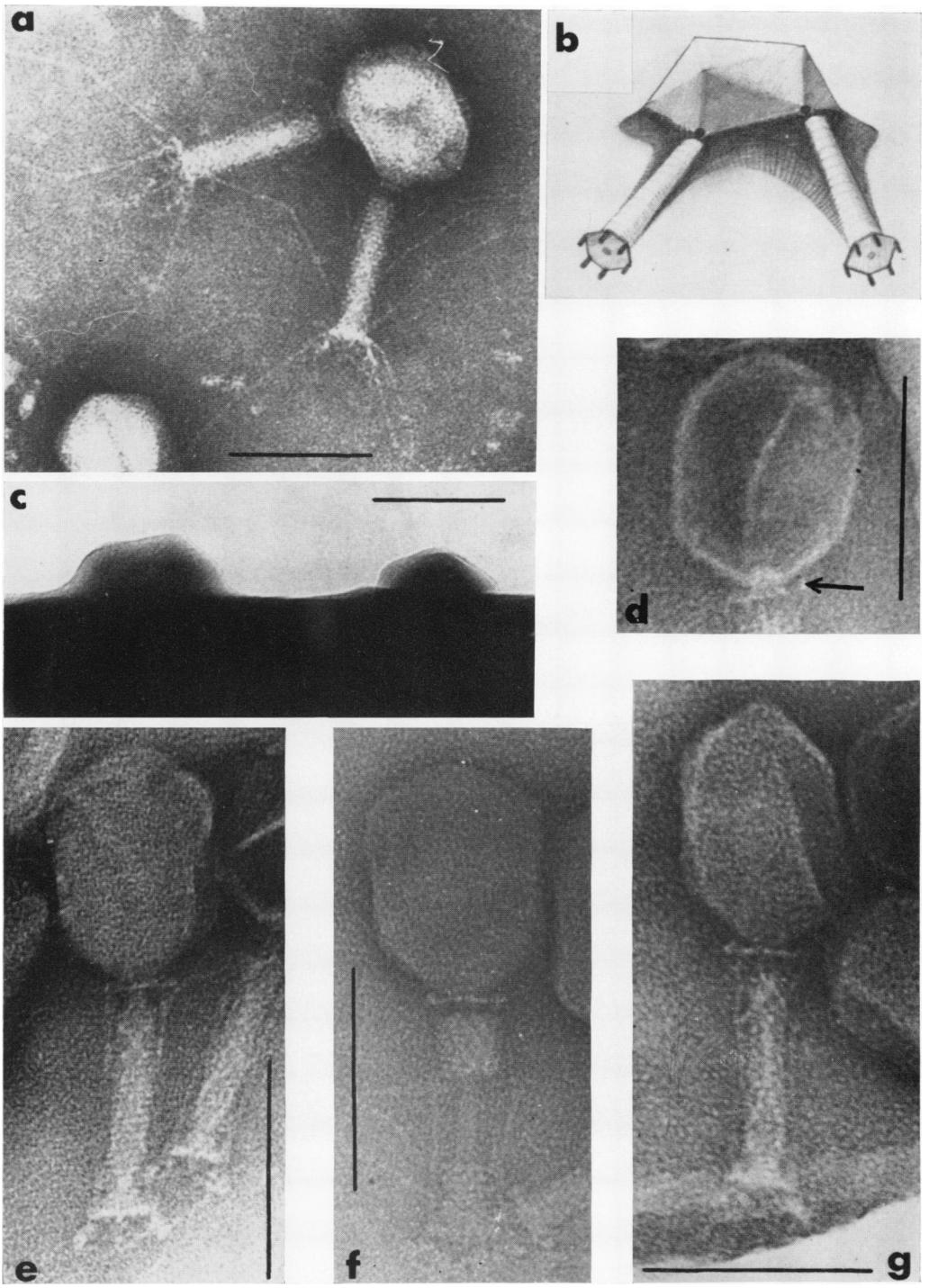


FIG. 17. *(a), (b) Abnormal T-even phage head, PTA, × 210,000, scale 1,000 A, after Boy de la Tour and Kellenberger (12), with permission of Virology, Academic Press, Inc. (c) Profiles of T-even phage heads treated with uranyl nitrate (21), × 200,000, scale 1,000 A. (d), (e), (f), (g) T4 in sodium molybdate (21), × 333,000, scales 1,000 A. (e) With permission of Academic Press, Inc.*

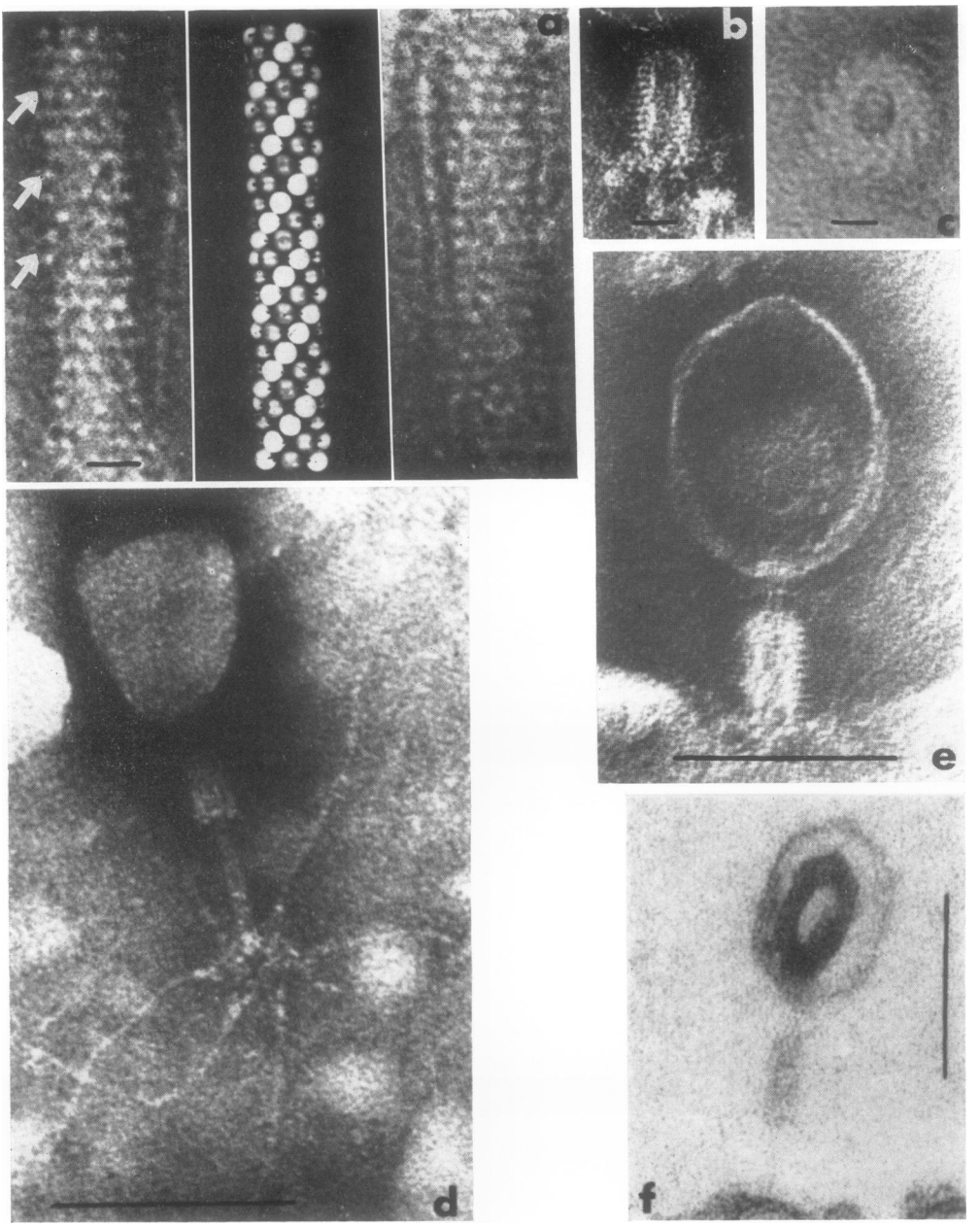


FIG. 18. *(a) Extended sheaths of T-even phages, PTA, × 700,000, scale 100 A; with permission of Academic Press, Inc. (b) Contracted sheath of T-even type phage 66t⁻ (15), PTA, × 333,000, scale 200 A. (c) T4 contracted sheath end-on, uranyl formate, × 600,000, scale 100 A; with permission of J. Roy. Microscop. Soc. (d) Base plate of T-even phage (9), potassium silicotungstate, × 333,000, scale 1,000 A, after Anderson and Stephens; with permission of Virology, Academic Press Inc. (e) Phage T4 adsorbed to cell wall, PTA, × 300,000, scale 1,000 A, after Almeida; with permission of J. Roy. Microscop. Soc. (f) T-even phage adsorbed to cell, section (141), × 250,000, scale 1,000 A; after Councilman Morgan.*

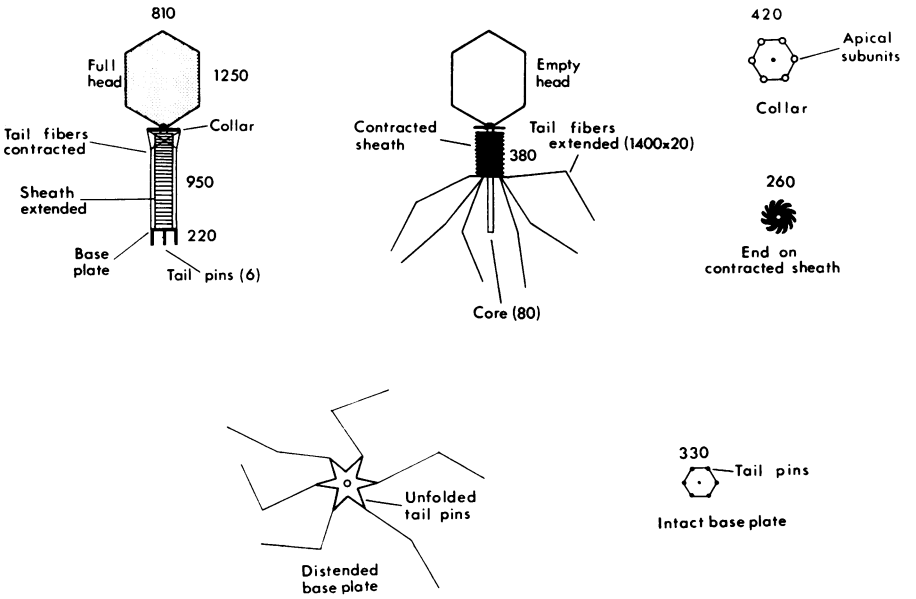


FIG. 19. *T-even phage components with dimensions in A units.*

obtain, the only one of any clarity being shown in Fig. 17c (right-hand particle). Bradley (21) considers that this micrograph suggests a sixfold long axis, though it is not conclusive since only half the virion is visible.

The phage head is joined to the tail at the apex of one of the pyramids. At this point, there is a small plug of protein lying within the head (Fig. 17d, arrow); it is not certain whether or not this acts as a mechanical joint to fix the tail to the head, as is the case with some other phages (28), or whether it is a symmetry adaptor, as suggested by Moody (155). A long (about 1,200 A) thin tube or core extends from this plug to the tip of the tail where it is attached to a platelike assembly. The core, down which the DNA passes on infection, is only 75 A in diameter with a 20 A central hole. The contractile mechanism, which has several components, is assembled round it. Directly beneath the head there is a thin disc or collar (Fig. 17e, f, g) which has a sixfold radial symmetry and possesses hollow subunits at the apices of the hexagon (21); it is only about 15 A thick. Between the collar and base-plate, there is a network of very fine fibers (Fig. 17e, f, g). Under appropriate conditions for phage adsorption, the link between these fibers and the collar breaks and they become splayed out from the base-plate to which they remain attached (29, 51, 116). Within the fibrous net ork lies the important contractile sheath. In the extended state, before adsorption and injection, this consists of a cylinder with 24 cross-striations formed by the arrangement of the capsomeres (Fig. 18a). The model in Fig. 18a, derived from the micrographs on either side, shows that the capsomeres are in a series of annuli with a sixfold radial symmetry. These morphological subunits (diameter, about 30 A) total 144. Some rows of them (arrowed) are whiter and form a coarse helix. The contracted sheath shown in Fig. 18b has a regularly serrated edge with 12 teeth. End-on (Fig. 18c), it appears as a cogwheel structure. Moody has discussed the structure of the sheath in detail (156). There have been a number of suggestions for the way in which the capsomeres rearrange themselves on contraction (21, 116, 117, 157, 172). Probably the most likely is the hypothesis of Moody (157): asymmetric sheath subunits, which form the helical chains of the extended sheath, rearrange themselves into the same number of helices but with a different pitch. This is achieved by changing their orientation. The base of the tail consists of a six-pronged plate which changes into a star-shaped object (Fig. 18d) when the sheath contracts (192).

Figure 19 summarizes these observations diagrammatically.

Infective Process of the T-even Phages

It has long been known that the first stage of the infective process, namely adsorption to the host cell wall, is carried out by the T-even phages tail first (5). Negative staining shows up more detail than the original shadowing, and reveals that the tail fibers turn away from the cell wall after adsorption and sheath contraction (Fig. 18e). It appears, in fact, that the tail fibers perform a necessary function in determining whether or not a phage particle can adsorb: it has been shown that if the tail fibers are extended the phage can adsorb, and if they are retracted it cannot (29, 116). The state of the tail fibers depends on environment, e.g., *p*H or the presence of tryptophan in the case of T4 (29, 51), or indole with T-even phages (29). Thus, the sequence of events is that, under suitable conditions, the tail fibers become extended and the phage attaches to the cell wall. This triggers the contraction mechanism already mentioned (156) and DNA is injected.

Margaretten et al. (141) showed a particularly interesting micrograph of a section through a virion adsorbed to a cell (Fig. 18f), in which the phage has supposedly injected its DNA. However, a ring-shaped structure remains; this could well be a portion of the DNA. The appearance suggests that the nucleic acid is coiled along the long axis of the head and is consistent with the appearance after uranyl formate staining (Fig. 16a).

A recent detailed study of adsorption and penetration has been made by Simon and Anderson (191), who studied adsorbed virions both sectioned and negatively stained. They were able to show that the initial attachment was made by the ends of the tail fibers. Next, the base-plate was brought up to the cell wall and sheath contraction occurred, the core penetrating about 120 A into the cell wall.

Once the DNA has entered the cytoplasm, there is a change in the cell's metabolic activity. It was evident from the first observations of sectioned infected bacteria that visible cytological changes occurred shortly after infection and long before the maturation of intracellular phage particles (8, 118, 139). Subsequent improvements in techniques allowed a more precise visualization of these changes, and a detailed picture of the whole intracellular multiplication process emerged (114, 119). Immediately after adsorption, the bacterial DNA is degraded so that its information is no longer available. After 5 to 7 min, the synthesis of phage DNA commences, followed 2 min later by the appearance of phage structural proteins. The first feature shown by the electron microscope is a pool of phage DNA (119) (Fig. 20a), in which the individual phage genomes condense or coil themselves up into compact cores, around which the head is next assembled. This is followed by the tail assembly. It is difficult to see the latter in electron micrographs, but the heads show up clearly (Fig. 20b).

Further details have been elucidated by the study of cells infected by conditional lethal mutants of phage T4 (63). The mutants, which contain a defective gene, cause the whole cell to produce, under nonpermissive conditions, mixtures of phage components, e.g., empty heads and cores with attached base-plates. Epstein et al. (63) and Kellenberger (114) were able to produce a genetic map of phage T4 locating some 50 genes; electron microscopy identified a particular phage component with its appropriate gene.

The lysis stage of the "life cycle" has been studied comparatively little with the electron microscope. It appears, however, that the cell wall ruptures and the contents are released. Cota-Robles (45) showed that this is accompanied by the formation of numerous extracellular vesicles which consist of cell membrane rather than cell wall material (Fig. 20c). His electron micrographs do not show how they are formed, but there is little doubt that they consist of rolled-up fragments of membranous material originating from cell envelopes rather than intracellular mesosomes; numerous cell membrane invaginations are not present in cells infected with T-even phages.

Structure of the Contractile Pseudomonas Phage PB-1

Phage PB-1 represents a very common morphological type, which grows on many different species of bacteria (15). It is distinguished from the T-even phages by its smaller size (750 A) and apparently octahedral head shape: the tail is about 1,500 A long. The form of the head is clear in Fig. 21a, and it can be seen that the tail sheath has a complex subunit structure. At the base of the tail, there are four fibers (Fig. 21b, arrow) suggesting that the whole tail assembly has a fourfold radial symmetry. This would fit onto the fourfold symmetry axis of the head (Fig. 21a). The tail fibers are sometimes retracted or folded up against the sheath (Fig. 21c, arrow). There is no obvious base-plate, but contracted sheaths show a discontinuity near their base (Fig. 21d, arrow). There are also longitudinal grooves which reflect the change in packing of the subunits on contraction, though the details of this process have not been worked out.

Preliminary observations on the DNA of phage PB-1 spread on a 0.1 M ammonium acetate

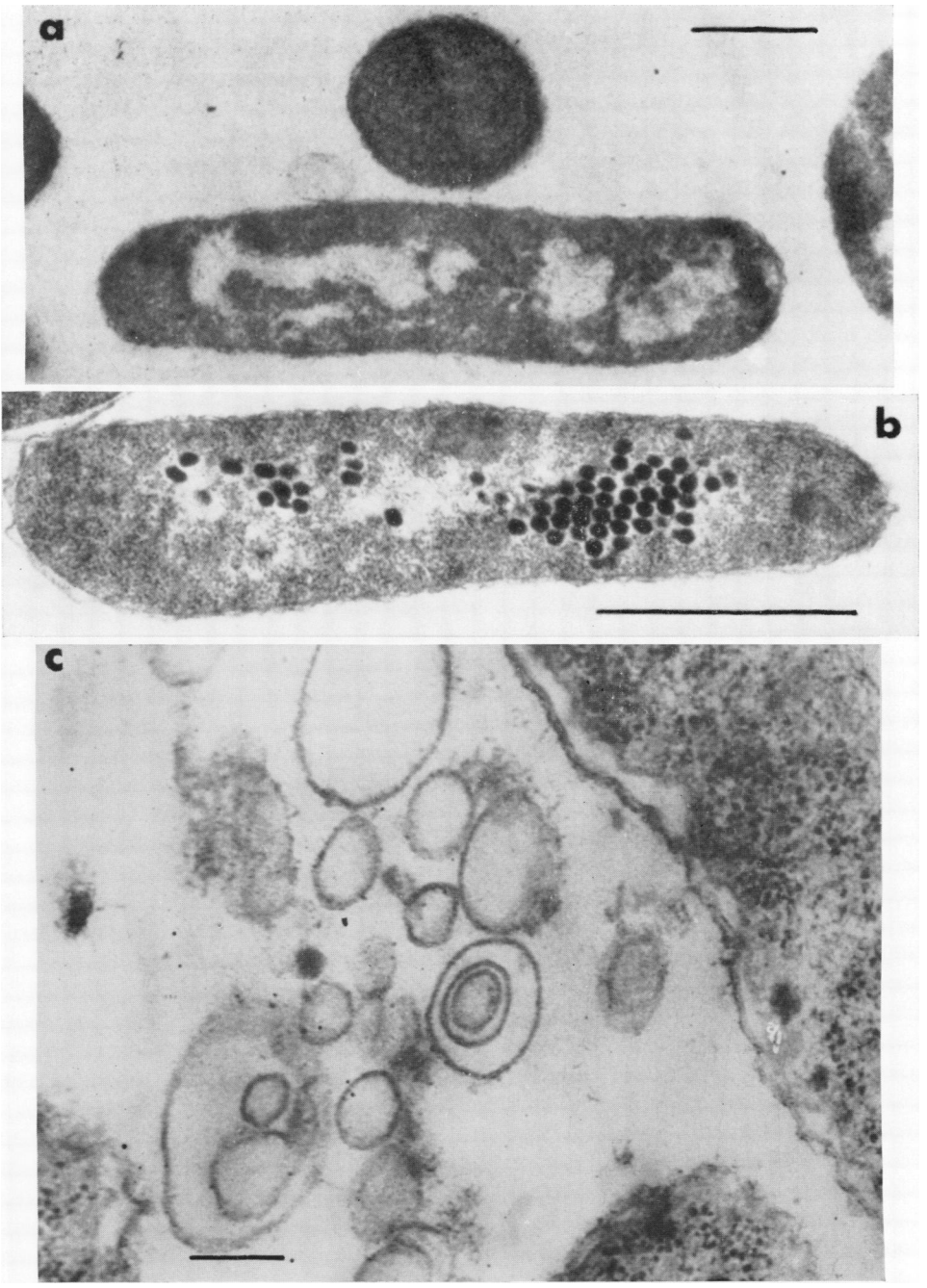


FIG. 20. *(a) Section of Escherichia coli 10 min after infection with T2 phage (119), × 17,000, scale 1 μ; after Kellenberger et al., with permission of Virology, Academic Press, Inc. (b) Section of E. coli 40 min after infection with T2 phage (113), × 35,000, scale 1 μ; after Kellenberger, with permission of Advances in Virus Research, Academic Press, Inc. (c) Section of cell debris after lysis of E. coli by T2 phage (45), × 120,000, scale 1,000 A, after Cota-Robles, with permission of J. Ultrastruct. Res.*

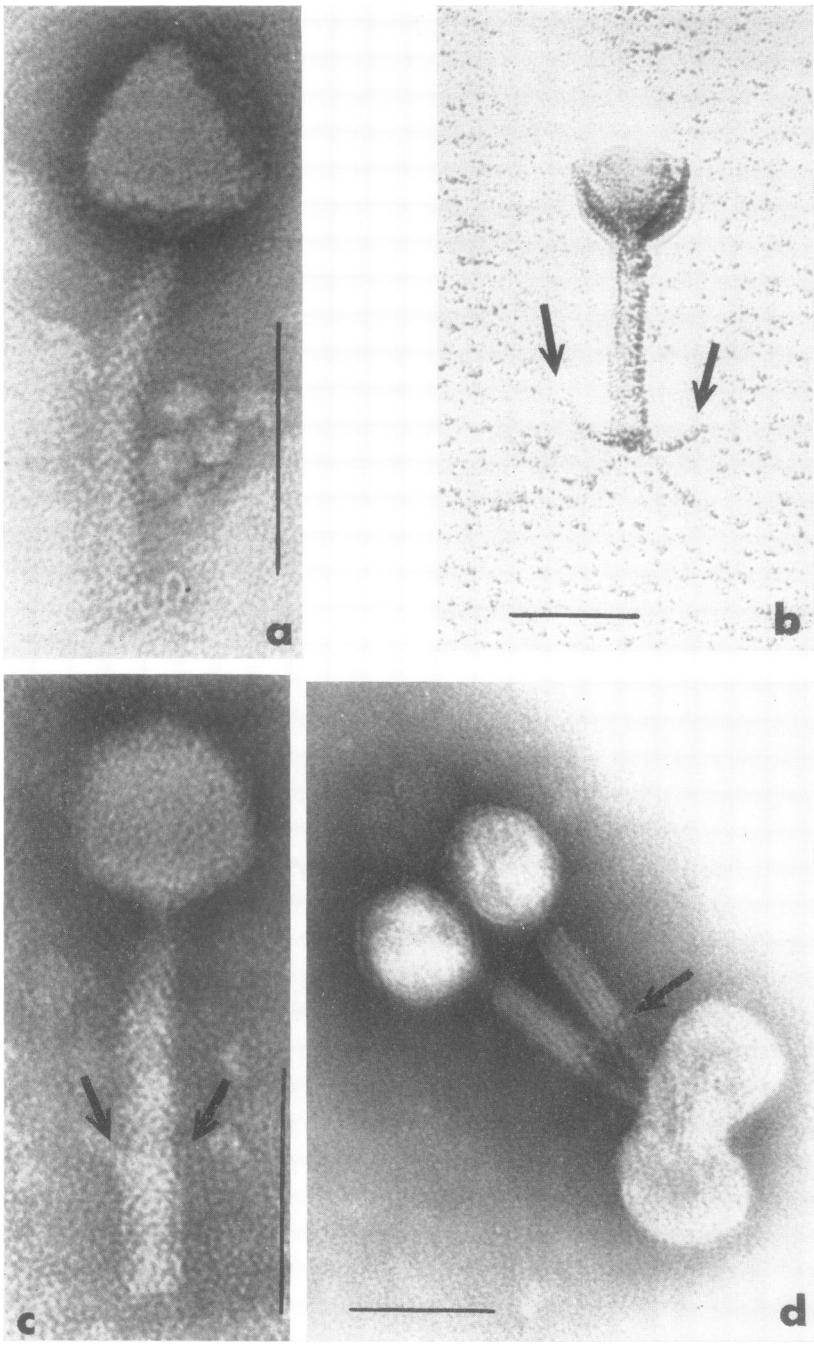


FIG. 21. *(a) Pseudomonas aeruginosa phage PB-1, PTA, × 333,000, scale 1,000 A. (b) Phage PB-1 from Langmuir trough preparation, × 167,000, scale 1,000 A. (c) Phage PB-1, PTA, × 333,000, scale 1,000 A. (d) Phage PB-1, PTA, × 185,000, scale 1,000 A.*

solution with the use of cytochrome *c* (124) indicate that it is a molecule about 25 μ long and is not in the form of a closed loop (Fig. 22). Acridine orange staining (23) shows that it is double-stranded. It is interesting to compare the total volume of the head, about 1.75×10^8 A^3, with that of the nucleic acid molecule, which is 7.9×10^7 A^3 (45%). The percentage of the head volume

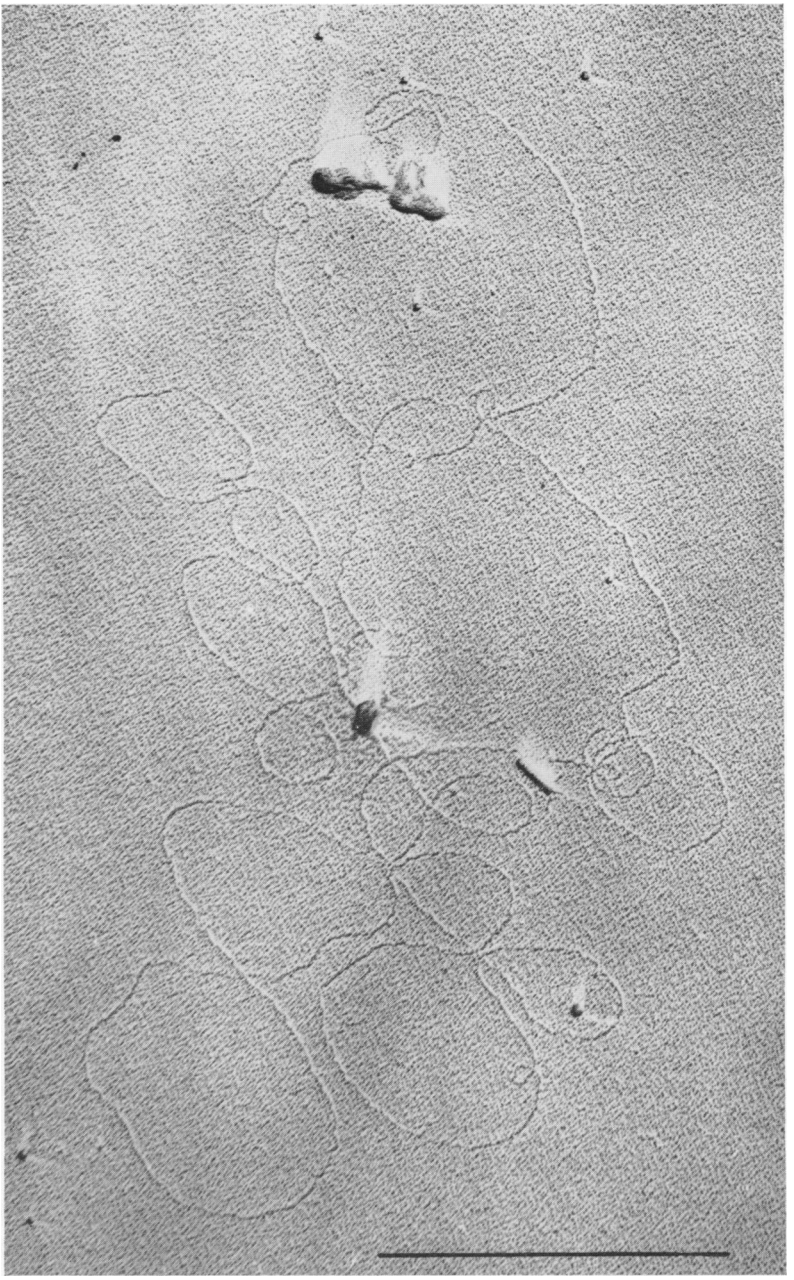


FIG. 22. *Phage PB-1 DNA spread by Langmuir trough method, × 45,000, scale 1 μ.*

occupied is almost the same with the T-even phages (43%).

Infective Process of Phage PB-1

As with other contractile phages, PB-1 adsorbs tail first, the tail fibers probably functioning in a similar manner to those of the T-even phages; they can be observed both extended and retracted. The sheath then contracts, and DNA is injected. Intracellular multiplication is illustrated in the following electron microscopic study of cells infected with phage PB-1. The micrographs can be compared with those of the T2 phage-infected cells of *E. coli* already described, although different fixation and embedding methods were used (119). A culture of *P. aeruginosa* was infected at

a multiplicity of 1:1, and samples were removed for fixation in glutaraldehyde and then OsO_4 before infection and at 10, 30, and 40 min afterwards. The cells were embedded in Vestopal, sectioned, stained in lead citrate, and examined in an electron microscope.

An uninfected cell (Fig. 23a) contains a fairly well dispersed nucleoplasm and many clearly defined dark particles, presumably ribosomes. The multiple layers of the cell envelope are clearly visible. Ten minutes after infection, there is little obvious change (Fig. 23b). The typical cell illustrated shows a slight "condensation" of the nucleoplasm. The most spectacular changes occur after 30 min, as shown in Fig. 23c. The nucleoplasm is less well defined and the cell contains many phage heads, some full and some empty. They are each surrounded by a narrow clear region. The particles are not confined to the nucleoplasm, some being embedded in dense masses of ribosomes in the cytoplasm. The clear areas are equally obvious whether the particle is in the nucleoplasm or among ribosomes. At this stage of infection, occasional bulges or blebs can be found at the cell surface, indicating structural changes in the cell wall. The rather swollen appearance of the cells also reflects this. At 40 min after infection, when the culture is in the process of lysing, many membrane-bound vesicles are visible (Fig. 24a). The cells themselves are in the process of lysing, large gaps appearing in the cell wall. There seems little doubt that the vesicles originate from the cell envelope. In Fig. 24b, a part of the cell wall is rolling up. One might expect cell wall and cell membrane fragments to form small tubes which would appear as vesicles in section.

It appears, therefore, that phage PB-1 behaves in a generally similar manner to the T-even phages in its infective process. Perhaps the most significant difference is the distribution of the mature phage particles throughout both the nucleoplasm and the cytoplasm; with T2, vegetative phage appears to be formed only within the DNA pool. It is not known what the clear areas are, but they could be small discrete pools of DNA, separated from the main body of the nucleoplasm.

Structure of Coliphage T5

Phage T5 belongs to the group with long noncontractile tails. Figure 25a shows that it has a flexible tail 2,000 A long and a head with a hexagonal outline about 900 A in size. Previous observations on shadowed preparations suggested that the head was an icosahedron, but an octahedron was not ruled out (15). In Fig. 25a and b, a number of facets are visible, corresponding well to superimposed models of octahedra. The tails exhibit a number of cross-striations (about 45 with a periodicity of 30 A) and a small thickened disc near the end (Fig. 25d, arrow). In Fig. 3d, 25c, and d, there are several very delicate fibers at the tail tip. As is so often the case, one cannot be certain of the head shape, but the negatively stained preparations shown here are more convincing than previous shadowed ones (15). This phage is typical of its morphological group (B), which has representatives for the majority of bacterial species.

Infective Process of Coliphage T5

As with other tailed phages, T5 adsorbs to the host cell tail first. Weidel and Kellenberger (216) studied T5 adsorption by isolating receptor sites from the cell wall and incubating them with the phage. Receptor molecules appeared as small spheres (Fig. 26a). After incubation with the virus, they became attached to the tips of the tails (Fig. 26b), and in some cases caused the virions to lose their nucleic acid. This last observation might seem to suggest that the energy for transferring the nucleic acid from the head, through the tail, and into the cell, lies within the phage particle. However, if this were the case, one would expect almost all the virions to be empty. It seems much more likely that nucleic acid transfer is achieved by a combination of forces from both bacterium and phage, as suggested by the work of Lanni (127–129) which has been described in detail above.

The intracellular multiplication of T5 has been described by Kellenberger (113), who illustrated the sequence of events shown in Fig. 27. At the time of infection, cells have a well-defined nucleoplasm (Fig. 27a), but after 5 min this tends to break up (Fig. 27b). After 10 min, it disappears altogether, and there is comparatively little change in appearance until the formation of vegetative phage (Fig. 27c) 30 min after infection. It would thus appear that there is no DNA pool, as is the case with T-even phages. Likewise, there is no transparent region surrounding the particles as has been described for the *Pseudomonas* contractile phage PB-1, although differences in fixation procedures might account for this latter dissimilarity.

Early light microscopic observations of T5-infected bacteria showed the formation of spherical cells several minutes before lysis (11), but these do not appear to have been observed in the electron microscope.

Structure of Tail-less Coliphages with Large Capsomeres

The best known of this morphological group (D), ϕX174, was first studied in detail by Sinsheimer (193). Various other isolates have

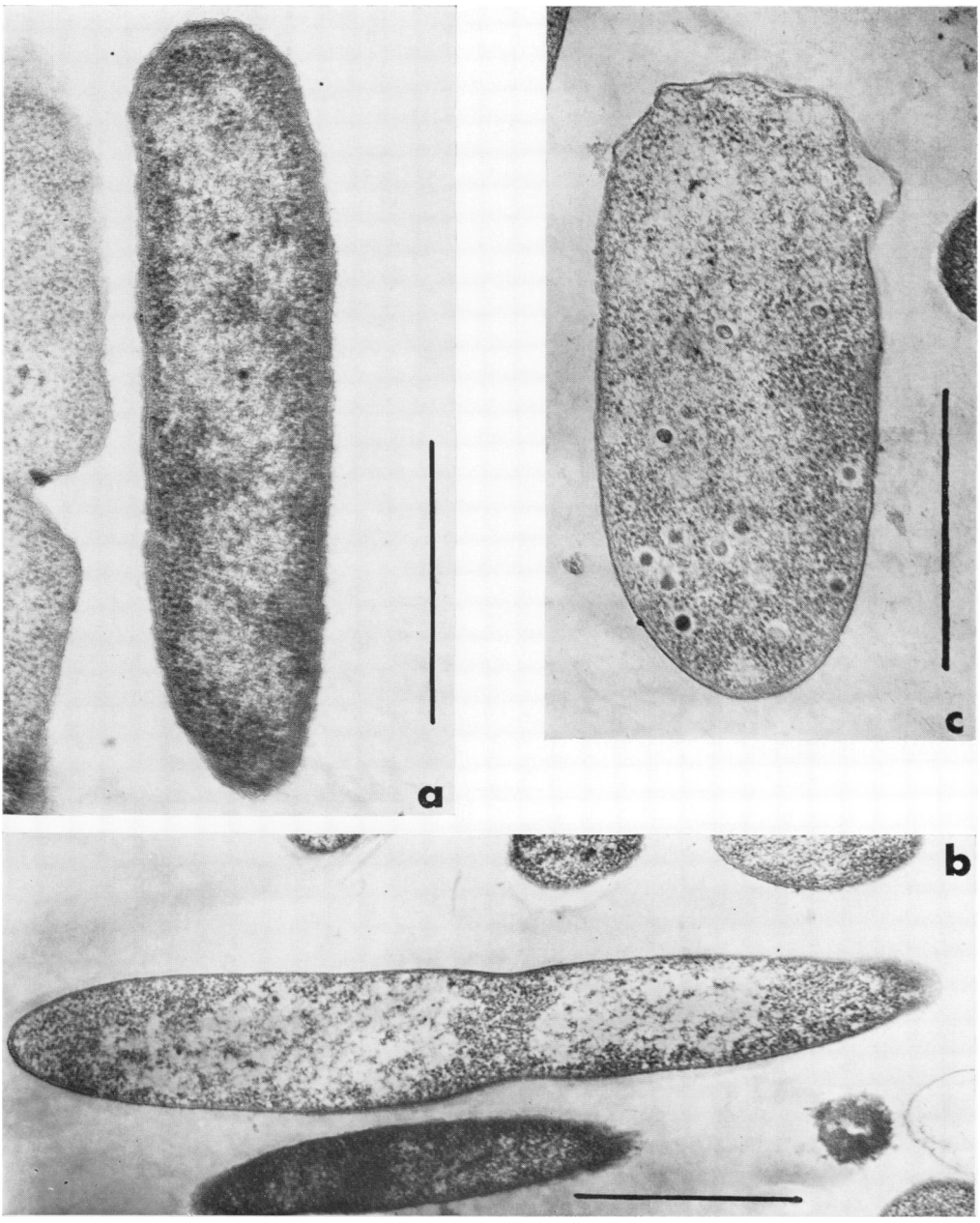


FIG. 23. *(a) Uninfected cell of Pseudomonas aeruginosa, section, × 37,500, scale 1 μ. (b) P. aeruginosa cell 10 min after PB-1 infection, × 30,000, scale 1 μ. (c) P. aeruginosa cell 30 min after PB-1 infection, × 37,500, scale 1 μ.*

also been described (66, 220). This type of phage is fairly common in sewage. The information given here is derived from both ϕX174 and ϕR (66). The first indication that large capsomeres were present was obtained from shadowed preparations (81). In Fig. 28a, it can be seen that the virion is sometimes hexagonal in outline (A) with a knob at each apex, and sometimes has a central subunit surrounded by five others, according to the orientation (B). This configuration can only be formed by an icosahedron with apical capsomeres. Supporting evidence was obtained by the shapes of the shadows cast by the virions (112). Tromans and Horne (211) deduced the icosahedral form

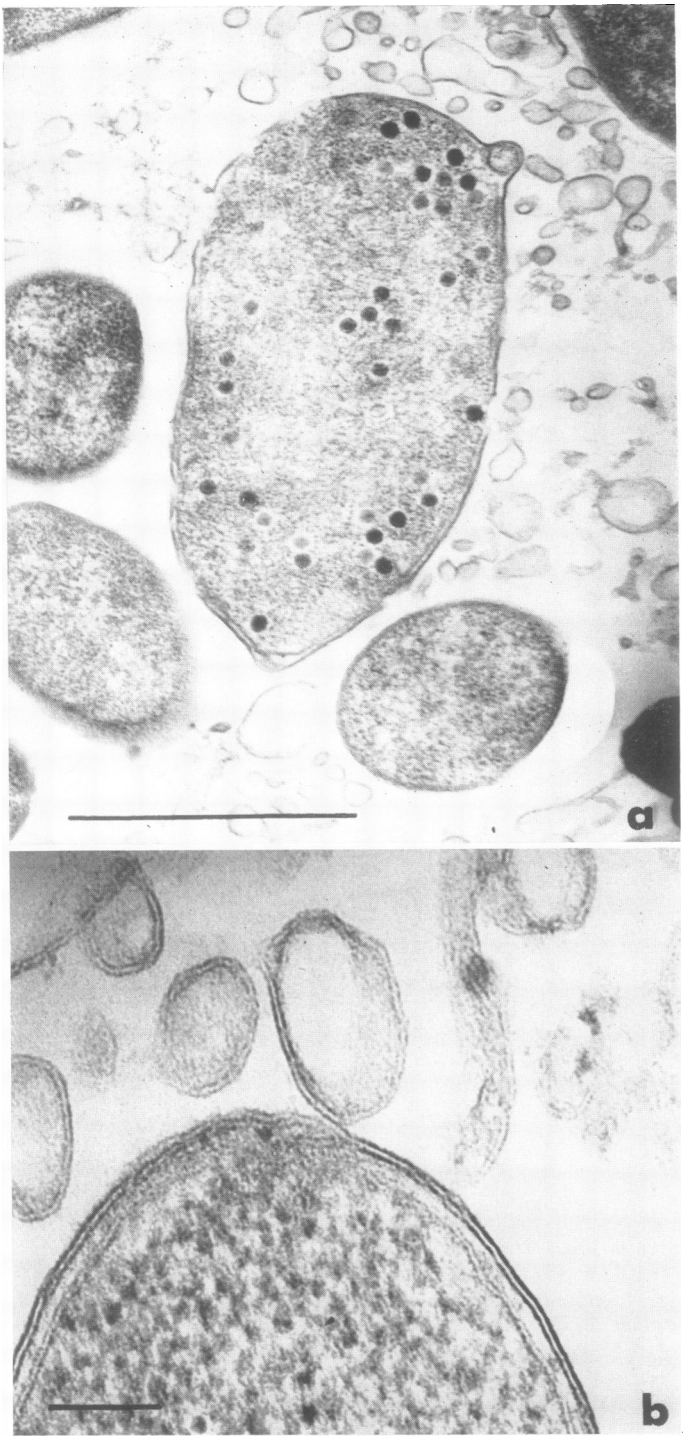


FIG. 24. (*a*) ***Pseudomonas aeruginosa cell 40 min after PB-1 infection, × 37,500, scale 1 μ. (b) P. aeruginosa cell 40 min after PB-1 infection, × 150,000, scale 1,000 A.***

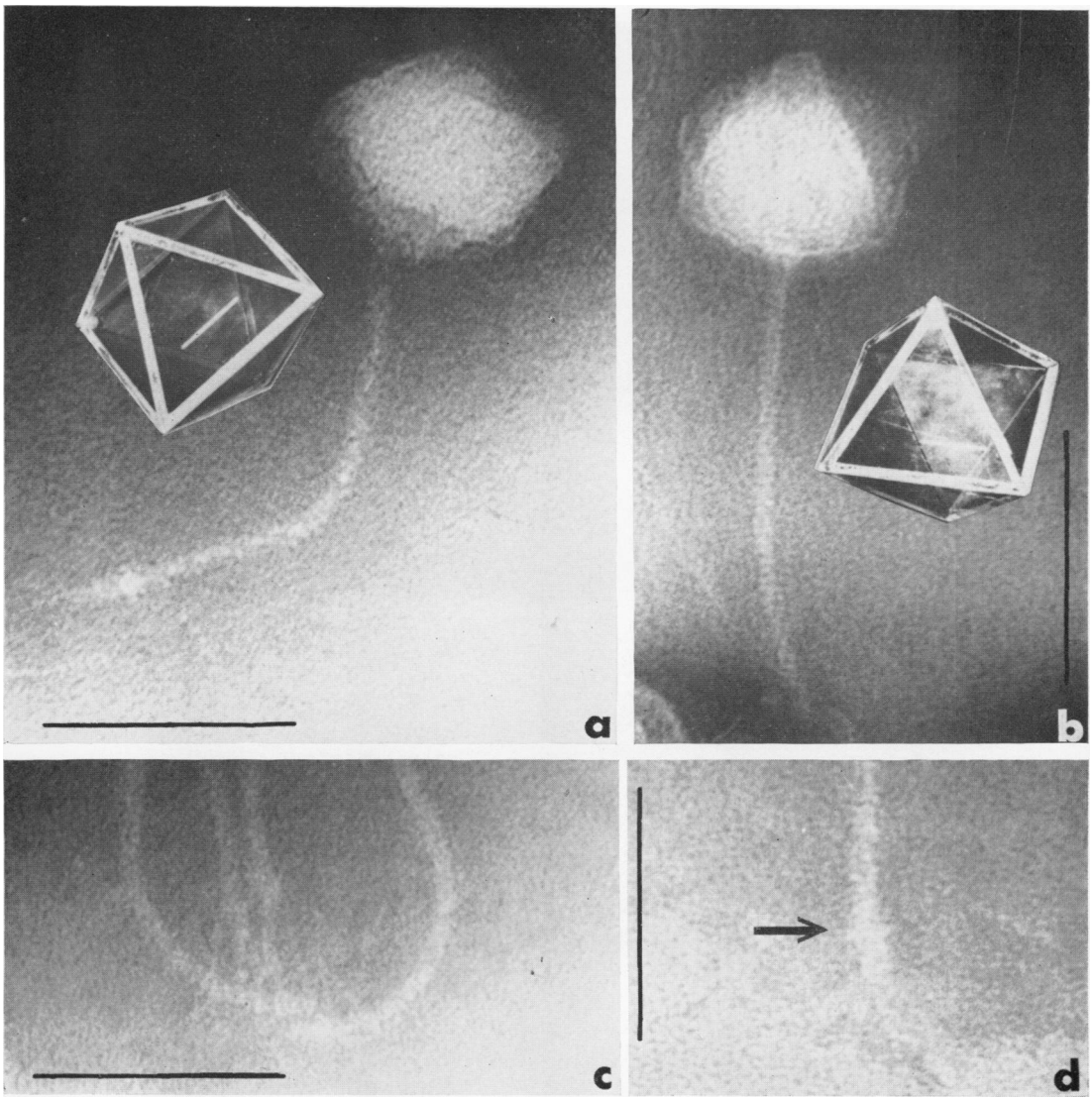


FIG. 25. *(a), (b), (c), (d) Coliphage T5, PTA, × 333,000, scales 1,000 A.*

from negatively stained preparations, which also revealed that the apical capsomeres appeared to be in the form of small dimers shown in Fig. 28b (112). They are only some 15 A in size, and there is no doubt from the electron micrograph that they are attached to the apices of the underlying capsid (13). This form of bacteriophage is therefore unique, and one might expect a special form of nucleic acid; it is in fact single-stranded DNA in the form of a closed ring (65). Its replicative form has been studied in an electron microscope (65); spreading on a Langmuir trough revealed a loop structure (Fig. 28c). Independent figures for the length are 1.64 μ (123) and 1.89 μ (39).

Infective Process of Tail-less Coliphages with Large Capsomeres

Little work has been carried out on the infective process of this morphological type. It is known that it adsorbs to the cell surface (203), and Kay (110) showed that the ϕR virion is univalent, suggesting that one of the capsomeres is different from the remainder and acts like a normal tail in the adsorption process.

Nothing is known of the cytological changes associated with intracellular growth. Sections of lysing cells suggest that there is a local rupture of the cell wall (143).

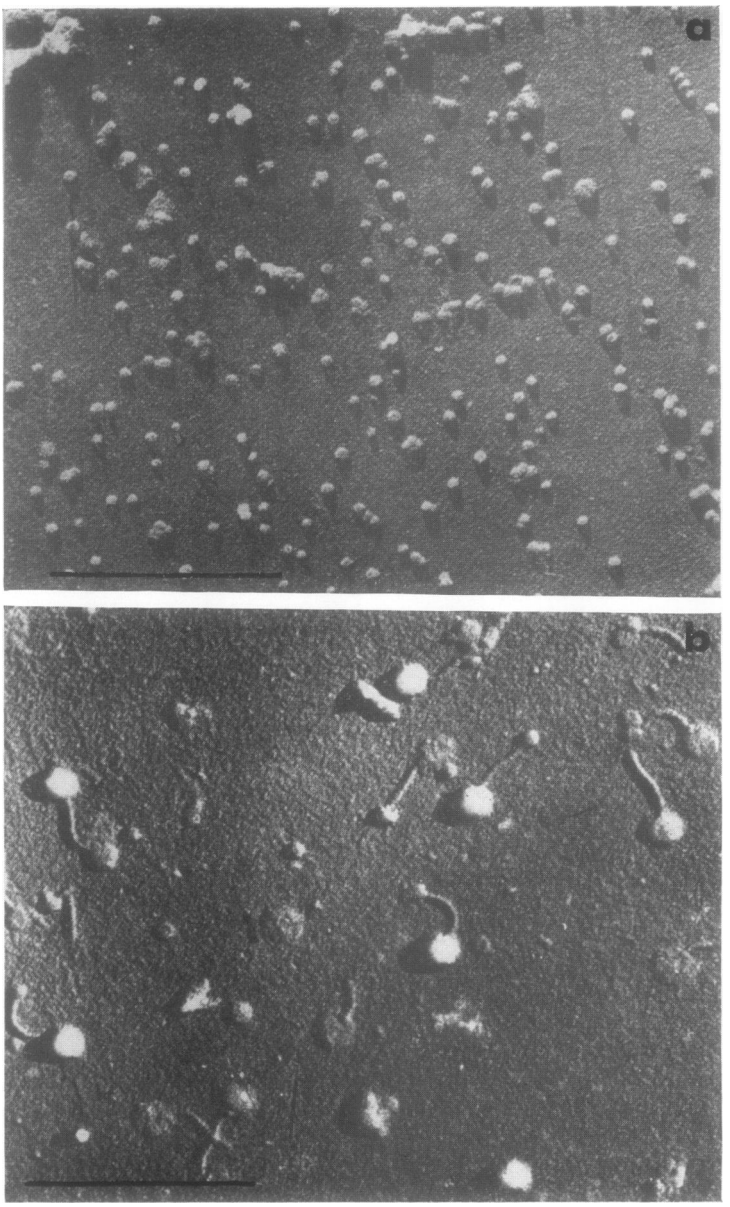


FIG. 26. *After Weidel and Kellenberger (216), with permission of Biochim. Biophys. Acta, × 60,000, scales 0.5 μ. (a) T5 receptors from Escherichia coli B, shadowed. (b) Phage T5 with receptors adsorbed to tail tips, shadowed.*

Structure of the RNA-containing Coliphages

Numerous isolates of RNA coliphages have been examined in the electron microscope (17, 134, 147), but it seems that they all have an identical appearance. The following description is based on the observations of Bradley (17) for the isolate ZIK/1. In negatively stained preparations, the virion has a characteristic semitransparent appearance (Fig. 29a), is tail-less with a hexagonal outline, and is 225 A in size. It belongs to morphological group E. Occasionally, completely transparent particles can be found (Fig. 29b); these are presumed to contain no RNA, having either lost it or been assembled without it. Little structure can be resolved on the capsid except in rare cases when an indication of capsomere arrangement may appear (Fig. 29c, d). By comparing a model of an icosahedron with such

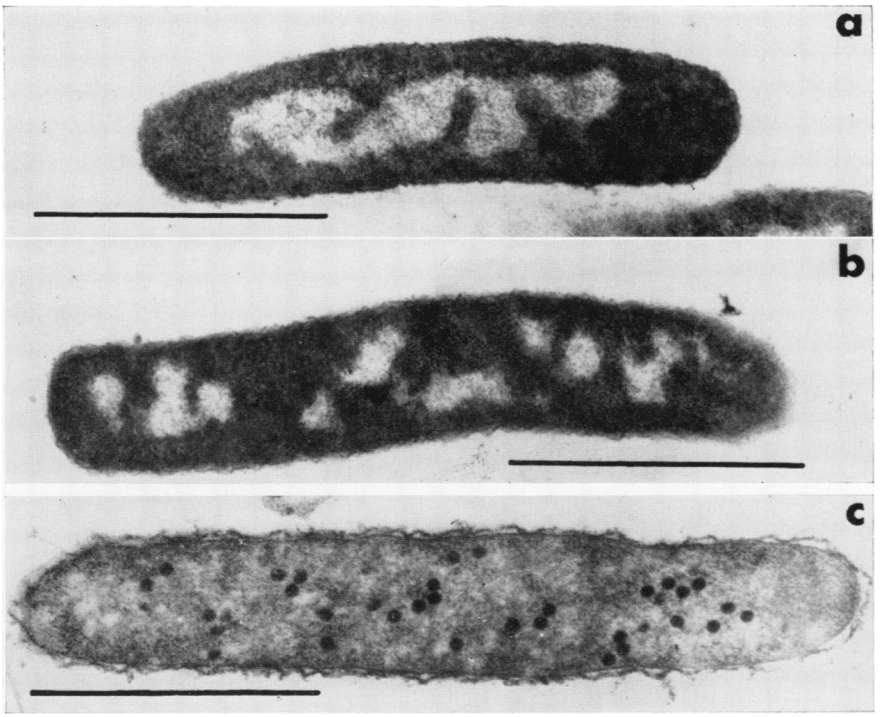


FIG. 27. *After Boy de la Tour (reference Kellenberger, 113, with permission of Advances in Virus Research Academic Press, Inc.), × 38,000, scales 1 μ. (a) Uninfected cell of Escherichia coli. (b) E. coli 5 min after T5 infection. (c) E. coli 30 min after T5 infection.*

micrographs, it is possible to show that this is the probable geometrical shape, as predicted by Crick and Watson (50), and to estimate the size and number of the capsomeres. On this basis, the isolate described here has 92 capsomeres packed with a center-to-center spacing of 45 A. The only other morphological feature visible is a small hole or pore in the capsid (Fig. 29e, arrow) about 20 A in size, the function of which is not known.

Infective Process of the RNA Coliphages

Unlike the majority of bacteriophages, those containing RNA do not adsorb to specific receptor sites in the cell wall. Crawford and Gesteland (48) showed electron micrographs of virions attached to fine filaments extending from the host bacterium. These filaments, or pili as they are called, to which RNA phages attach, were characterized by Brinton (33) and examined by Lawn (130) by use of negative staining; they were shown to be specific for male strains of *E. coli* (those carrying the F or fertility episome). The phages did not adsorb to any other form of pilus: they are thus male-specific. An example of this adsorption is shown in Fig. 29f. Initial adsorption to pili is reversible (59, 214); that is to say, the application of shear by blending removes viable phages which can reattach. While all the evidence points to infection via F-pili, the fact is not yet definitely established.

The intracellular multiplication stage of infection by phage f2 has been studied by Schwartz and Zinder (188) using thin sections; they demonstrated that large intracellular crystals form just before lysis (Fig. 30). A more recent and detailed study by Franklin and Granboulan (68) showed that there was no change in cellular organization until after the latent period (45 min after infection by phage R17) when fibrillar arrays surrounded by ribosomes became visible. After 70 min, crystals became visible in a few cells, rapidly increasing in frequency until 90 min after infection when almost all cells contained them. The initial fibrillar arrays were considered to be an RNA pool. Lysis by RNA coliphages is not accompanied by spheroplast formation, as is the case with *Pseudomonas* RNA phages; it is manifested by the presence of many intact empty ghosts (170) rather than badly ruptured cell envelopes.

Structure of the RNA-containing Pseudomonas Phages

Two isolates of RNA *Pseudomonas* phages have been described, 7s (64) and PP7 (24). Phage

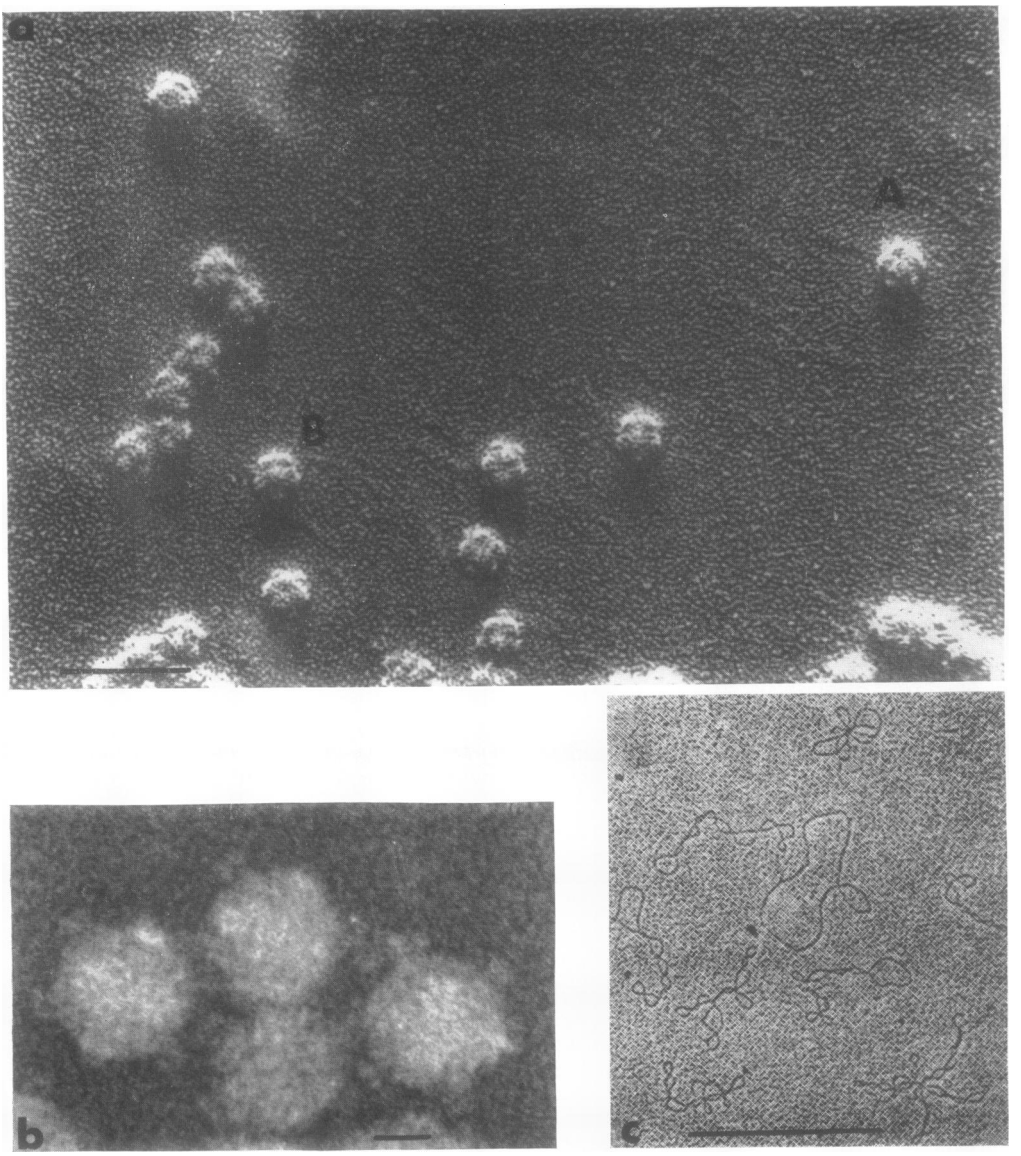


FIG. 28. *(a) Coliphage φR, shadowed (112), × 170,000, scale 1,000 A, after Kay and Bradley. (b) φR in PTA (15), × 750,000, scale 100 A. (c) φX174 replicating-form DNA spread on Langmuir trough (39), × 25,000, scale 1 μ, after Chandler et al. (Science* **143:** *47–49 1964), with permission of the Editors.*

7s (Fig. 31a) has a regular structure in the capsid and PP7 (Fig. 31b, c) does not. This could reflect a difference in the capsid or in the negative staining conditions. The latter seems a more likely explanation, since the two isolates are serologically related (24) and are therefore likely to be morphologically similar. Hence, a comparison to provide an overall picture is probably valid. The appearance of phage 7s in the electron microscope (Fig. 31a) indicate from the number and arrangement of the faces, rather than the arrangement of the individual capsomeres, that the capsid is icosahedral in form. It can be seen from the micrographs included here that the virion has the typical semitransparent appearance of the RNA coliphages, its icosahedral form giving a hexagonal outline about 250 A across. Empty virions are occasionally seen (Fig. 31c) and, rarely, individual capsomeres with a spacing of about 30 A can be discerned (Fig. 31b, c, arrow

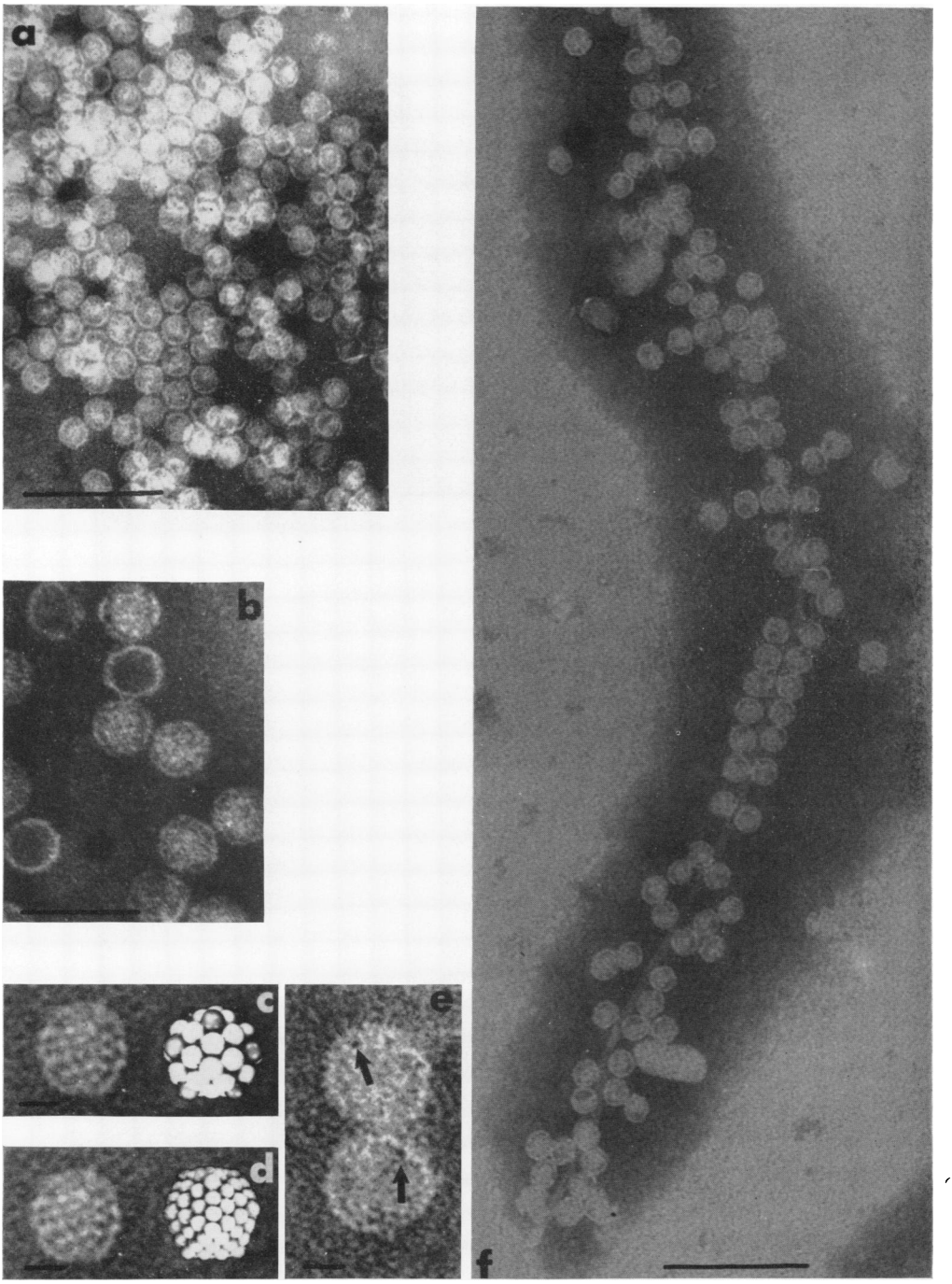


FIG. 29. *(a) RNA coliphage ZJ/1 (17), PTA, × 200,000, scale 1,000 A. (b) RNA coliphage ZIK/1 (17), PTA, × 333,000, scale 500 A. (c), (d), (e) Phage ZIK/1 (17), PTA, × 500,000, scales 100 A. (f) Phage ZIK/1 adsorbed to F pilus (17), PTA, × 200,000, scale 1,000 A.*

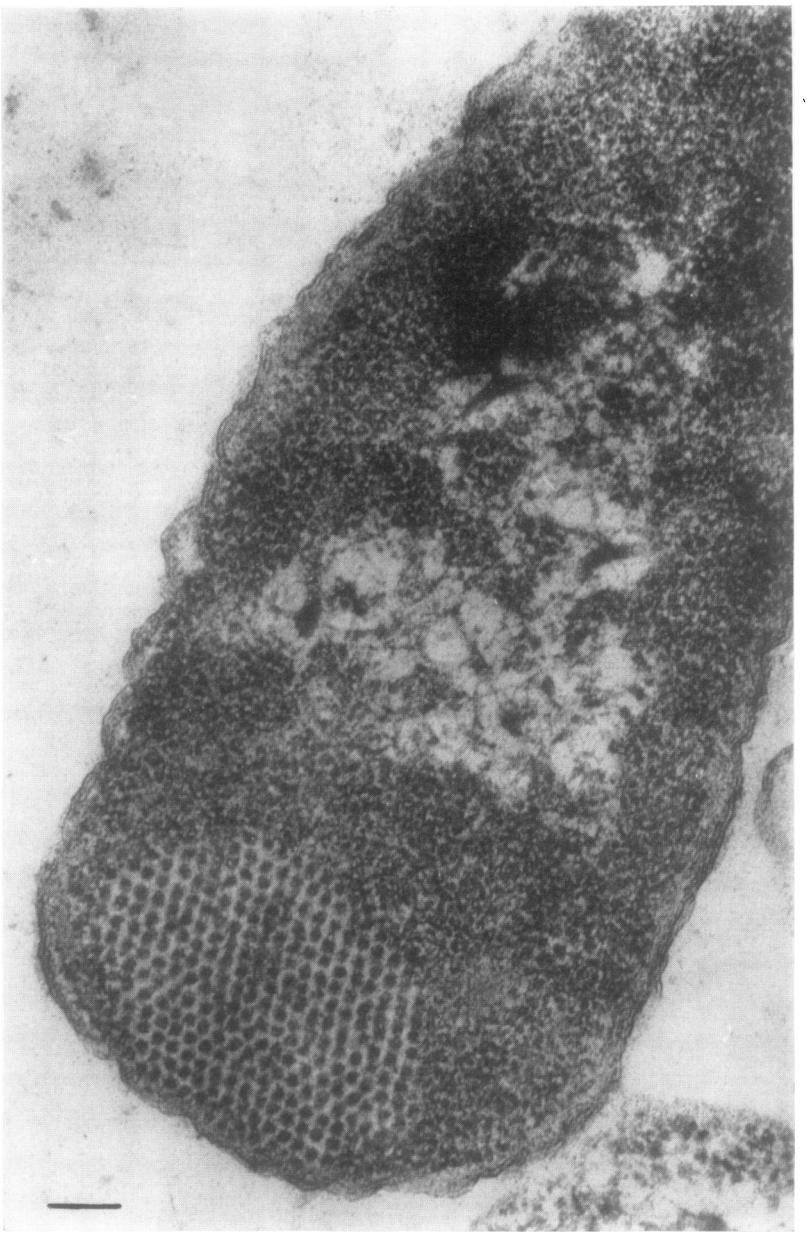


FIG. 30. *RNA coliphage f2 in infected host cell, section (188), × 95,000, scale 1,000 A, after F. M. Schwartz (first published in J. Roy. Microscop. Soc.).*

C). This would give 252 for an icosahedron with an edge of 180 A. As with the RNA coliphages, a pore may be present in the capsid (Fig. 31b, c, arrow P). Because of the differences in capsomere size, phages of the two host species are morphologically distinct.

Infective Process of Pseudomonas RNA Phages

As with coliphages, the RNA *Pseudomonas* phages adsorb to polar pili (Fig. 32a), though unpublished evidence indicates that these are not associated with the FP episome (*Pseudomonas*

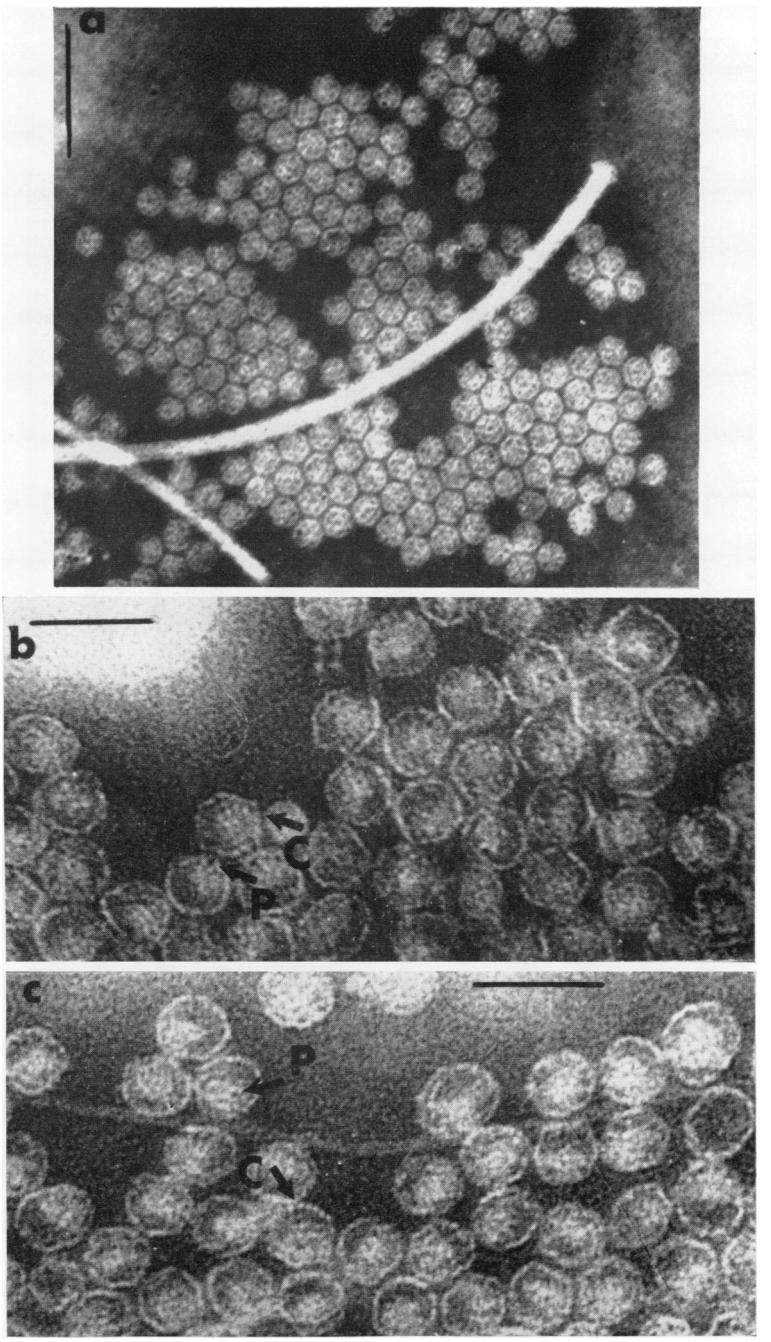


FIG. 31. (*a*) *Pseudomonas aeruginosa RNA phage 7s(64), PTA, × 180,000, scale 1,000 A, after Feary et al., with permission of J. Bacteriol.* (*b*), (*c*) *P. aeruginosa RNA phage PP7 (24), PTA, × 333,000, scales 500 A.*

fertility factor), nor are the phages male-specific. Apart from the nature of these extracellular receptors, the adsorption process is probably identical to that of coliphages. The fact that ribonuclease blocks plaque formation suggests that the injection process is also similar.

Marked intracellular changes can be seen during the course of phage maturation in the host.

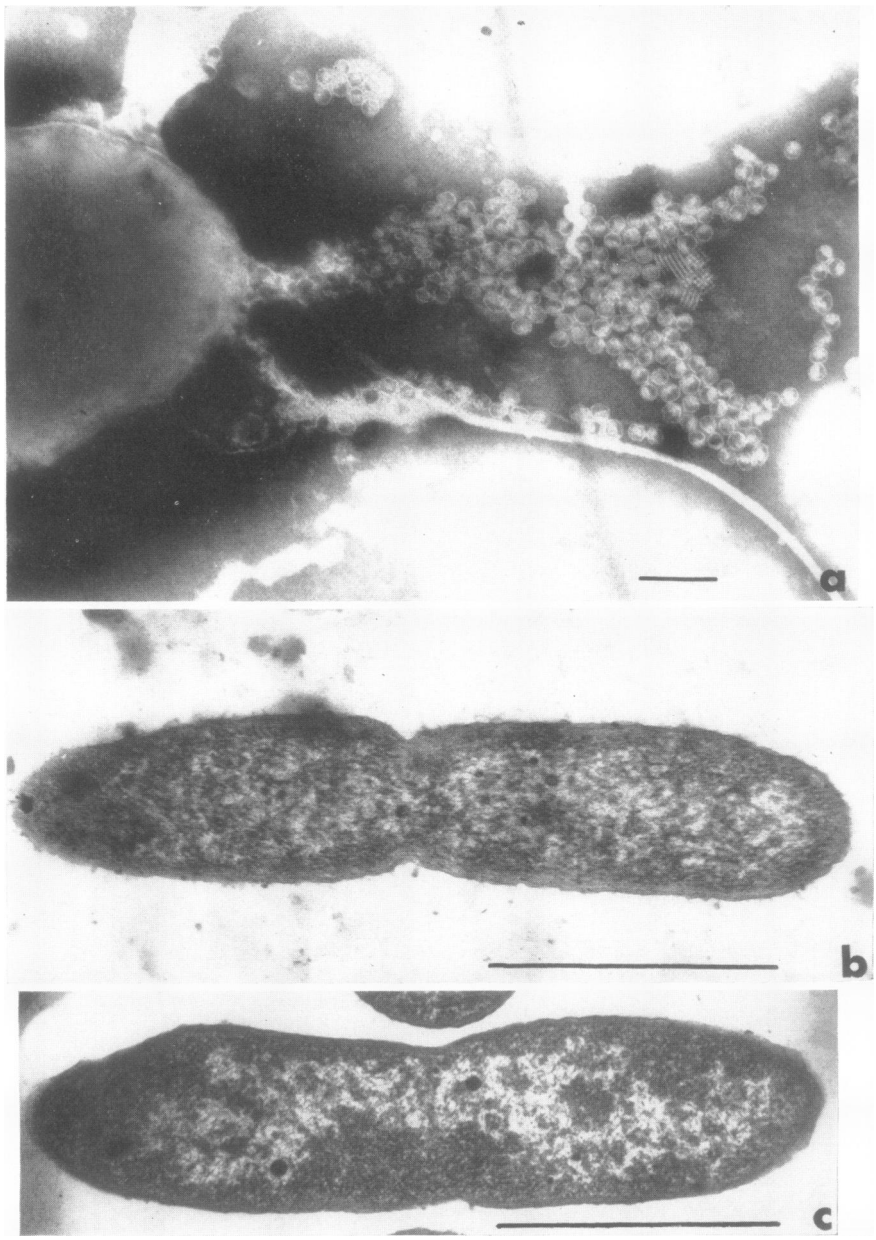


FIG. 32. *(a) Pseudomonas aeruginosa RNA phage PP7 adsorbed to pili (20), PTA, × 100,000, scale 1,000 A, with permission of J. Roy. Microscop. Soc. (b) Section of uninfected cell of P. aeruginosa, × 37,500, scale 1 μ (c) Cell of P. aeruginosa 20 min after infection with RNA phage PP7 (24), × 37,500, scale 1 μ.*

The uninfected cell (Fig. 32b) appears, from single sections, to have a more or less evenly distributed nucleoplasm, but 20 min after infection it is invaded by dense regions of the cell contents which subsequently differentiate into crystals (Fig. 32c). This happens about 40 min after infection, and is first manifested by the appearance of fine striations much smaller than the size of an individual virion (Fig. 33a). At about the same time spheroplast formation occurs (Fig. 33b); this is most strikingly visible in the light microscope. Fully differentiated crystals appear simul-

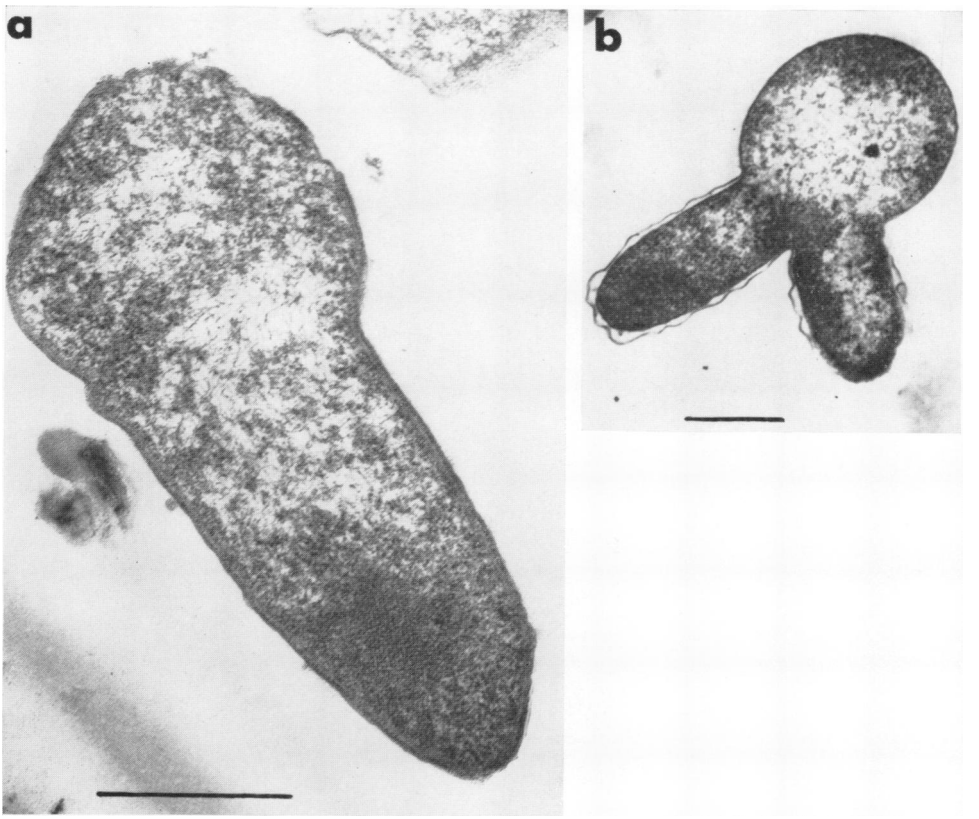


FIG. 33. *(a) Section of Pseudomonas aeruginosa cell 40 min after phage PP7 infection (24), × 50,000, scale 0.5 μ. (b) P. aeruginosa cell 80 min after phage PP7 infection (24), × 12,500, scale 1 μ.*

taneously, and they sometimes completely fill the arm of a spheroplast (Fig. 34). In this example, the plane of the section contains about 1,200 virions, which would mean a burst size of about 36,000, assuming a circular cross-section. Lysis is brought about by the rupture of the spheroplast wall (Fig. 35), causing the cell to split and thus releasing mature virions and the remains of the cytoplasm. This mechanism for lysis is unique for bacteriophages, but a small bacterial parasite for *Pseudomonas*, to be described below, has much the same effect.

Structure of the Filamentous Coliphages

The various isolates of filamentous coliphages all have an identical morphology (17, 91, 147, 221), and a complete picture can be built up from the different descriptions. The basic structure consists of a closed ring of 1-DNA (148) with a protein capsid. The length of the virion is quoted as being between 8,000 and 8,500 A and its width about 68 A (Fig. 36a). The latter measurement is based on electron microscopy of close-packed arrays of filaments: X-ray diffraction shows that the virions crystallize with a minimal spacing of 56 A (146). This is in reasonably close agreement. The filaments show little obvious structure, but again X-ray evidence points to a 32.2 A repeat structure, probably in the form of a supercoiled α-helix. The only visible detail in the fine structure of the virion in negatively stained preparations is a dark line down the long axis (Fig. 36b). Various interpretations have been applied to this; Marvin (146) considered that it indicates a two-strand arrangement and proposed a model "analogous to a circle of string pulled taught from opposite sides of its circumference, where the string represents single-stranded DNA coated with protein." On the other hand Bradley and Dewar (26) suggested a structure based on micrographs of transverse sections through the virion, which show a small circle with a central black dot (Fig. 36c). Assuming that the black dot represents the same structural feature as the axial line seen in negatively stained preparations, they suggested that the virion is in the form of a long open-ended tube, not unlike the tail of a noncontractile phage (morphological group B); the DNA lies in the

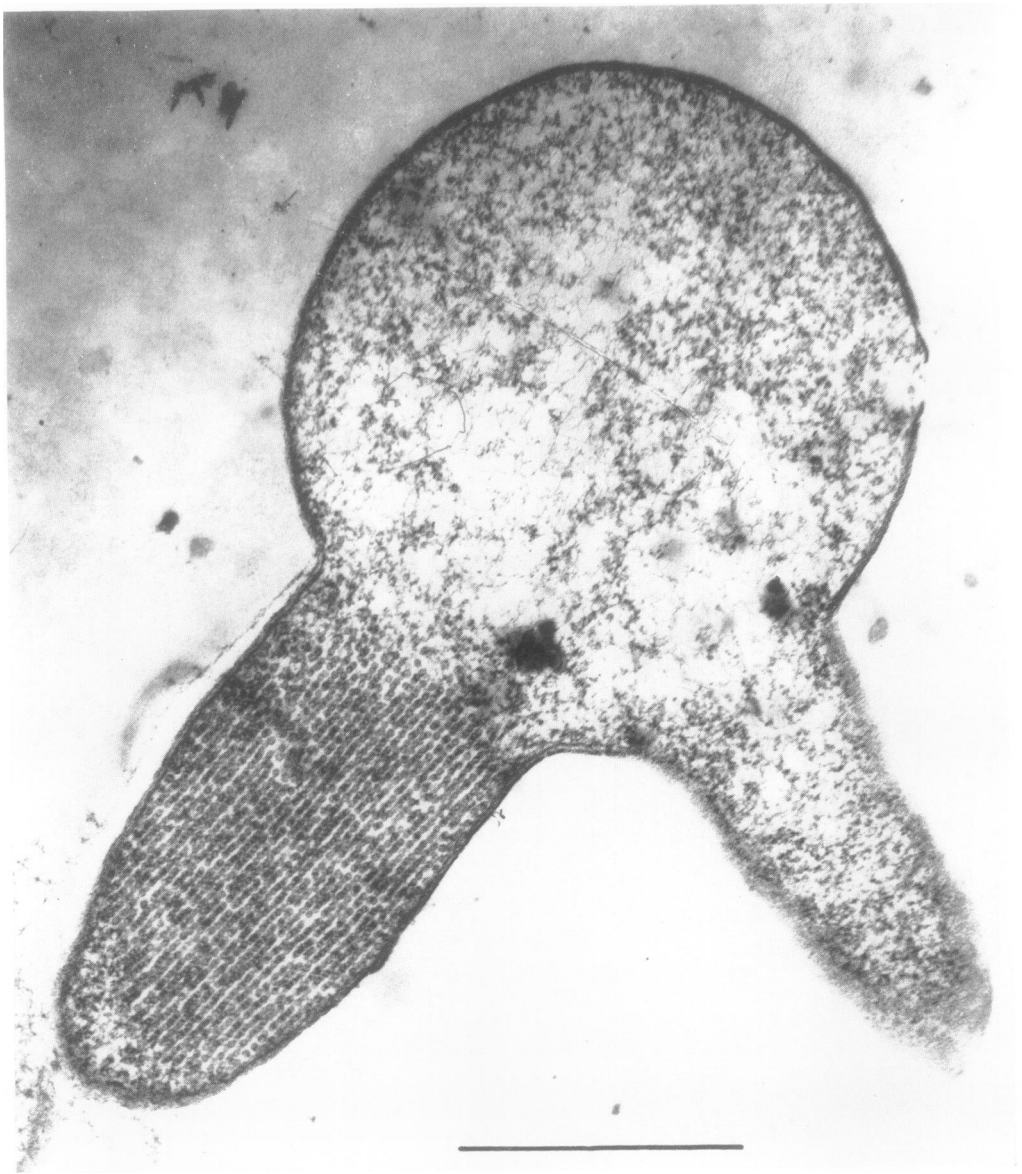


FIG. 34. *Section of Pseudomonas aeruginosa cell 80 min after RNA phage PP7 infection (24), × 37,500 scale 1 μ.*

core of the tube surrounded by, but not integrated with, the helical capsid. The dark line in negatively stained preparations would be explained by the penetration of the negative-staining chemical into a virion which had lost its nucleic acid. The picture thus emerges of an extremely long, headless phage tail.

The nucleic acid is 1-DNA with a molecular weight of 1.3×10^6 to 2.3×10^6. This, remarkably, is the same as that of the 1-DNA of ϕX174; Sinsheimer (194) quoted 1.7×10^6. Also, as is to be expected, both nucleic acid molecules have the same sedimentation coefficients. The length of the ϕX174 closed ring of 1-DNA is between 1.64 μ (123) and 1.89 μ (39). The average of these two values is twice the observed length of the filamentous phage virion. Add to this the fact that the 1-DNA of the filamentous virion is a closed ring structure like that of ϕX174 (148) and the picture is complete; the DNA must lie like a

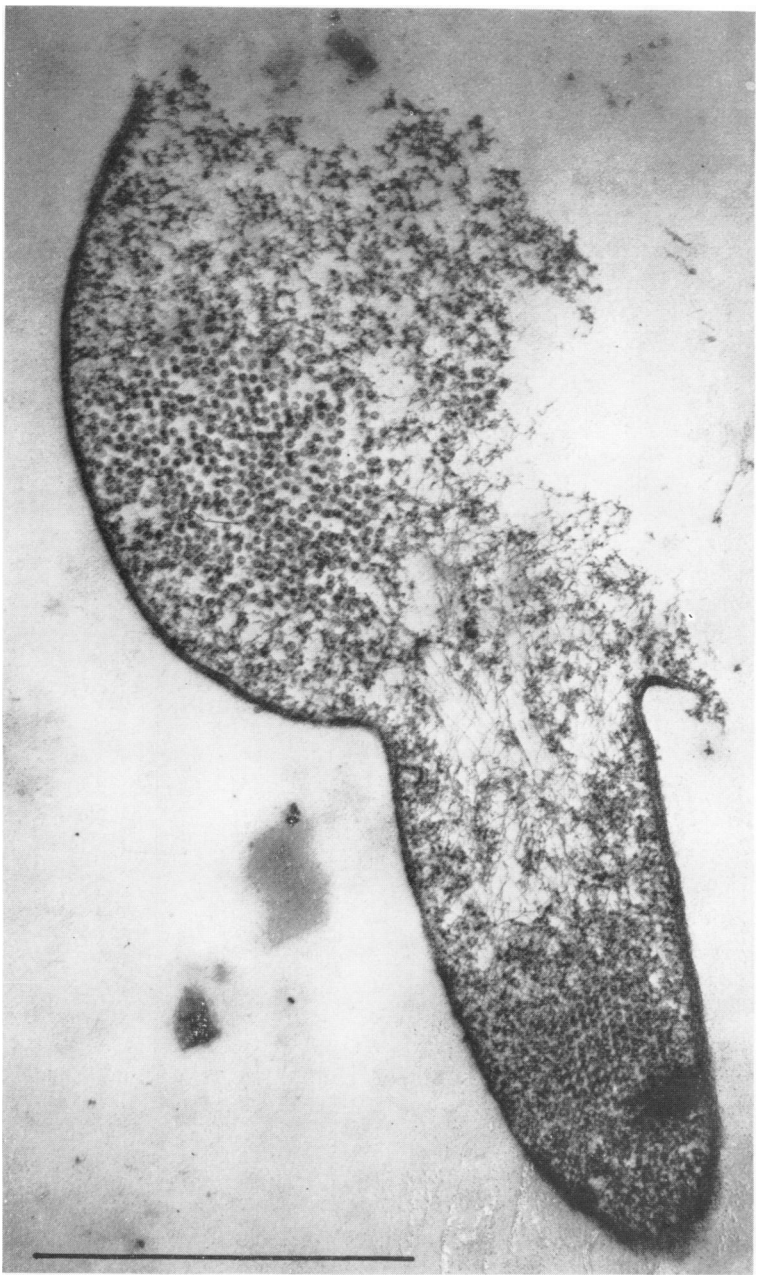


FIG. 35. *Section of Pseudomonas aeruginosa cell 50 min after RNA phage PP7 infection (24), × 50,000, scale 1 μ.*

taughtened circle of string, the two strands lying parallel or only very loosely coiled. Too tight a coil would mean that the virion would have to be significantly shorter than half the length of the φX174 1-DNA molecule. This arrangement would presumably fit into the tube of the capsid.

Infective Process of Filamentous Coliphages

The observation of the adsorption process of filamentous coliphages was not achieved until several years after their isolation. Like the RNA coliphages, they are male-specific, and it was

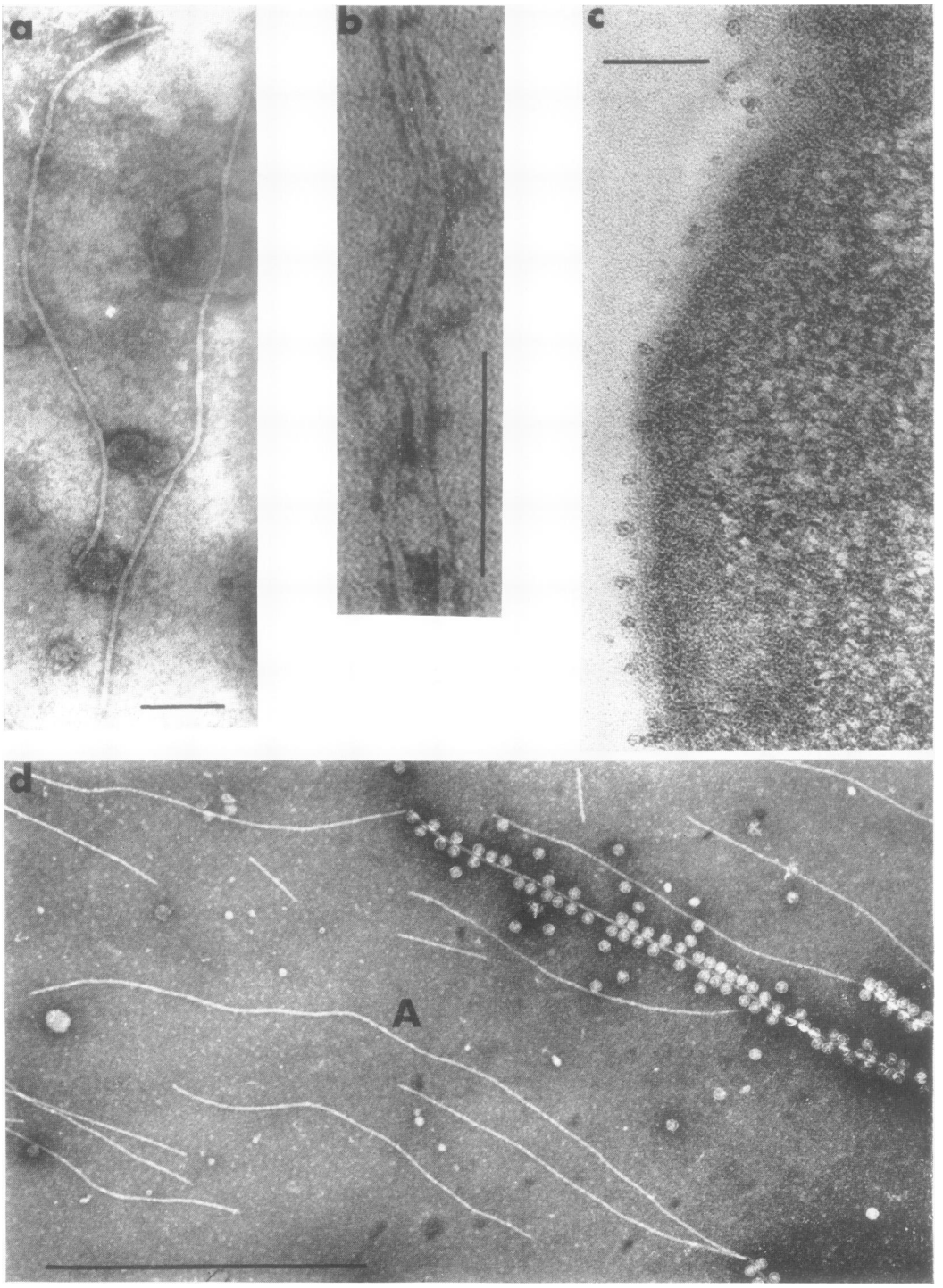


FIG. 36. *(a) Coliphage ZJ/2 (24), PTA, × 125,000, scale 1,000 A. (b) Coliphage ZJ/2 (17), PTA, × 333,000, scale 1,000 A. (c) Sections of coliphage ZJ/2, × 150,000, scale 1,000 A, after Bradley and Dewar (26), with permission of J. Gen. Virol. (d) Coliphage f1 adsorbed to RNA phage labeled F-pili, × 47,000, scale 1 μ; after Caro and Schnös (37), with permission of Proc. Natl. Acad. Sci. U.S.*

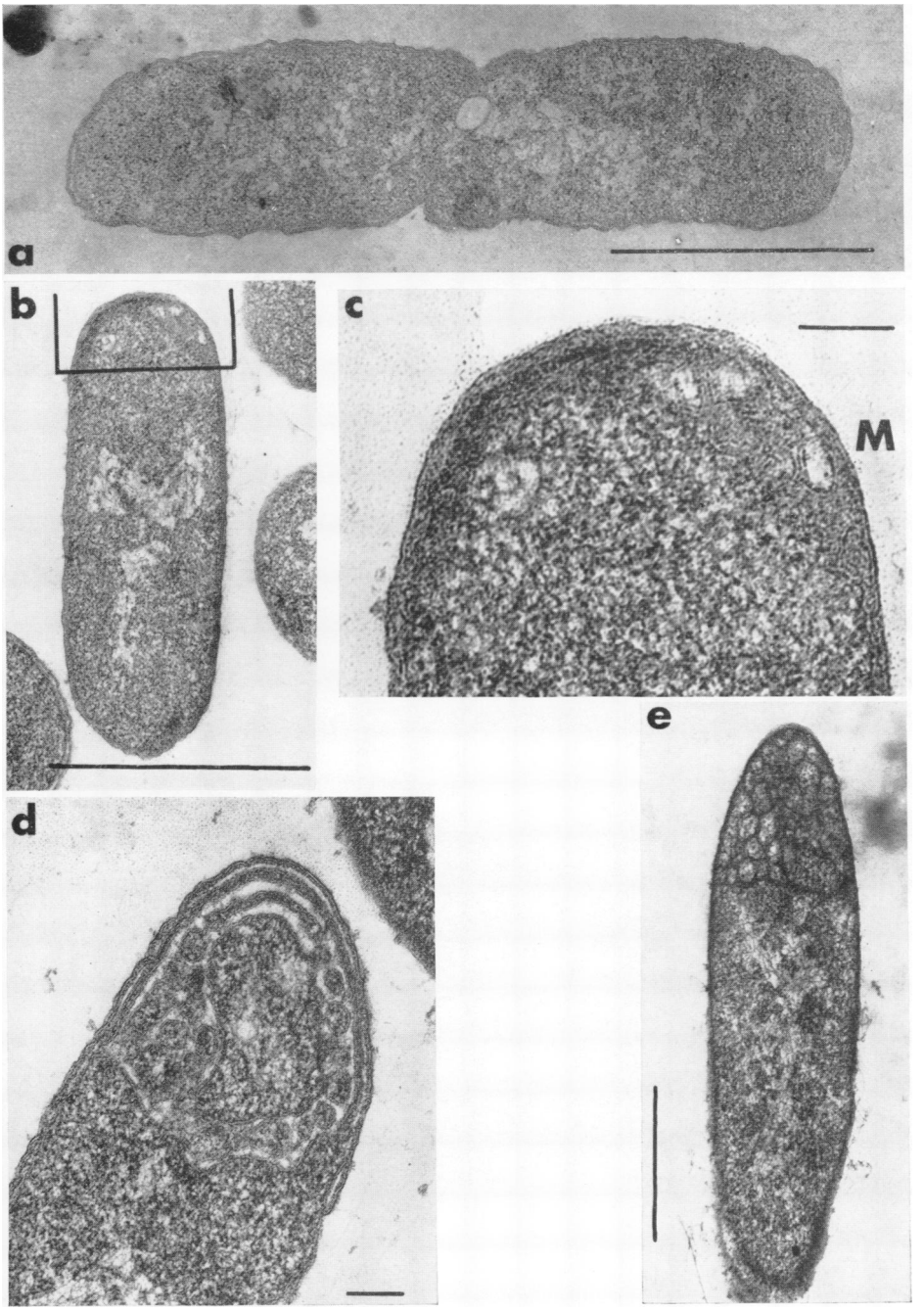


FIG. 37. *After Bradley and Dewar (26), with permission of J. Gen. Virol. (a) Section of uninfected cell of Escherichia coli strain C+/L, × 35,000, scale 1 μ. (b) E. coli C+/L cell 20 min after ZJ/2 infection, × 35,000, scale 1 μ. (c) Marked area from (b) enlarged to × 120,000, scale 1,000 A. (d) E. coli C+/L 60 min after ZJ/2 infection, × 70,000, scale 1,000 A. (e) E. coli C+/L 100 min after ZJ/2 infection, × 20,000, scale 1 μ.*

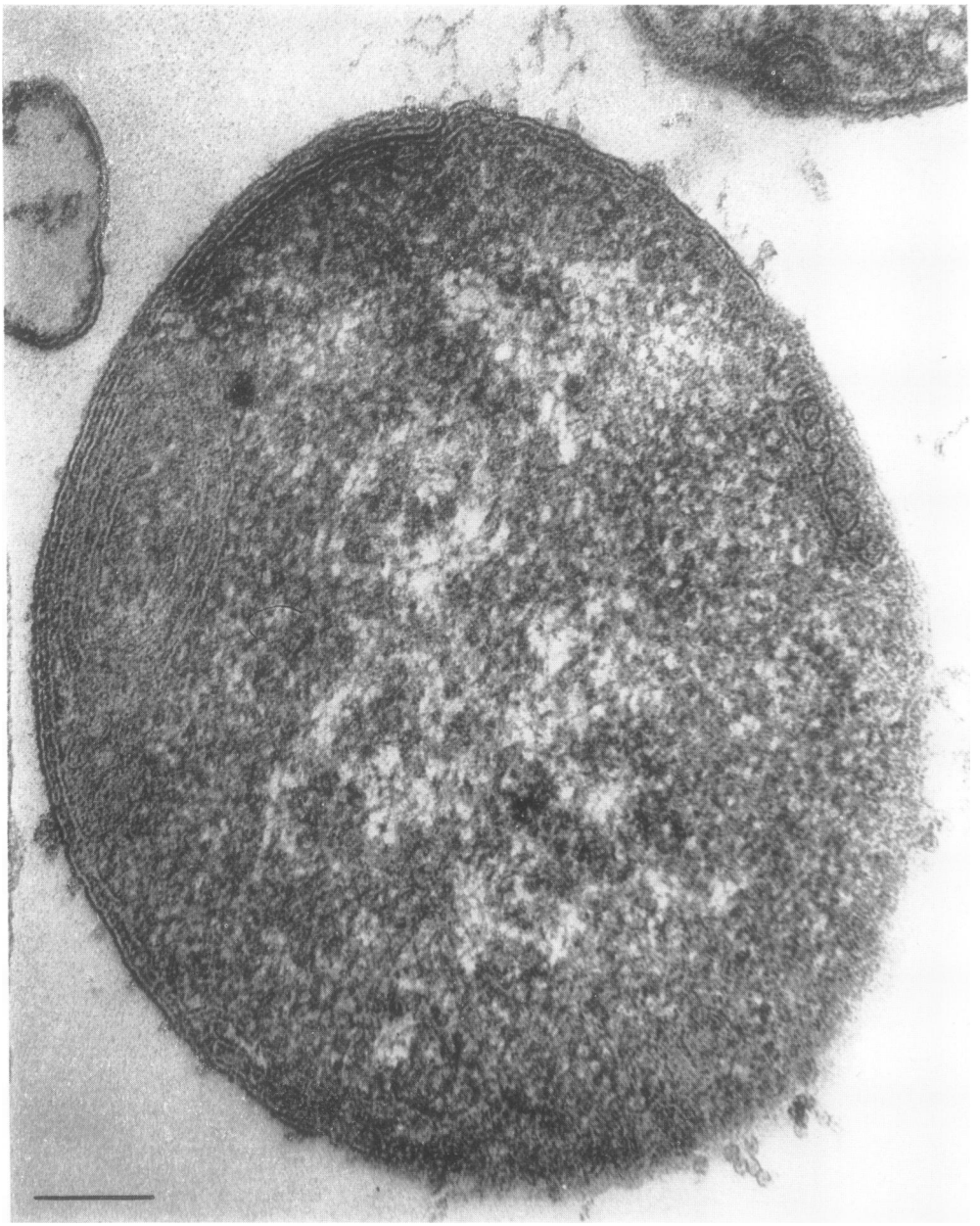


FIG. 38. *Transverse section of Escherichia coli C+/L 60 min after ZJ/2 infection, × 160,000, scale 1,000 A; after Bradley and Dewar (26), with permission of J. Gen. Virol.*

logical to assume that they adsorbed to F-pili or to another receptor coded for by the F episome. In 1966, Caro and Schnös, using the electron microscope, demonstrated unequivocally that the virions attached to the tips of F-pili (37). It can be seen from Fig. 36d (37) that either one or two particles can attach to the tips of the F-pili which are labeled with adsorbed RNA phage virions. The filament A is double the normal length, and probably two virions are attached together. A significant point illustrated by the micrograph is that the adsorption sites of the RNA and filamentous phages are at different loci on the pilus, the sides and tip, respectively; one

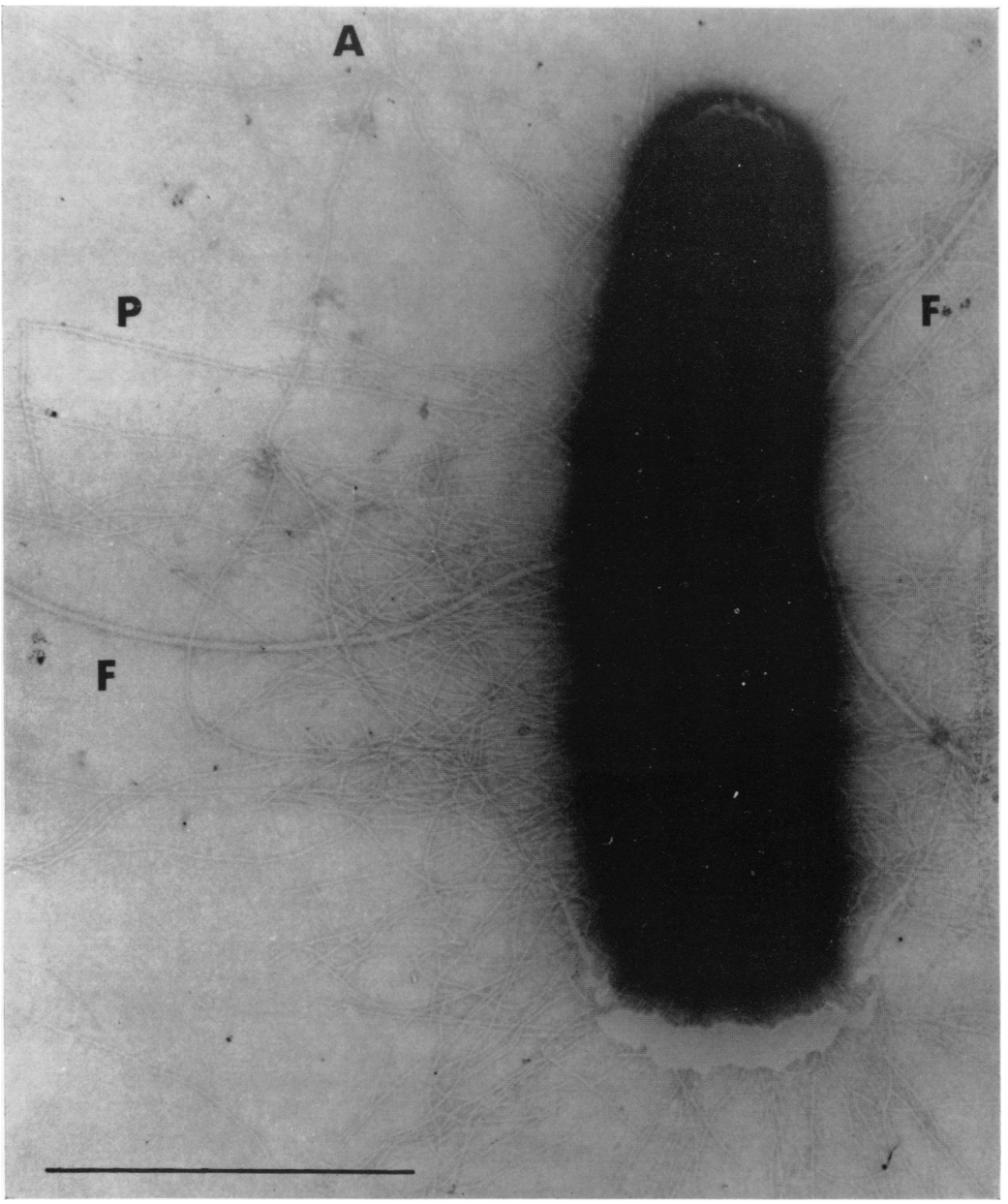


FIG. 39. *Escherichia coli C+/L 120 min after ZJ/2 infection, fixed in glutaraldehyde, negative stained in PTA, × 50,000, scale 1 μ, after Bradley and Dewar (26), with permission of J. Gen. Virol.*

phage does not, therefore, block adsorption by the other. Adsorption to fragments of pili indicates that only one end of a pilus or portion of a pilus is available for adsorption.

These results explain the observations of Tzagoloff and Pratt (213), who found only two or three receptor sites per cell for the isolate M13. It has been proposed that DNA penetration occurs via the F-pili. If so, the spread in penetration time observed by Tzagoloff and Pratt (213) could reflect the different lengths of the F-pili as suggested by Caro and Schnös (37).

After penetration, the DNA initiates marked cytoplasmic changes within the host cell (26).

Some 20 min after initial infection, there is a marked contraction of the nucleoplasm, as shown in Fig. 37b, which can be compared with the uninfected cell in Fig. 37a. At the same time, small mesosomes are formed at the pole (Fig. 37c, M). At 1 hr after infection, the mesosomes are more marked and extend further into the cell (Fig. 37d, e). They also occur at the poles of dividing cells. Figure 38 shows that these mesosomes probably originate from invaginations of both cell wall and cell membrane. Extracellular phages appear concurrently with mesosome formation. Negative staining shows that the host cell is surrounded by a mass of filaments (Fig. 39), the thicker ones being F-pili (P) or flagella (F); two phages appear to be adsorbed to the tip of the pilus at A. In sections, the extracellular phages are less well defined except in transverse section (Fig. 40a, A). They cannot be confused with pili since these do not appear in sections of this strain of *E. coli* (26); they are probably lost during the fixation and embedding process. In no case has it been possible to resolve intracellular phage clearly, but there is an indication in Fig. 40b, which is an enlargement of the marked area in Fig. 40a. At points B, C, and D, extracellular filaments join the cell wall and seem to continue inside (arrows). This micrograph suggests that phage synthesis occurs near the cell surface. The formation of extracellular phage is not accompanied by lysis (26, 92, 183); cell division continues for perhaps one generation afterwards. The remaining contents of the cell then leak out, presumably through the many holes left by extruding virions, to leave an empty ghost containing varying numbers of mesosomes (Fig. 40c). These eventually break up into vesicle-like structures similar to those obtained with T-even phage lysis (Fig. 20c). Two unique features in this process are that the virions are released without actual lysis, and that phage infection initiates the formation of excess cell wall and cell membrane material (26).

Structure and Infective Process of the Blue-green Alga Virus

The discovery in 1963 of a virus for a blue-green alga provided a valuable comparison for virus structure and infection outside, but close to, the bacterial kingdom (166, 181, 182, 187). The virion, which attacks *Plectonema* spp., *Lyngbya* spp., and *Phormidium* spp., closely resembles bacteriophages of the group with short noncontractile tails (166, 187). Electron micrographs of shadowed virions (181) show that they possess undoubted 5:3:2 symmetry, indicating an icosahedral form (Fig. 41a, b) about 660 A in size. Negatively stained virions (196) have a short tail and hexagonal outline, giving an appearance almost identical to coliphage T3 (Fig. 41c). Subsequent studies by Smith et al. (197, 198) indicated that the tail was longer than at first thought (Fig. 41d), though still slightly shorter than the head diameter. The full and empty virions, shown in Fig. 41d and e, appear similar to bacteriophages.

The infective cycle initiates, as with bacteriophages, by adsorption to the cell surface tail first (Fig. 42a). Next, the viral DNA is presumably injected, but no obvious intracellular changes can be observed in the early stages of the infective process (Fig. 41f, 20 min after infection). The micrograph shows a longitudinal section with the photosynthetic lamellae lying parallel to the cell walls, completely surrounding the perimeter of the individual cell. The nucleoplasm is rather diffuse. One hour after infection, small helical structures (Fig. 42d, arrow H) are formed between the now displaced photosynthetic lamellae (Fig. 42d, L). They then move into the space between the displaced lamellae and the cell wall (called the virogenic stroma). In Fig. 42d, many helices can be seen in the virogenic stroma (VS). Helices at high magnification in the virogenic stroma (Fig. 42b, A) have a membrane around them. According to Smith et al. (197), they become compressed to form what are presumed to be spherical mature virions (Fig. 42b, B). This interpretation is based on the observation that, in comparable sections, the elongated helices predominate 1 hr after infection and the spherical particles 16 hr after infection.

A detailed picture has thus been built up of the whole infectious cycle, which consists of the following principal events. First, adsorption to the cell surface occurs; the virions attach, like bacteriophages, by their tails. After injection, virus precursors, in the form of small helices, appear between the photosynthetic lamellae. The latter become displaced and the virogenic stroma is formed; the virus precursors move into this area where they obtain capsids. They then change their elongated shape to the nearly spherical configuration (in sections) of the mature virion. Cell lysis finally occurs after about 16 hr, leaving a skeleton of photosynthetic lamellae with a few virions embedded in it (Fig. 42c).

Multiplication of the Parasite Bdellovibrio bacteriovorus in Susceptible Bacteria

B. bacteriovorus (201, 202) is a small highly motile bacterium which is parasitic on species of *Erwinia* and *Pseudomonas*. Its life cycle bears a certain resemblance to the infective process of a bacteriophage, so that a description, as given by Starr and Baigent (199), is included here for comparison.

The *Bdellovibrio* cell itself is a very small

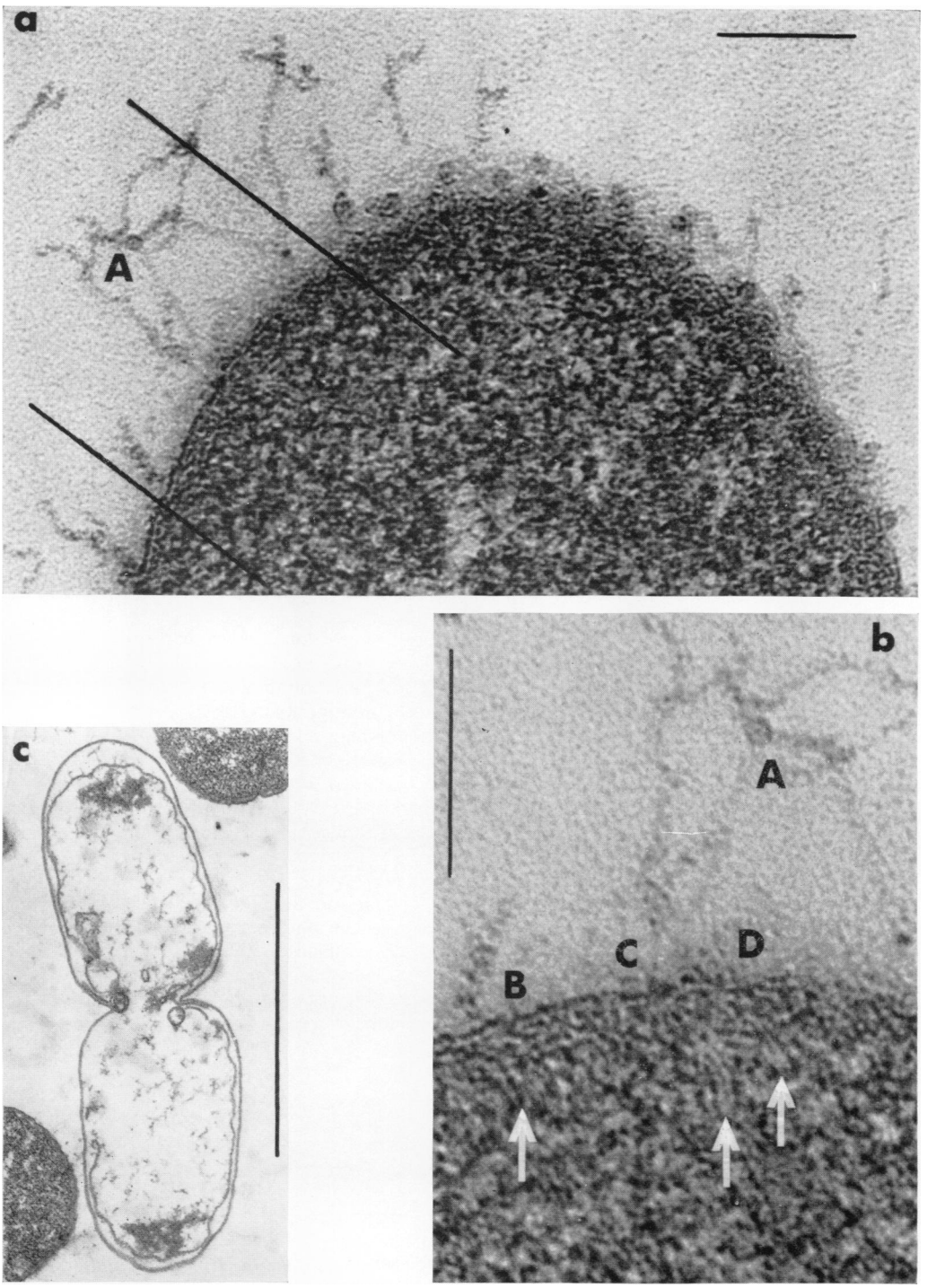


FIG. 40. *After Bradley and Dewar (26), with permission of J. Gen. Virol. (a) Section of cell of Escherichia coli C+/L 60 min after ZJ/2 infection showing extruding filamentous phages, × 200,000, scale 1,000 A. (b) Marked area from (a), × 333,000, scale 1,000 A. (c) Empty cell of E. coli C+/L 80 min after ZJ/2 infection, × 40,000, scale 1 μ.*

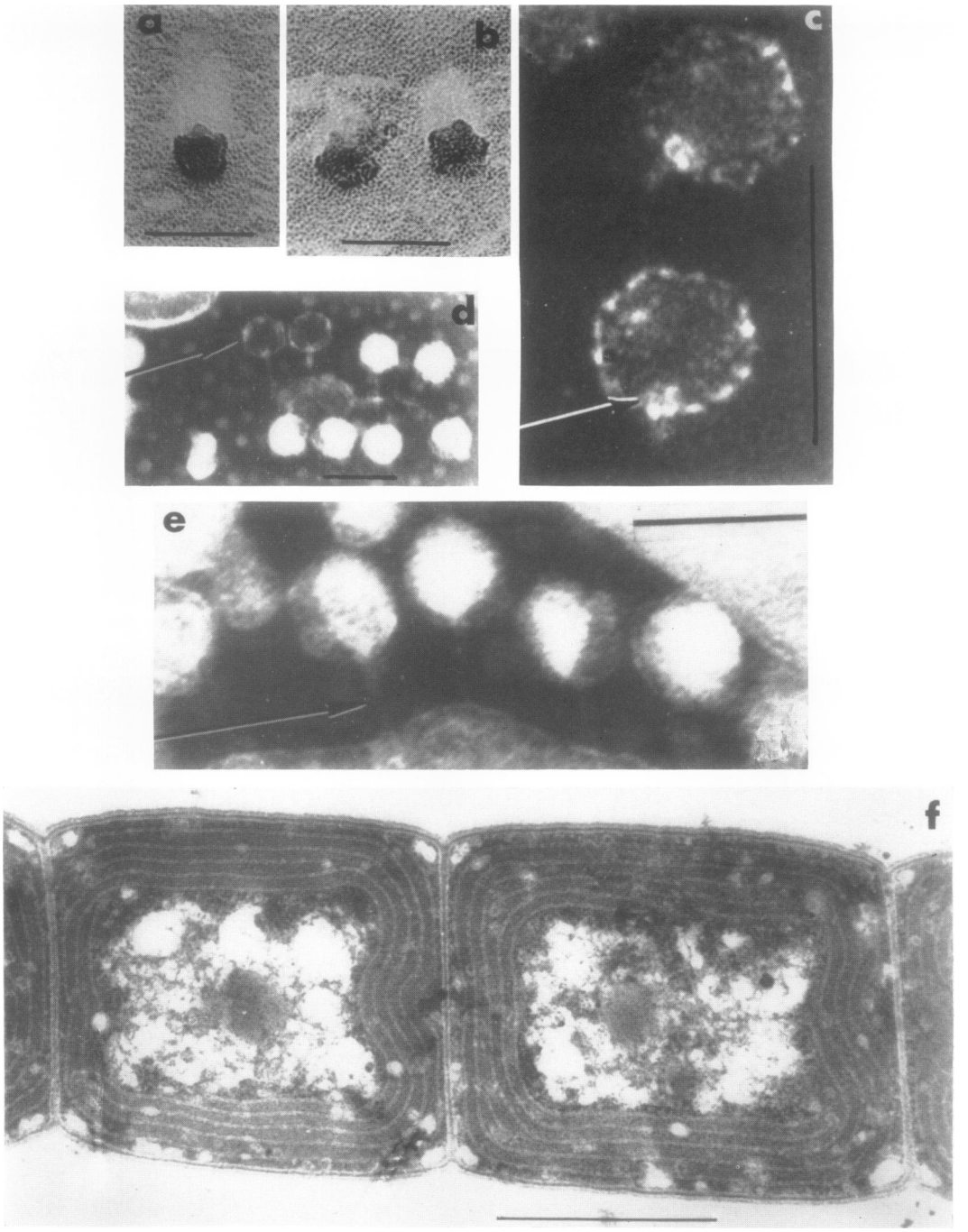


FIG. 41. *(a), (b) Shadowed particles of algal virus LPP1 (181), × 155,000, scales 1,000 A; after Safferman and Morris (Science* **140:** *679–680, 1963), with permission of the Editors. (c), (d), (e), (f) With permission of Virology, Academic Press, Inc. (c) Negative-stained algal virus, protein tail plug arrowed, × 400,000, scale 1,000 A, after Smith et al (196). (d) Algal virus negative stained in PTA, empty virions arrowed, × 100,000, scale 1,000 A; after Smith et al. (198). (e) As (d), × 250,000, scale 1,000 A. (f) Section of Plectonema boryanum 20 min after infection with algal virus, × 30,000, scale 1 μ; after Smith et al. (196).*

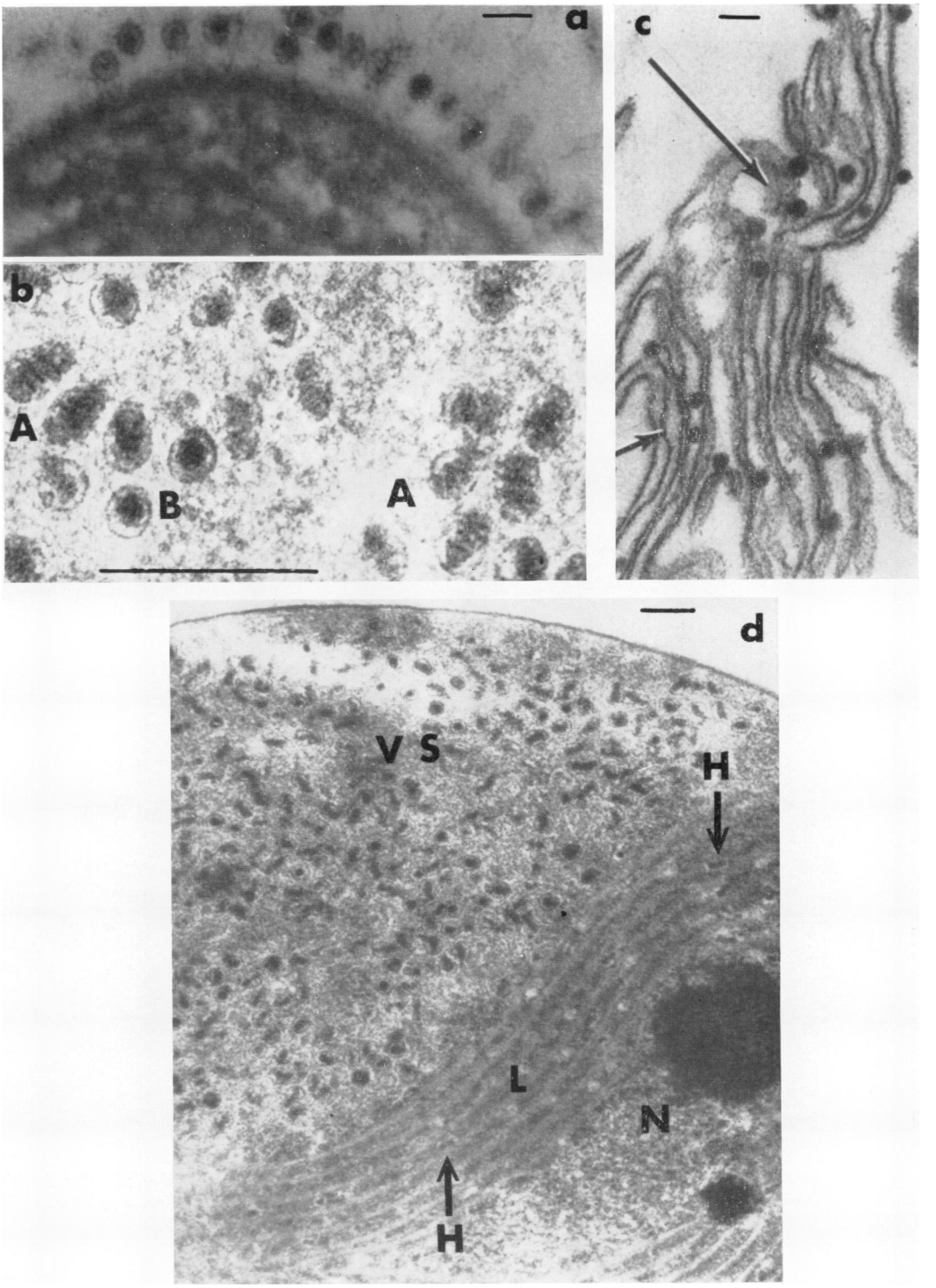


FIG. 42. *(a) Algal virus adsorbed to cell surface, section, × 70,000, scale 1,000 A, after K. M. Smith. (b), (c), (d) With permission of Virology, Academic Press, Inc. (b) Algal virus helices, × 333,000, scale, 1,000 A, after Smith et al. (197). (c) Remains of lysed cell of Plectonema boryanum, virions are arrowed, × 60,000, scale 1,000 A, after Smith et al. (196). (d) Section through cell of P. boryanum 60 min after infection; VS, virogenic stroma; N, nucleoplasm; H, helix; × 80,000, scale 1,000 A. After Smith et al. (197).*

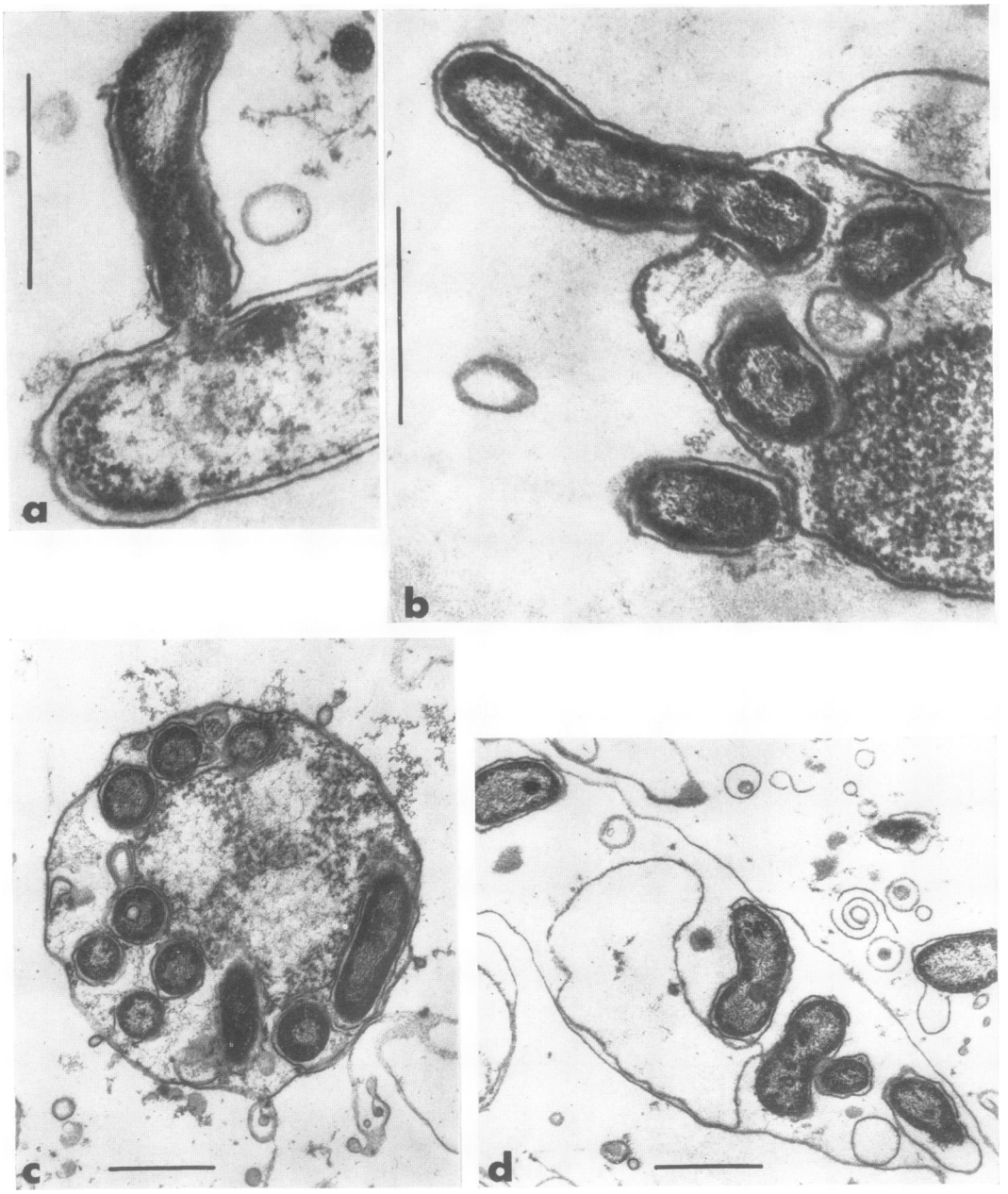


FIG. 43. *After Starr and Baigent (199), with permission of J. Bacteriol. (a) Pseudomonas tabaci cell with adsorbed Bdellovibrio, × 60,000, scale 5,000 A. (b) Bdellovibrio organism entering Erwinia amylovora cell, × 60,000, scale 5,000 A. (c), (d) Infected P. tabaci cells, × 30,000, scales 5,000 A.*

bacterium only 0.8 to 1.0 μ long. It has a well-defined nucleoplasm surrounded by densely packed granules which are presumably ribosomes. Occasional mesosomes are present. The predatory cycle starts by the violent contact of the *Bdellovibrio* organism with its much larger host cell; this initial attachment is reversible. Next a pore is formed in the host cell wall (Fig. 43a) and the parasite passes into the cytoplasm; a cell in the process of penetration is shown in Fig. 43b. It can be seen from Fig. 43c that several complete parasites enter the host, the contents of which

soon become completely disorganized, the nucleoplasm disappearing and the cytoplasm loosing its original coherence. At the same time, the host cell membrane shows signs of disintegration, and it finally disappears, leaving the cell wall to form a spheroplast. After invasion, the original *Bdellovibrio* organism increases its size to three or four times its original volume as it digests the cell contents; two or three divisions then occur. The parasites remain inside the host until the whole of the contents have been absorbed. An empty cell envelope containing the *Bdellovibrio* organisms is all that is left (Fig. 43d). The former lyses to release the parasites for another cycle. The cell wall debris is similar in appearance to that formed by the bacteriophage lysis of *P. aeruginosa* and *E. coli*.

The resemblance between phage and *Bdellovibrio* infection is only superficial. These two totally different biological entities utilize the host cell contents in very different ways. The cellular parasite uses it as a nutrient medium and divides normally, whereas the phage employs the cell metabolism for the synthesis of its own DNA and protein. One process is nutritional and the other genetic. Lysis is very similar in both cases; spheroplast formation occurs as with RNA *Pseudomonas* phages, and the cell contents are released by the breakdown of the cell wall.

COMPARISON OF BACTERIOPHAGE INFECTIVE PROCESSES

It is clear from the foregoing that a great deal of variation exists in the ways in which different phages carry out the basic stages in the infective process. The adsorption of phage particles depends upon the existence of specific receptors and these are of two fundamentally differing types: those which are integral with the cell wall, and could be called somatic receptors, and those which are not (F-pili, and flagella). The type of phage which adsorbs directly onto the cell wall has a tail of some sort, which presumably "selects" the receptor; the one exception is ϕX174 (morphological group D). Even this phage does not, however, have a smooth capsid, since each apex of its icosahedron has an external capsomere, one of which probably acts as a tail. Phages which attach to extracellular receptors are of three different morphological types. The first, adsorbing to flagella (108, 154), has a long non-contractile tail (group B), and the remaining two are the RNA and filamentous types (groups E and F). The small round RNA virions can pack in large numbers along the length of the pilus, which might ensure that any abortive injection of nucleic acid is quickly replaced by a successful one. The filamentous types are morphologically similar to their receptors and lock firmly onto the pilus tip; the method used by the virions in "selecting" the tip of the F-pilus must be extremely effective.

Too little is known about the actual process of nucleic acid injection to make a valid comparison between the various types of phages, but it seems likely that a different process will be involved for somatic receptors compared with extracellular ones. The passage of nucleic acid down an F-pilus, if such occurs, may be stimulated in a manner similar to that involved in the transfer of DNA in bacterial conjugation: according to one theory, mating between male and female cells involves the transfer of DNA through the F-pilus.

Whereas the intracellular multiplication of all phages is basically the same, the visible cytological effects vary greatly. In most cases, the nucleoplasm undergoes some contraction or condensation. Mature phage particles may form within it, as with T-even types, or in the cytoplasm, like the RNA forms, which occur at a sufficiently high concentration to aggregate into crystals. However, the majority are more or less evenly distributed throughout the cell. The formation of intracellular phage is not usually accompanied by any other gross cytological changes until just before lysis. The exceptions to this are the *Pseudomonas* RNA phages and the filamentous coliphages. In the first case, phage maturation is accompanied by a weakening of the cell wall and spheroplast formation; in the second, excess cell wall and membrane material appears to be produced. These functions seem to be associated with specialized methods of phage release not found with other types.

The release of phage progeny is usually accompanied by lysis of the cell, the envelope disintegrating to liberate the contents. In some cases, including the RNA and filamentous coliphages, complete break-up of the cell envelope does not occur until long after the contents have leaked out through small holes. With T-even and some other phages, however, lysis is accelerated by the action on the cell wall of lysozyme-type enzymes synthesized after infection. With RNA *Pseudomonas* phages, lysis seems to be brought about by the weakening of the cell wall followed by spheroplast formation and osmotic rupture, as with the parasitic bacterium *Bdellovibrio*.

It is evident that there are indications of a pattern linking the same morphological types of phages to particular variations in their "life cycle." The tailed phages follow more or less the same lines, regardless of morphological type; even the blue-green alga virus adsorbs tail first and lyses its host in the normal way. The RNA

and filamentous phages seem to have characteristic effects on the host.

There are two physiological processes associated with bacteriophages which are quite distinct from the cycle of the virulent phages just described. In the first (temperate phage infection or lysogenization), adsorption proceeds in the normal way according to morphological type, but, after injection, the phage genome attaches itself to the bacterial chromosome and does not cause the formation of intracellular phage: the viral genome replicates in synchrony with the bacterial chromosome in the form of an episome. Vegetative phage is formed only under a metabolic stimulus when normal intracellular multiplication and lysis follow. The second process, known as "lysis from without" (55) is largely confined to contractile phages (comparatively few have been studied). It amounts to the mechanical perforation or possibly the chemical degradation of the cell envelope followed by its lysis by osmotic shock. It is caused by the adsorption of large numbers of virions; at high multiplicities of infection, (about 200:1) cultures of host bacteria are lysed in a few minutes. In some cases, spheroplasts are formed (115). Cota-Robles and Coffman (46) showed by electron microscopy that the lysis from without of *E. coli* B by phage T2 was an explosive effect causing the release of the cell contents. They found no visible changes in the cell wall.

NATURE AND REPRODUCTION OF BACTERIOCINS

Bacteriocins are a group of specific bactericidal particles, each apparently acting on the cell in more or less the same manner. It is evident, however, that there are two very distinct basic types. One is a small molecule, which is usually thermostable, cannot be sedimented in the ultracentrifuge, and has not been resolved in the electron microscope. The other is very much larger, is easily sedimented, and appears in the electron microscope as a phagelike object or phage component such as the tail assembly. The common feature of the two types is that they are protein in nature (175). The only detailed work on chemical structure has been carried out on colicins (bacteriocins of *E. coli*) and has been discussed by Cocito et al. (41) and in reviews by Fredericq (73) and Ivánovics (102). The two bacteriocin types are not necessarily associated with different bacterial species. For example, colicin V forms a large clear area on agar and is dialyzable, thus belonging to the first group. Colicin 15, on the other hand, has a molecular weight exceeding 200,000, is sedimentable, and can be seen in the electron microscope to consist of a mixture of empty phage heads and intact, tailed phagelike objects (184).

Another common feature of all bacteriocins is the manner in which they are perpetuated in nature. Their genetic determinants exist in the cell as extrachromosomal elements analogous to those associated with temperate bacteriophages (107). They replicate in phase with the bacterial chromosome and are thus maintained as long as the bacteriocinogenic strain exists. Active bacteriocin is released in quantity by induction with metabolic inhibitors, or lysis by phage infection or other agents. However, a small amount of bacteriocin is present in normal cultures of the organism; it is produced, presumably, by the spontaneous lysis of a few cells by some unknown factor.

C factors or *col* factors (episomal elements or plasmids for colicins) may be compared with F (fertility) factors and temperate phages. While closely resembling these two types of episome, there are some differences. Temperate phages are attached to the bacterial chromosome, the phage genome becoming integrated with it, but the F factor can exist either attached to the bacterial genome or in an autonomous state in the cytoplasm. Some C factors, on the other hand, exist entirely in the autonomous state. It is a point of similarity that in some cases both C factors and F factors can be simultaneously transferred during mating between male and female strains of *E. coli* K-12 as extrachromosomal elements (40, 74, 161). Nagel de Zwaig and Anton (160) demonstrated the simultaneous transfer of the colicinogenic plasmids which control the production of colicins V and I, and the F factor. Undoubtedly, a close relationship exists between the C-factor and the F episome, but Nagel de Zwaig (159) was able to separate the two. The transfer of C factors during mating constitutes a mode of proliferation in a bacterial population.

NOMENCLATURE OF BACTERIOCINS

The classification, and hence the nomenclature, of bacteriocins has room for improvement. The existing names, which are given to the various bacteriocin families, are based on the classification of their bacterial hosts. Due perhaps to the high degree of specificity of bacteriocins, the name is almost always based on the specific rather than the generic name of the host organism. Thus, colicins are bacteriocins of *E. coli*, monocins of *Listeria monocytogenes*, and so on. A list of principal names is given in Table 6. So many colicins have been isolated and studied that Fredericq found it necessary to subdivide them, basing his classification on the spectrum of re-

TABLE 6. *Names of principal bacteriocins*

Host organism	Bacteriocin	Reference
Escherichia coli	Colicin	78
Aerobacter aerogenes	Aerocin	84
Serratia marcescens	Marcescin	82
Pasteurella pestis	Pesticin	10
Pseudomonas aeruginosa (= *P. pyocyanea*)	Pyocin	105
Listeria monocytogenes	Monocin	83
Bacillus cereus	Cerecin	85, 151
Staphylococcus spp.	Staphylococcin	69, 85

sistance of various resistant mutants of *E. coli* against colicins (70). Arising from this is a code of nomenclature in which the number of the producer strain is followed by the original letter designation given by Fredericq (70). For example, colicin CA23-D is a type D colicin (70) produced by *E. coli* strain CA23.

PRODUCTS OF THE INDUCTION OF BACTERIA

One of the most efficient ways of producing large amounts of bacteriocin for electron microscopy or other studies is by inducing the bacteriocinogenic strain with a suitable metabolic inhibitor, one of the best being mitomycin C. The addition of a small amount of this antibiotic to a log-phase culture causes the release of bacteriocin together with numerous other substances. Clearly, it is most important to distinguish the bacteriocins from other proteins, a task in which the electron microscope is of considerable value. However, it must be remembered that both temperate and genetically defective bacteriophages can also be produced by induction. Infectivity tests are required to recognize temperate phages, but defective phages are virtually indistinguishable from large bacteriocins. In view of the extensive use of the induction process in producing bacteriocins, a description is included here of the various kinds of particles which are likely to be encountered in such preparations. Although those described originate from different species of bacteria, it has been found that in many cases they have a similar appearance.

Particles Obtained from Some Enterobacteriaceae

The lysis of *Aerobacter cloacae* produced a great variety of particles from one strain. After the removal of bacteria, the contents of the culture fluids were centrifuged and resuspended in 0.1 M ammonium acetate for electron microscopy. The most numerous objects were oval transparent "phage heads" (Fig. 44a). Occasionally full "heads" of more or less the same shape could be found (Fig. 44b). These closely resembled the heads of a *Staphylococcus* phage (Fig. 10c, d), but there was no long tail, the empty particle merely having a small knob at one end. Another lysate of the same species yielded a concentrated suspension of rod-shaped particles (Fig. 44c, d) which are similar to but not identical with a number of pyocins to be described below. In addition to the rods, Fig. 44d shows some thin hollow tubes and small round objects (arrow). The rods are considered to be analogous to the headless tails of contractile phages, and the tubes are probably the cores which belong to them. The small round particles are morphologically identical to RNA-polymerase molecules seen end-on (43, 75). Occasionally, small striated rectangles could be found (Fig. 44e) similar to RNA-polymerase molecules lying on their sides. The structure of identical particles obtained from *P. aeruginosa* is described in detail below (see Fig. 46c). The strain of *A. cloacae* examined here was not active against an indicator (strain 623.60) provided by Hamon (Institut Pasteur, Paris, France), but the possibility that it is bacteriocinogenic cannot be ruled out. Three cloacin-producing strains of *A. cloacae* (2403.60, 1400.60, 6510.60), also provided by Hamon, again produced rod-shaped headless tails and numerous empty "heads," usually showing a hexagonal outline like those in Fig. 45d. A series of aerocin-producing strains of *A. aerogenes* gave a similar range of particles. With both species, it was found that some strains produced one type of particle, and others two types, both phagelike components. The RNA-polymerase-like objects were universally encountered. One aerocin (1022.58, Hamon) proved to be nonsedimentable and thermostable, and therefore to belong to the "small molecule" group of bacteriocins. It was not possible to determine whether or not the phagelike components were bactericidal towards the other strains.

The induction of a strain of *Alcaligenes faecalis* produced a suspension of phagelike objects with long noncontractile tails (Fig. 44f). Since a strain of *A. faecalis*, against which they were biologically active, could not be found, it was not possible to classify them as phages, killer particles, or bacteriocins.

Several colicinogenic strains of *E. coli* were also lysed with mitomycin C and the culture fluids were prepared for electron microscopy. As was to be expected, a large number of different types of particles were encountered. Strain 3000 (17), an F^+ male, produced curious tail-like assemblies (Fig. 45a), not unlike the rods already

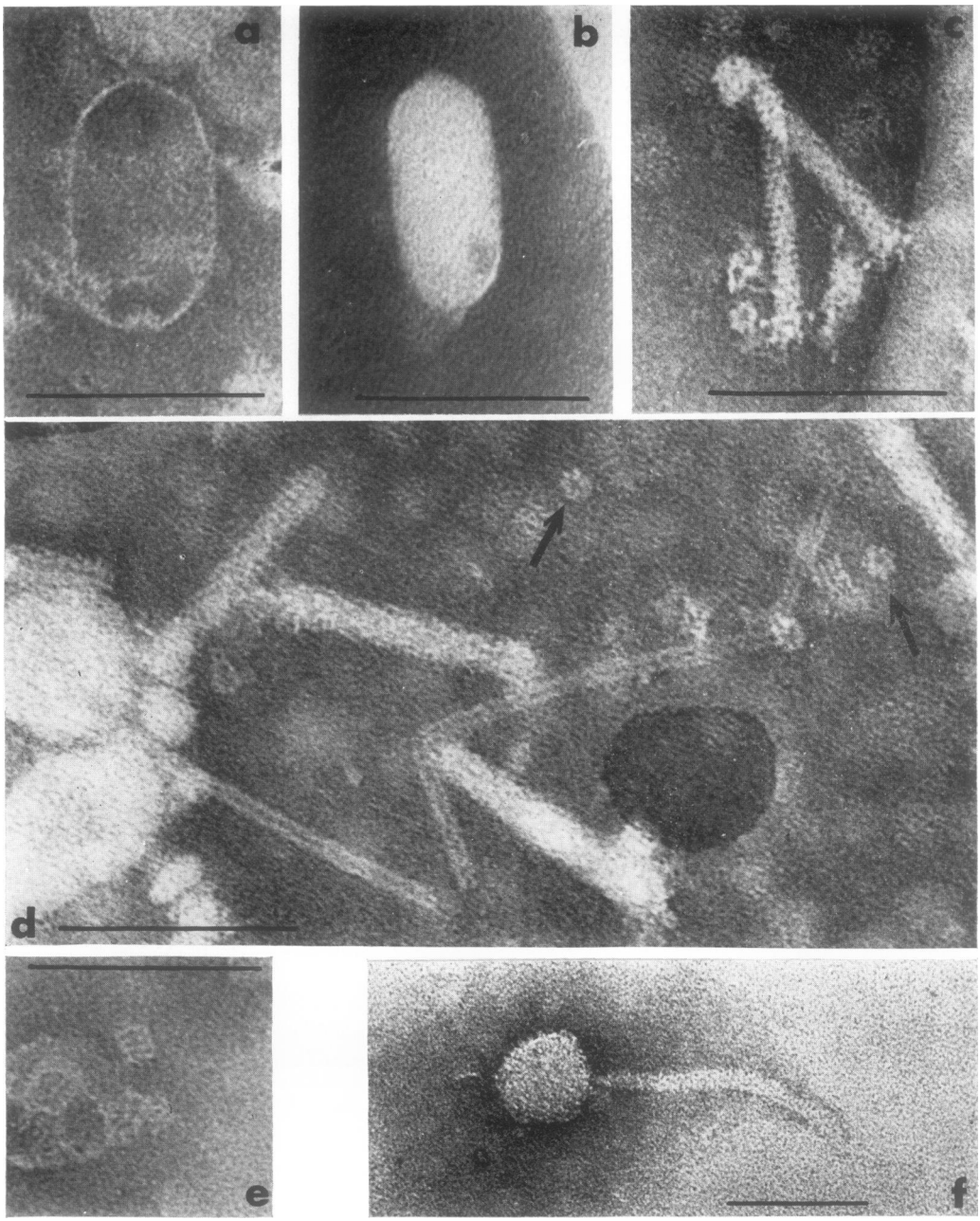


FIG. 44. *(a), (b), (c), (d), (e) Products of mitomycin C induction of Aerobacter cloacae, PTA, × 333,000, scales 1,000 A. (f) Phage-like particle from mitomycin C induction of Alcaligenes faecalis, × 200,000, scale 1,000 A.*

described but with contracted sheaths. Strain CA 23, producer of colicin D, gave a small phagelike object (Fig. 45b) which is of particular interest because of the small head size. The smallest known head size for a normal bacteriophage with a long noncontractile tail is about 500 A, whereas in this case it is only 300 A. The only other tailed phagelike particle with such a head diameter is specific for *Bacillus* spp. and behaves like a bacteriocin (189). No activity could be associated

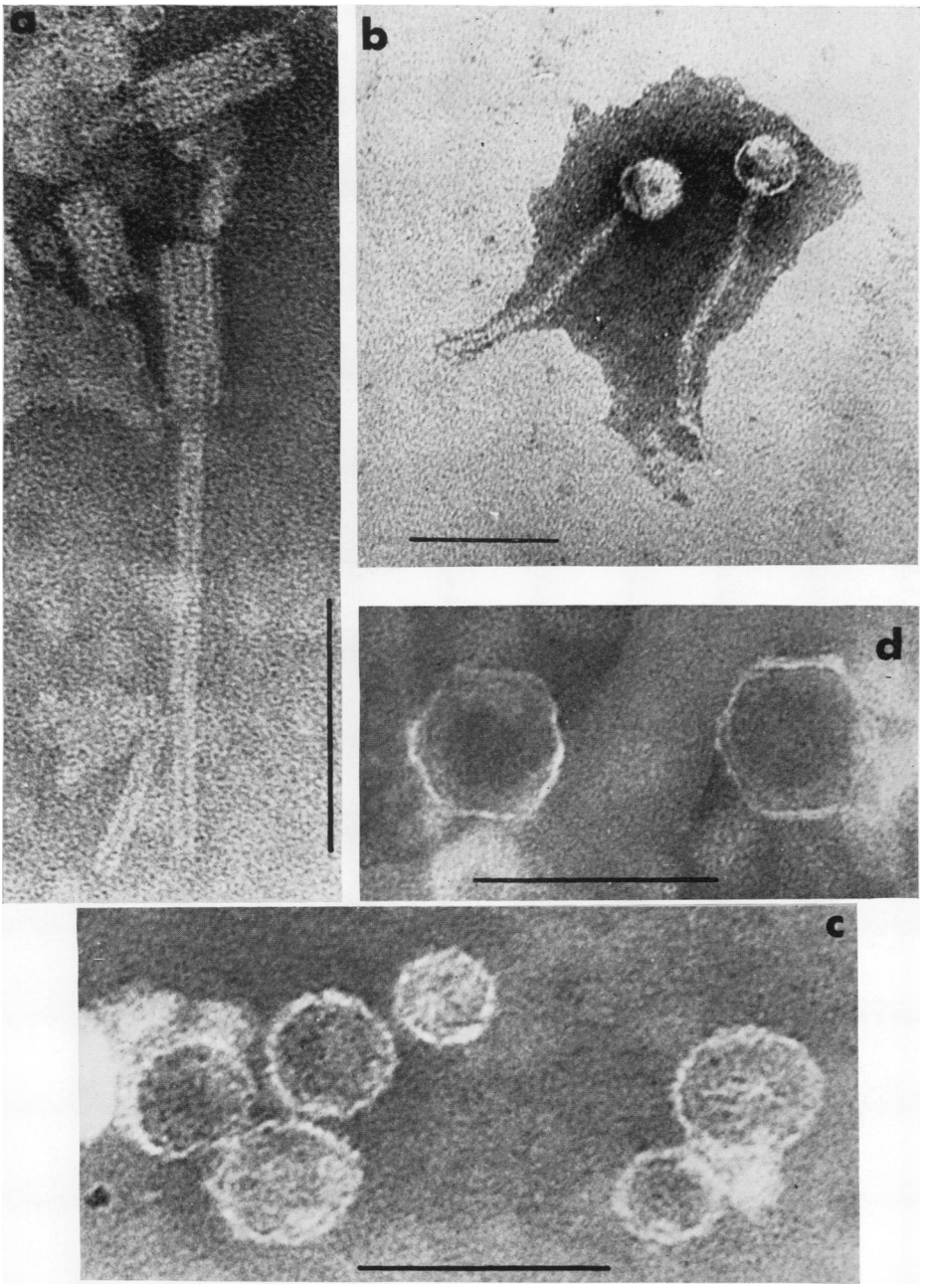


FIG. 45. *PTA, scales 1,000 A. (a) Products of mitomycin C induction of Escherichia coli strain C3000, × 333,000. (b) Phage-like objects from mitomycin C-induction of E. coli strain CA23, × 200,000. (c) Crinkled spheres found in many mitomycin C-induced lysates of various E. coli strains, × 333,000. (d) Empty hexagonal phage-like objects produced by inducing Pseudomonas aeruginosa with mitomycin C, × 333,000*

with the particle from strain CA23 with any degree of certainty. The same strain produced large numbers of RNA-polymerase type particles and small rings like those illustrated in Fig. 46b for *P. aeruginosa*. A particularly common feature of *E. coli* lysates was the empty round objects looking like phage heads (Fig. 45c). Their main distinguishing feature was a crinkled appearance.

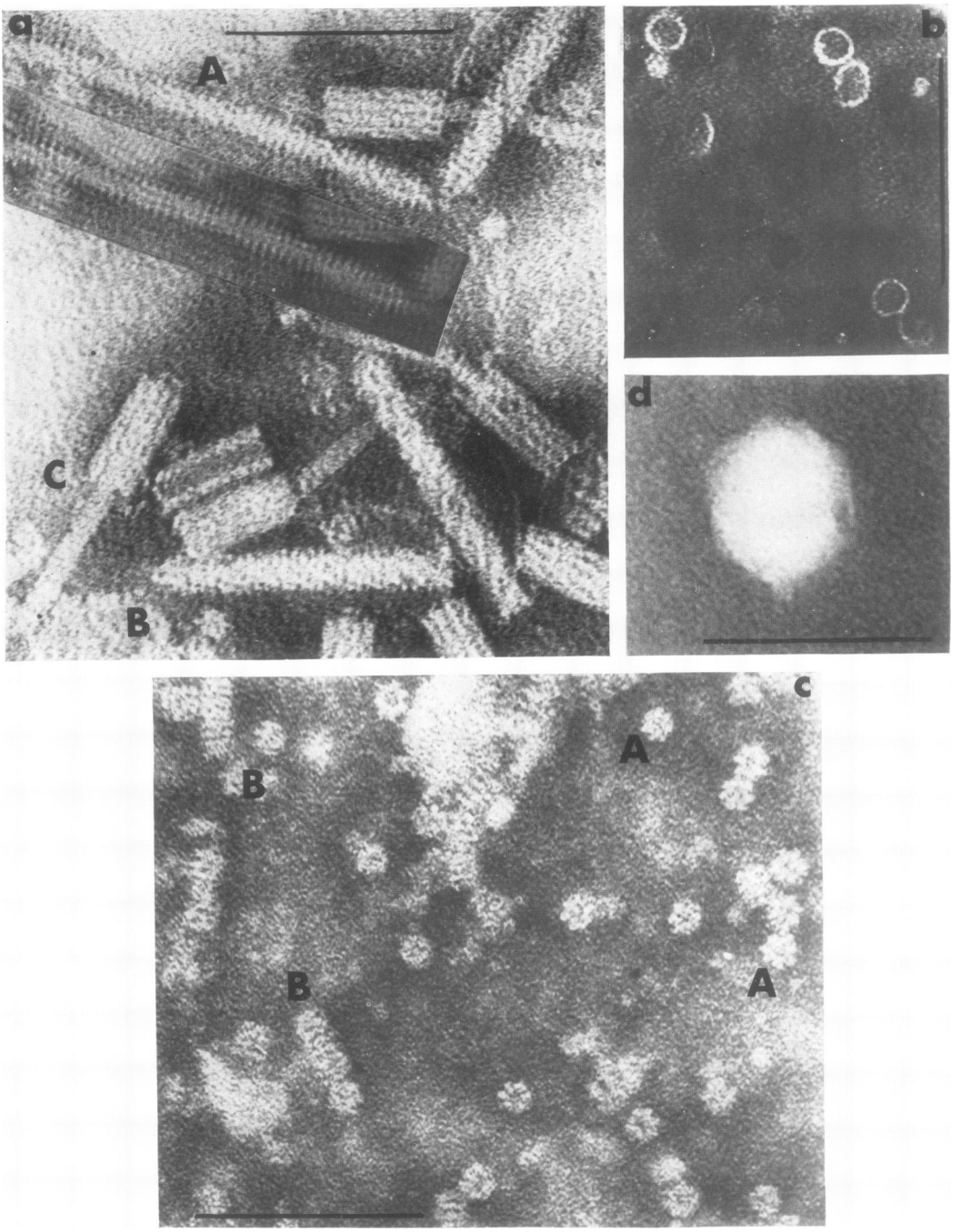


FIG. 46. *PTA, × 333,000, scales 1,000 A. (a), (b), (c) Products of mitomycin C induction of Pseudomonas aeruginosa strain Götze (22); with permission of Maruzen Co. Ltd., Tokyo. (d) Phage-like object from mitomycin C induction of Streptococcus faecalis.*

Though usually monodisperse, a few smaller or larger ones could be found in the same preparation, as shown in the micrograph. Their importance will be mentioned below. Hexagonal empty "heads" like those in Fig. 45d for *P. aeruginosa* were also produced.

Particles Obtained from Pseudomonas spp.

Mitomycin C lysates of a number of fluocin-producing strains of *P. fluorescens* produced three types of particles: rod-shaped headless contractile tails more or less similar to those described above, empty hexagonal tail-less heads, and RNA-polymerase type particles. Mixtures were often encountered, but the two phage-like components were also found separately.

An electron microscopic examination of the products of the mitomycin C lysis of the pyocin-producing *P. aeruginosa* strain Götze (from Hamon) gave a complete picture of the range of objects most often produced by this species (22). The commonest type of particle was the familiar headless contractile tails (Fig. 46a), both extended (B) and contracted (C). In addition, headless long noncontractile tail-like objects were found (A, inset printed by linear integration). A further compoment, with a sedimentation coefficient of only 8*S* (Fig. 46b) was considered a segment of subunits from a contracted sheath. RNA-polymerase type particles were produced in particularly large amounts (Fig. 46c). Their structure and dimensions compared very closely with those of RNA-polymerase molecules obtained from *E. coli* (43, 75). Particles at A in Fig. 46c are end-on with an apparent sevenfold radial symmetry (22), whereas those at B are short chains in side view. Since the sedimentation coefficient of 22*S* measured from this preparation is close to the value of 23.7*S* quoted by Fuchs et al. (75), and the morphology is identical, it is reasonable to assume that the micrographs shown here represent the same enzyme. Several other pyocin-producing strains of *P. aeruginosa* gave empty hexagonal "heads" (Fig. 45d). It is evident from these observations that the products of mitomycin C induction are generally similar in appearance for both *Pseudomonas* species mentioned.

Particles Obtained from Streptococcus faecalis

The induction of *S. faecalis* causes the production of several kinds of particles unlike those described for gram-negative bacteria. The commonest type of particle found in a lysate of strain NCIB 8256 (NCTC 8175), which produces a bacteriocin (34), is shown in Fig. 46d. It looks like coliphage T3, has a short noncontractile tail, and occurs both full and empty. The next most frequent is a headless tail (Fig. 47a); it is only about 1,000 A long and 70 A thick, with extremely fine cross-striations and a collection of fibers at one end. While resembling a phage tail, it is quite different from anything described so far. The lysate also contained a small number of intact phagelike objects (Fig. 47b) with contractile-type tails and empty heads. Even more infrequently, hollow tubes could be found (Fig. 47c); these could well be polymerized sheath material from the previous particle.

Identification of the Bactericidal Principle

It can be seen from the foregoing that it is not an easy matter to determine which of the many objects released from a bacterium is responsible for bacteriocin activity. With bacteriophages, which multiply and provide more or less homogeneous preparations, it is a simple matter to distinguish the characteristic particle present in infective cultures by its absence in noninfective ones, as observed in an electron microscope. Bacteriocins, however, do not proliferate, so that the active principle cannot outgrow any inactive particles with which they might be confused. Biological purification procedures such as plaque-picking cannot therefore be employed, and one must resort to physical and chemical methods.

One of the earliest attempts at identifying a colicin in the electron microscope (121) employed the direct electron microscopic examination of lysing induced bacteria, but it was not possible to determine the active principle. Sandoval et al. (184) adopted the following procedure in a study of colicin 15 (158). Bacteria-free induced lysates were precipitated with ammonium sulfate and the centrifuged pellets were suspended in water. This solution was then chromatogaphed on a DEAE (diethylaminoethyl cellulose) column. Fractions from a stepwise elution were tested for activity against sensitive indicators; the most active fractions were pooled and the chromatography was repeated. Biological activity in the eluate closely followed the optical absorbance intensity at 280 mμ, and electron microscopy of the active fractions revealed a mixture of empty tail-less heads and intact contractile-type phage particles. The particulate form of the active principle was confirmed by differential centrifugation and gel filtration in a Sephadex column. In the latter, the excluded phase contained most of the active principle, which again showed phage components in the electron microscope. Since no bacteriophage activity could be detected, it was concluded that either the empty heads or the intact phagelike particles were the active constit-

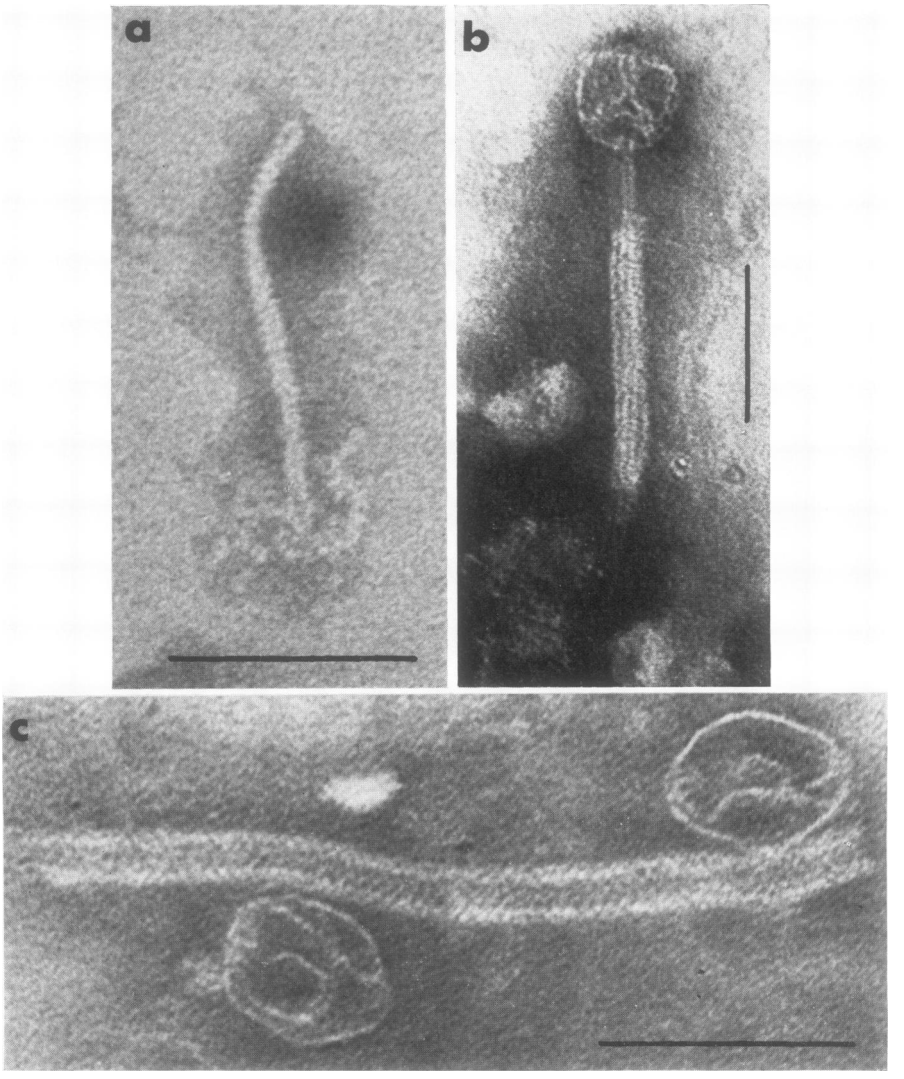


FIG. 47. *Products of induction of Streptococcus faecalis by mitomycin C, PTA, scales 1,000 A. (a), (c) × 333,000. (b) × 200,000.*

uent of the colicin. The only way to separate them would have been by gradient centrifugation.

It will be recalled that there are two basic kinds of bacteriocin, one small with low molecular weight and one much larger; the latter is sedimentable and the former is not. The recognition of this fact means that it is at least a simple matter to place a given bactericidal principle into the appropriate category without the comparatively complicated purification mentioned above. Bacteria are removed from an induced culture by low-speed centrifugation, and the supernatant fluid is centrifuged again at about 40,000 × *g* for 3 hr, sedimenting any phage components, but not small molecules. After washing several times with intermediate low-speed runs to remove coarse debris, a suspension of the pellet can be examined in an electron microscope and tested for activity. If there is no activity, whatever objects may be found in the electron microscope are obviously not the bactericidal principle, which must reside in the supernatant fluid from the original centrifugation. This is again centrifuged at 40,000 × *g* for 3 hr and decanted for a high-speed run at 150,000 × *g* for 6 hr or more. If a spot test from the top of the centrifuge tube is active, the principle clearly belongs to the small molecule type.

If a sedimentable active principle appears

homogeneous in the electron microscope, there is a possibility of proper identification. Care must, however, be exercised in determining homogeneity, since in the electron microscope a concentration of 10^7 to 10^8 particles per ml will only be represented by about one per grid square with the negative-staining technique. Such a concentration could give marked activity, so that the particle concerned might easily be overlooked among a large number of predominant objects. Proper separation of a mixture can be achieved only by gradient centrifugation.

Although this simple procedure of differential centrifugation has not been widely tested, it has been found to work on some colicins and pyocins to be described below and on the streptococcin already mentioned. In the latter case, most of the activity was removed from the supernatant fluid by centrifugation at 40,000 $\times$ *g* for 3 hr, further demonstrating the effectiveness of the procedure. As will be described, two active principles were found to be produced by one colicinogenic strain (*E. coli* K-235), one centrifugeable and one not. One point seems fairly certain: the ubiquitious RNA-polymerase type particles have no bactericidal activity.

STRUCTURE OF BACTERIOCINS

Comparatively little work has been done on the physical structure of bacteriocins, perhaps because of the difficulty of positive identification in the electron microscope. However, sufficient information is available to indicate the prevalence of the two basic forms, and the structure of some pyocins has been studied in detail.

Colicins

An early electron microscopic study of colicins ML, E, V, and K by Kellenberger and Kellenberger (121) did not produce any definitive results concerning their morphology. These authors did, however, make a particularly important observation: colicin ML had two active constituents. One was sedimentable at 25,000 $\times$ *g*, chloroform-resistant, and very thermolabile. The other was chloroform-sensitive and could not be sedimented. These constituents fit into the two groups of bacteriocins which have been suggested, but the authors were not able to identify the sedimentable component as part of a bacteriophage. One must remember, however, that negative staining was not available at the time and they had to rely on the comparatively inefficient shadow-casting technique. Colicins E, V, and K could not be identified in the microscope, but it was suggested that colicin K was a very small molecule since it could not be sedimented in an ultracentrifuge. Colicin K has been well studied since then, because it was thought to be a structural component of coliphage T6; however the observations of Goebel et al. (77) indicate otherwise. They purified the active constituent, a solution of it having the following properties: it contained one electrophoretic component but it was heterodisperse in the ultracentrifuge; it was thermostable, basically protein, and shared no chemical or immunological properties with coliphage T6. Goebel et al. stated that it was in fact the somatic or O antigen of the producer strain *E. coli* K-235. These studies show that this colicin is one of the small -molecule group. Colicin V is also the O somatic antigen of its producer strain (95, 96).

In the course of a preliminary investigation, Bradley (*unpublished data*) made the following observations on colicin K. A pellet obtained after centrifuging a mitomycin C lysate of *E. coli* K-235 for 3 hr at 40,000 $\times$ *g* was highly bactericidal, as was the supernatant fluid. Activity was retained in the pellet after washing with several cycles of centrifugation, and the electron microscope revealed a very high concentration of rough spherical particles shown in Fig. 45c and a few with a smooth hexagonal outline (Fig. 48b). No other obvious particles of uniform appearance were found. The active supernatant fluid from the first centrifugation resisted boiling for 3 min without measurable loss of activity and the principle could not be sedimented at 150,000 $\times$ *g* for 6 hr. It is clear that one component is the O somatic antigen obtained by Goebel et al. (77), that is, the nonsedimentable small molecule. The sedimentable component is one of the objects illustrated; the reason why it was not found by Goebel et al. (77) is that it was probably removed in the course of their purification process. These particles are of particular interest; the rough ones resemble objects found by Bradley (17) and Karamata et al. (109) in association with *E. coli* strains C 3000 and K-12, respectively. Whereas the high concentration of rough particles suggests that they are the active agent, the mere presence of the smooth, angled ones which are identical to empty phage heads, means that they cannot be ruled out. Colicin K certainly appears to have a component belonging to each basic type, but further investigation is required to ascertain the exact nature of the sedimentable component.

Bradley and Dewar (25) studied an active sedimentable fraction of colicin H (from *E. coli* CL 10 supplied by Hamon) obtained from the fluids of a noninduced culture of the producer strain. This consisted mainly of intact phagelike objects (Fig. 48a) closely resembling the coliphage E1 (15). The heads were full, and some of the tails

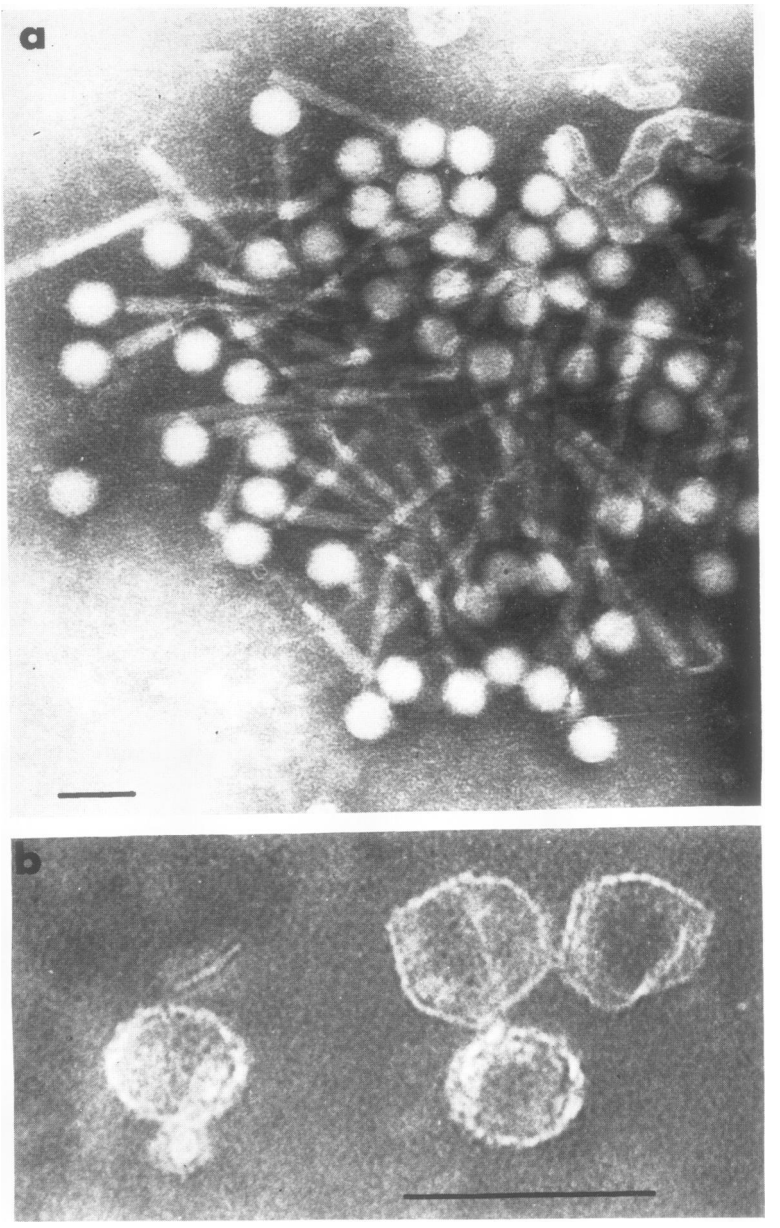


FIG. 48. *(a) Phage-like active principle from noninduced Escherichia coli strain CL 10, PTA, × 100,000, scale 1,000 A. (b) Particles from colicin K preparation (mitomycin C induction), PTA, × 333,000, scale 1,000 A.*

were contracted. These were also similar to one of the components found by Sandoval et al. (184) for colicin 15, though these authors also obtained many empty heads without tails.

Colicin 15 is of interest in that its producer strain, *E. coli* 15, is one of the few that gives only a sedimentable component on induction. Endo et al. (62) correlated its appearance with bactericidal activity as follows. After induction, a culture of *E. coli* 15 was centrifuged at 7,000 rev/min for 10 min, and the pellet was resuspended in one-tenth of its original volume of phosphate buffer, this being designated fraction I. The supernatant fluid was centrifuged at 54,000 × *g* for 1.5 hr, and the pellet was suspended in the same volume of buffer as fraction I, to con-

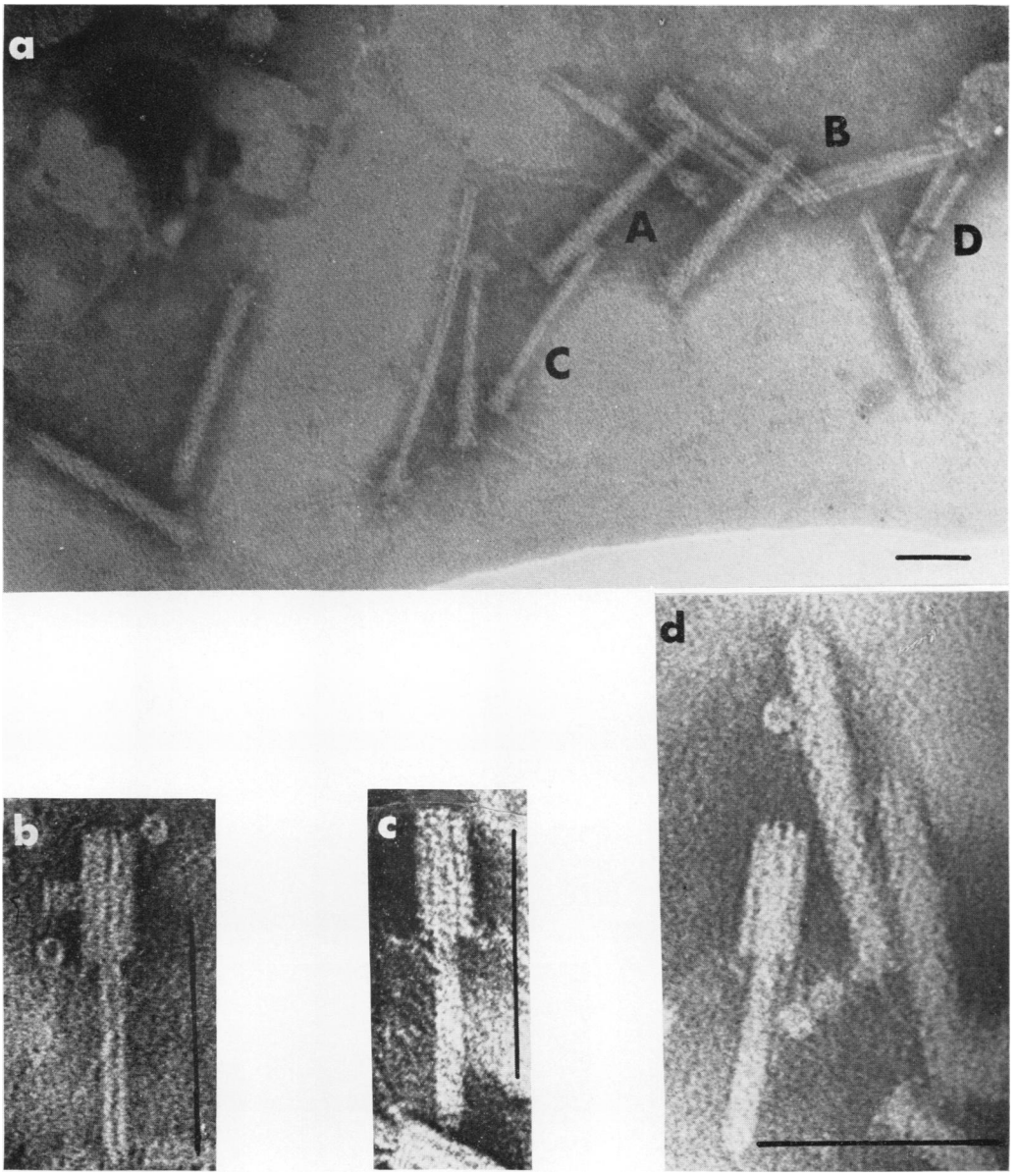


FIG. 49. *(a) Pyocin C 10, UV-induced, PTA, × 100,000, scale 1,000 A. (b), (c), (d) Pyocin Götze, mitomycin C-induced, PTA, × 333,000, scales 1,000 A.*

stitute fraction II. The supernatant fluid from this centrifugation was precipitated with ammonium sulfate, the pellet obtained after centrifugation being resuspended in phosphate buffer, dialyzed to remove ammonium sulfate, and made up to the same volume as the other fractions; it was labeled fraction III. Each fraction was examined in an electron microscope and tested for bactericidal activity. A few phagelike particles were found in fractions I and III, but fraction II contained a very high concentration of them. Bactericidal activity resided in fraction II. It was concluded that the phagelike particles were the active principle. Mennigmann (153) carried out more detailed electron microscopy and concluded that, because of its appearance (it resembled a small contractile phage), colicin 15 was a defective bacteriophage, thus confirming the observations

of Sandoval et al. (184). These observations raise the important question as to the difference between a genetically defective bacteriophage and a bacteriocin; this is better discussed after describing other bacteriocins.

One can conclude from the above observations that colicins can be of either basic type, the sedimentable ones so far examined being phage-like components.

Pyocins

Several pyocins (bacteriocins for *P. aeruginosa*) have been studied in the electron microscope, and a consistent pattern has emerged. Ishii et al. (99) examined a purified active preparation from a mitomycin C lysate of *P. aeruginosa* strain R. They found rod-shaped contractile structures identical with those in Fig. 46a, which were obtained from the pyocinogenic *P. aeruginosa* strain Götze. Bradley (*unpublished data*) studied the original pyocin C10 (supplied by A. C. Paterson, Aberdeen University) of Jacob (105) and found it identical. The preparation illustrated was purified by precipitation and centrifugation from an ultraviolet-induced culture of the producer strain (*P. aeruginosa* C10). Bactericidal activity resided in the centrifuged pellets, indicating that the preparation examined in the electron microscope was the active principle. The following description includes micrographs of different pyocins.

The basic form is the striated rod (Fig. 49a, A), and the remaining particles (except for some cell debris) are parts of it: the tubes at B are hollow

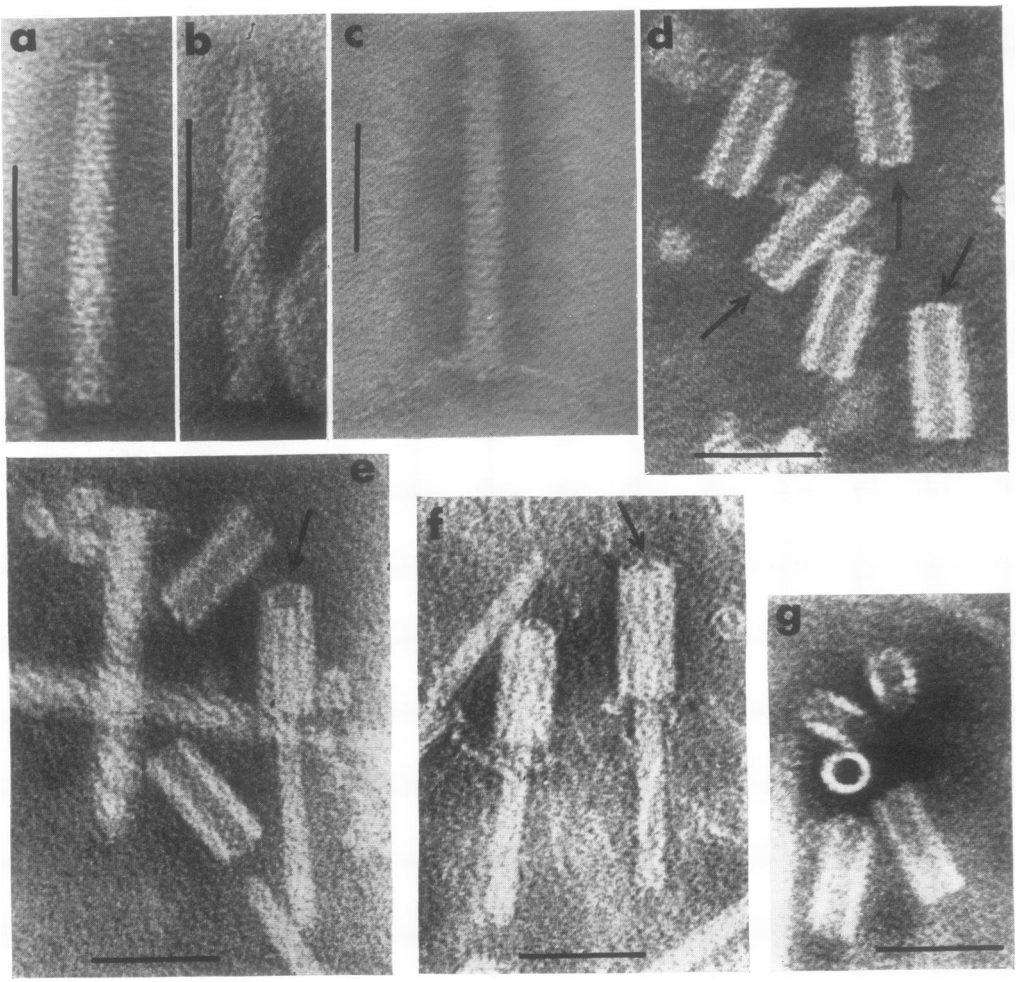


FIG. 50. (*a*), (*b*), (*c*) *Pyocin C 10, mitomycin C-induced, PTA,* × *333,000, scales 500 A.* (*d*), (*e*), (*f*), (*g*) *Pyocin Götze, mitomycin C-induced, PTA,* × *333,000, scales 500 A.*

cores; those at C are solid cores and still have some sheath material attached. At D there are two lengths of isolated contracted sheath. Pyocin C10 appears to be a stable structure, most of the sheaths being in the extended state. Strain Götze, however, contained much more contracted sheath material, mostly attached to cores (Fig. 49b and c). It will be noticed in these micrographs that the particle in Fig. 49b has a hollow core and that in Fig. 49c has a full one. This feature was found in pyocin C10 (Fig. 49a, c). The inference is that the cores contain some substance, which could be a short strand of nucleic acid. The extended rods in Fig. 49d (Götze) appear solid for most of their length, but at one end a length of about 200 A is hollow, again indicating the presence of some substance. The structure of the extended sheath of pyocin C10 is shown in Fig. 50a and b. In the first it appears as fine striations formed by hollow tubular capsomeres, and in the second, due to an orientation difference, as a coarse helix. At one end of the rod, which is flattened into a small base-plate, there are a few fibers (Fig. 50c). Contracted sheaths which have been parted from their cores (Fig. 50d, Götze) are particularly interesting, since they are closed at one end (arrow) and open at the other. This is even visible when the cores are still present (Fig. 50e, f, arrow). The particles in Fig. 50f have two partly open flaps at the top. End-on contracted sheaths (Fig. 50g) present an appearance reminiscent of isolated T-even contracted sheaths (31). The dimensions of rods obtained from different strains are similar: overall length, 1,250 A; length of contracted sheath, 450 A; diameter of extended sheath, 180 A; diameter of contracted sheath, 200 A; number of striations, about 36. A point of considerable interest arises from these figures; the diameter of the contracted sheath is only very slightly greater than that of the extended sheath, whereas the length decreases by almost two-thirds. One can only presume that the extended sheath is a very loosely packed collection of subunits. Contraction would be achieved by rearrangement to a new lattice where space is more efficiently utilized, so that the same number of subunits can pack into a much smaller volume. It is interesting to compare the structure of the rods with the tail of the *P. aeruginosa* phage PB-1 described above (Fig. 21a); here the length is 1,500 A with the sheath extended. In the contracted state, the sheath is about 700 A long, but the diameter of 150 A is the same for extended or contracted sheaths. Once again, there is a reduction in volume on contraction. In conclusion, it seems likely that the pyocins are derived from, or are the forerunners of phages basically similar to PB-1. The only pyocin so far found with a different morphology from those described above is numbered 28 (209). Figure 51 (209) shows that it is in the form of long flexible rods of variable length. They are striated and usually hollow, though several of those shown are partly solid (arrow). A number of rings the same diameter as the rods are visible, and are assumed to be broken segments. They are certainly not RNA-polymerase because of their appearance and smaller size. The identity of the rods with the active principle was established by its centrifugeability, the purification procedure used (precipitation and DEAE chromatography), and the absence of any other particles in the active preparation. Takeya et al. (209) correlated the activity with the number of particles visible in the electron microscope, and there is little doubt that they are the active principle.

Pyocin 28 clearly constitutes a phagelike object; it closely resembles the tail of a phage of group B with a long noncontractile tail. It is interesting to note that it is also thermolabile, being inactivated at 60 C for 10 min. It seems reasonable that most phage components are easily degraded by heat, and this might well be a means of confirming their identity as bacteriocins.

Pyocin-type particles identical to those described above appear to be associated with many strains of *P. aeruginosa*. Bradley (*unpublished data*) has obtained them in large quantities from the male and female derivatives (FP^+, FP^-) of Holloway strain 1.

Bacteriocins of Bacillus

The morphological identity of *Bacillus* bacteriocins was uncertain until 1964 when Seaman et al. (189) described what they termed a "protophage" which was obtained by inducing *B. subtilis*. It was considered to be some form of defective temperate bacteriophage (it could only kill a host cell, not multiply within it) rather than a bacteriocin; this view was shared by Ionesco et al. (98), who described a similar particle. Both were phagelike in appearance with small heads and long contractile tails. Bradley (18) and Stickler et al. (200), who studied similar particles, lean towards their classification as bacteriocins. This is mainly a problem in semantics and is best discussed after their description.

The morphology of all the particles described by the above authors (18, 98, 189, 200) is similar except for the length of the tail, so that the description of isolate GA-2 (18) and also one from *B. licheniformis*, 749/C (supplied by M. R.

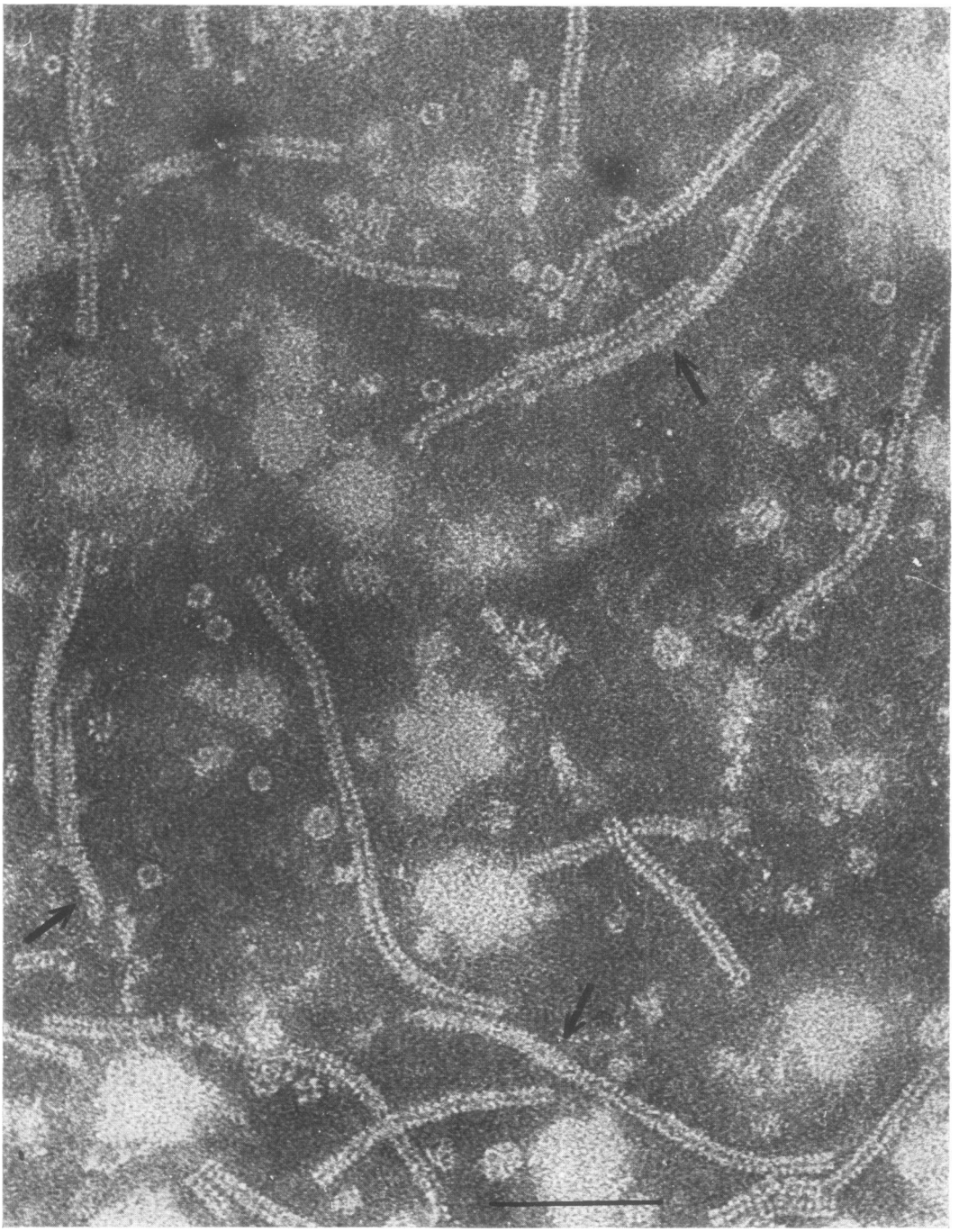


FIG. 51. *Pyocin 28, PTA, × 250,000, scale 1,000 A, after Takeya et al. (209), with permission of Virology, Academic Press, Inc.*

Pollock, Edinburgh University, *unpublished data*), will apply generally. *B. licheniformis* and *B. subtilis* are taxonomically very close, and their bactericidal particles are similar. The basic form can be seen in Fig. 52a from *B. licheniformis*; the polyhedral head is about 380 A in size, and the tail with its sheath extended is 2,000 A long with some 50 striations about 40 A apart. In

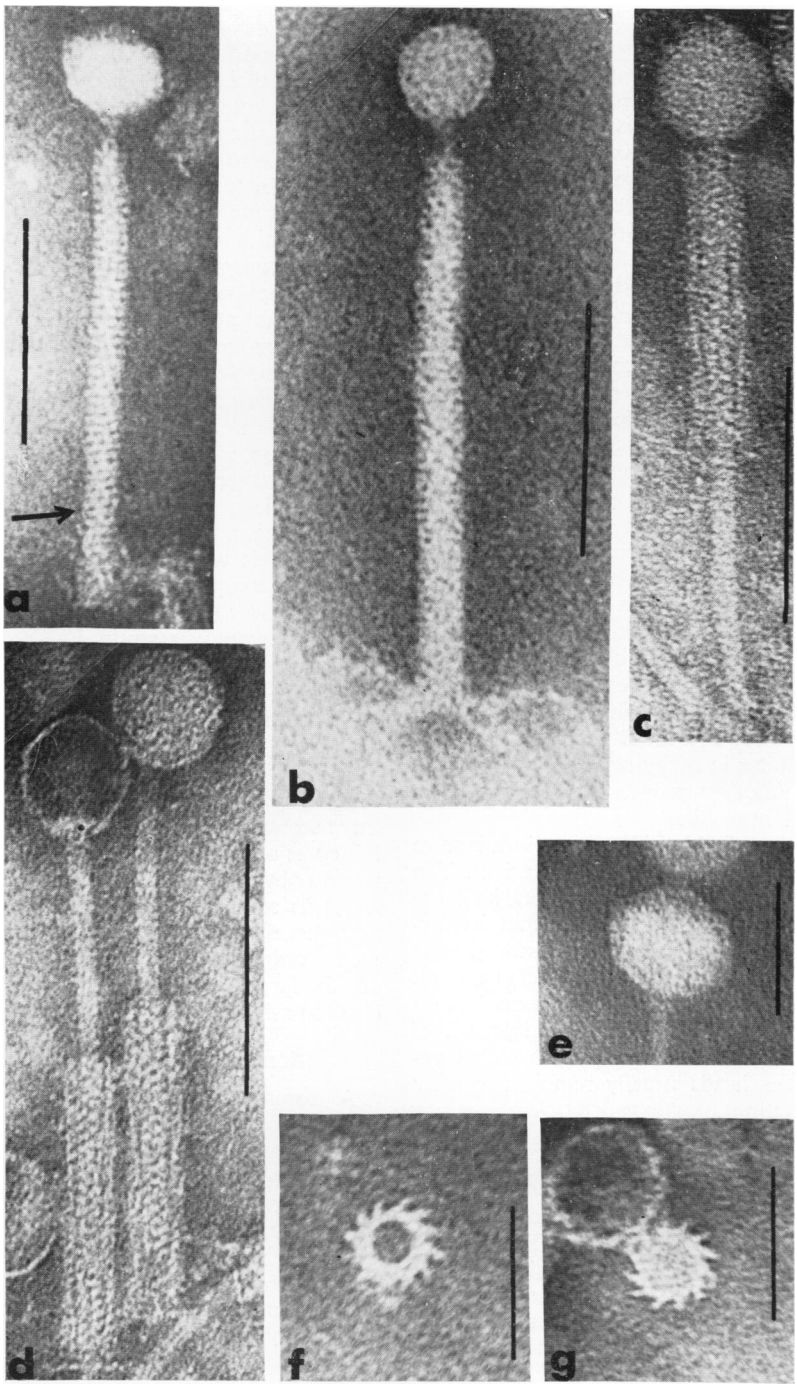


FIG. 52. *Bacillus killer particles in PTA. (a) From B. licheniformis, × 300,000, scale 1,000 A. (b) From B. subtilis (18), × 333,000, scale 1,000 A. (c), (d), (e) From B. licheniformis, × 333,000, scales (c) 1,000 A, (d) 1,000 A, (e) 500 A. (f), (g) End-on contracted sheaths from B. licheniformis, × 400,000, scales 500 A.*

certain orientations, mainly at the base of the sheath, the capsomeres show up as clear circles indicating a tubular form (Fig. 52a, arrow). They are arranged in a series of annuli. The *B. subtilis* particle in Fig. 52b is similar, and a few fibers, which probably number six according to Stickler et al. (200), are visible at the tail tip. The contracted sheaths of the *B. licheniformis* particles in Fig. 52c and d are about 1,200 A long, the subunits having rearranged themselves into longitudinal rows. End-on views of contracted sheaths (one hollow, Fig. 52f, and one solid, Fig. 52g) show a cogwheel structure with 12 teeth, presumably corresponding to the longitudinal rows. As with contractile pyocins, the widths of the extended and contracted sheaths are nearly the same (about 160 and 200 A, respectively), whereas contraction is almost 50%. In Fig. 52c, the thin core projects below the contracted sheath, but in Fig. 52d the sheath has remained at the distal end of the core. It is not possible to ascertain the head shape from micrographs such as Fig. 52a and e, which show a somewhat irregular hexagonal outline. It can be seen from Fig. 52d that both full and empty heads occur; all the isolates so far examined have been found to contain 2-DNA. Seaman et al. (189) showed that the DNA of their particle (PBSX) is similar in many respects to the DNA of its host. Another property of their particle is that it is thermolabile.

All but one of the killers mentioned were produced by inducing the host bacterium. The exception, GA-2 (18), was liberated in large numbers from normal cultures, so that its formation seems to be similar to temperate phages and also bacteriocins. According to Seaman et al. (189), the particle kills a sensitive bacterium on contact before the DNA can be injected, if injection takes place at all (injection seems unlikely since the heads usually remain full after sheath contraction).

It is evident from the foregoing that *B. subtilis* and *B. licheniformis* killer particles have many of the physiological characteristics of a bacteriocin but they also have the morphological characteristics of a bacteriophage. Their classification really depends upon the definition of a bacteriocin, a matter which will be discussed.

Little detailed work has been carried out on bacteriocins of other *Bacillus* species, but in retrospect it seems that the particle described by Kellenberger and Kellenberger (120) for *B. cereus* was similar to those mentioned above. A killer particle for *B. megaterium* (142) turned out to be a 550 A sphere in the electron microscope, suggesting a phage head.

Monocins

Hamon and Péron provided an indication of the nature of *Listeria monocytogenes* bacteriocins when they noted their thermolability (86). From the information already described, this property would seem to suggest that they are phage components. These authors also stated that "monocins and monocinogenic factors have some characteristics which are not found in other bacteriocinogenic species"; those relevant to this description are thermolability, trypsin resistance, and precipitation at a lower concentration of ammonium sulfate compared with bacteriocins. In a later study (87), they noted that the lytic principle of monocins 31, 783, and 9000 were centrifugeable at 144,000 $\times$ *g* for 3 hr. Electron microscopy revealed the presence of phage components, and it was concluded that a substantial proportion of monocins are likely to be of this nature. Hamon and Péron did not illustrate this work, so the structures obtained cannot be compared with those shown by Bradley and Dewar (25).

L. monocytogenes strain 1896X produced an abundance of bactericidal phagelike objects without induction; most of them were headless tail assemblies of a highly complex nature (Fig. 53a), though some heads were present. A smaller number of particles with empty heads attached to a complete tail were found (Fig. 53b). While there seemed to be no nucleic acid in the heads, tail cores appeared both solid and hollow. There was no obvious sheath contraction, though headless tails seemed to have a different capsomere arrangement than those with heads, as can be seen from Fig. 53a and b. It seems that these are the largest bacteriocins so far studied in the electron microscope.

MODE OF ACTION OF BACTERIOCINS

A common feature of bacteriophages and bacteriocins is that they adsorb to specific receptors on the cell surface. With the exceptions of the RNA and filamentous phages, these receptors are located within the cell wall. After adsorption, a cell "infected" by a bacteriocin can often be "rescued" by the action of trypsin. This proteolytic enzyme probably degrades the bacteriocin particle before it can have a lethal effect. Trypsin rescue is presumably restricted to bacteriocins which are trypsin-sensitive; these seem to be the small-molecule type (e.g., colicin K), phage components such as monocins being trypsin-resistant (87). These observations could suggest that some bacteriocins at least are bacteriostatic rather than bactericidal. Reeves (175) stated that, with K and E_3, trypsin rescue is

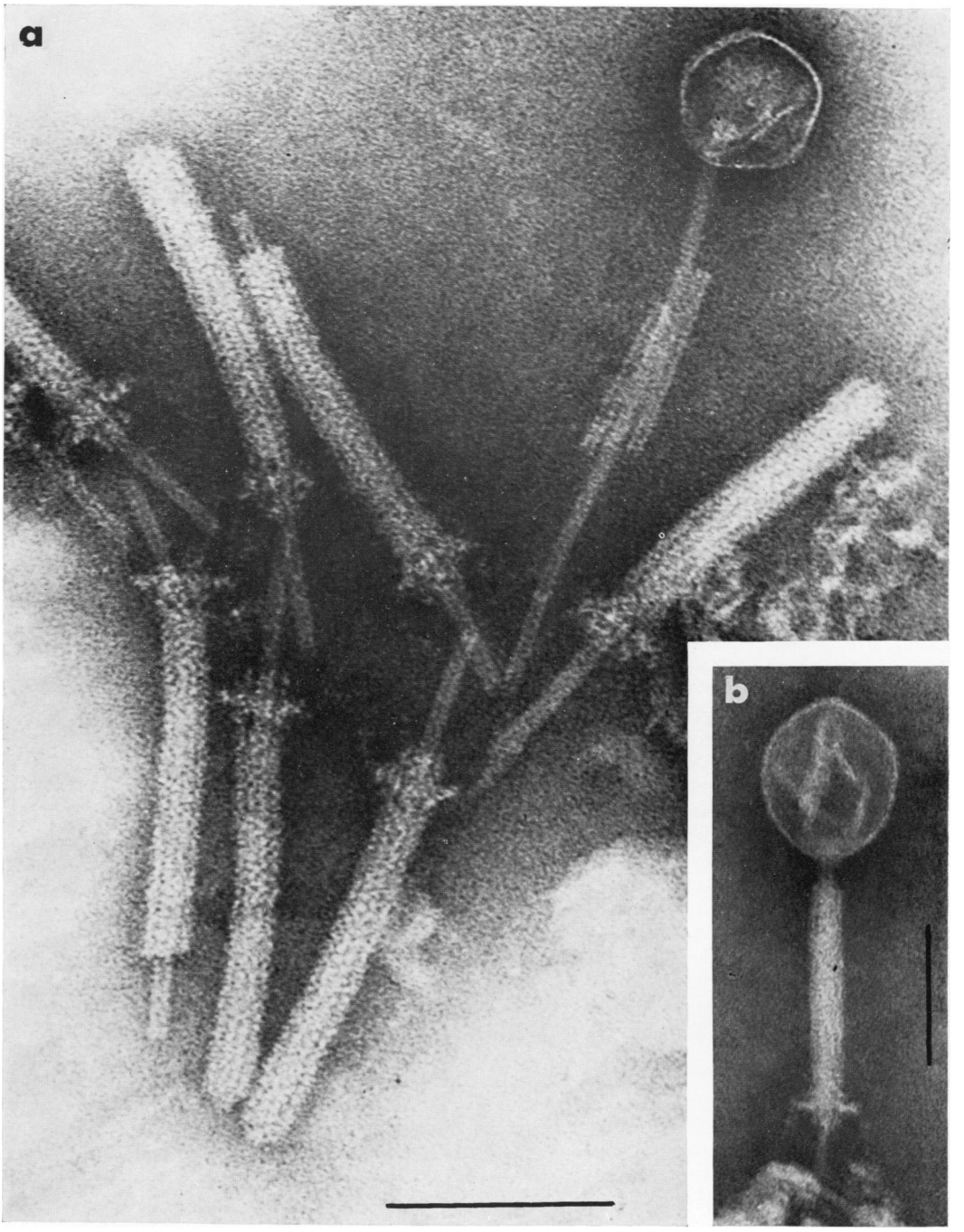


FIG. 53. *Monocin 1896X (25), PTA, scales 1,000 A. (a) × 333,000. (b) × 200,000.*

still possible long after the addition of the colicin.

Unfortunately, little work has been carried out on the effects of bacteriocins on the cell except in the case of colicins. Reeves (175) has reviewed colicin action in detail, so that only the more important points are included here. Colicin K stops the synthesis of DNA, RNA, and

protein in a sensitive cell (163). Colicin ML effectively does the same; respiration is checked, and then falls (106); the multiplication of a virulent phage is also immediately suppressed. CA42-E_2 closes down the cell metabolism completely in 15 min (178). The action of all these colicins is thus basically similar, though there may be some variation in detail. Pyocin C10 appears to act in a similar manner to colicin K (105). One particle adsorbed to the cell wall is sufficient to kill. Two megacins have been studied. Megacin 216 causes a drop in respiration (103) followed by leakage of the cell contents through the cell wall (104). Megacin C causes the DNA of the sensitive cell to break down, and temperate phages are induced (93); RNA and protein synthesis cease as with some colicins.

Obviously one cannot generalize about bacteriocin action, but there are some points of interest. The pyocin C10 phage-type tail appears to affect the cell in exactly the same way as the small molecule of colicin K. Pyocin C10 appears to contain some substance (possibly DNA) in its core which may or may not be injected. At all events, it seems unlikely that this substance, which occupies only about 800 A of the core, could have any effect if it entered the cytoplasm. Little is known about the effect of the DNA-containing *Bacillus* killer particles on the cell, save that they kill rapidly on contact. In general, it is probably only the protein part of phagelike bacteriocins which is lethal. Bacteriocins have no genetic action at all and are unable to lysogenize bacteria. A comparison can be made between the action of bacteriocins and that of T-even phage ghosts. They can kill in a manner apparently identical to bacteriocins (56, 90, 164). All that can be said with any degree of certainty is that the small-molecule types of bacteriocin act in a more or less similar fashion to phagelike objects, and indeed to virulent phage protein. There is thus a fundamental physiological link between these three entities.

RHAPIDOSOMES

Rhapidosomes are perhaps one of the most interesting phagelike particles to be described, since they are in the form of headless contracted tails and contain RNA. They appear to have no biological activity and are produced by the autolysis of the gliding organism *Saprospira grandis*. They were first observed by Lewin (132) and studied in the electron microscope. Two kinds of particle were found, the first (Fig. 54a) in the form of a thick-walled hollow tube strongly reminiscent of polymerized contracted sheath material. These resemble the so-called microtubules found in *Proteus* by van Iterson et al. (101), which are believed to be of bacteriophage origin. According to Correll and Lewin (44), this was the most numerous component. The second kind of object was an apparently similar thick tube with a thin one protruding from it (Fig. 54b). The possibility of the bacteriophage origin of rhapidosomes is demonstrated by comparing the second component to a monocin particle which has lost its base-plate (Fig. 54c). Two joined pieces of contracted sheath material from a pyocin are shown in Fig. 54d for comparison with the first type of particle. Rhapidosomes with an identical morphology have also been obtained from myxobacteria (176).

CLASSIFICATION OF BACTERIOPHAGES AND BACTERIOCINS

Although there is an obvious morphological link between some types of bacteriocins and bacteriophages, the former do not behave like true viruses; i.e., they are not propagated by infection. The current view, therefore, is that the two groups of particles should be classified separately, bacteriophages being integrated into a general scheme of virus classification.

Classification of Bacteriophages

There have been many attempts at classifying bacteriophages, but the majority are of little value. A most reasonable approach was made by Adams (1, 2), who considered various criteria, such as morphology and serological relationships, and concluded that a Latin binomial nomenclature based on a Linnaean form of classification was premature. Furthermore, he was of the opinion that bacteriophages should be classified outside any system used for other viruses, since, if they were integrated, it would imply that all viruses had "a common origin remote in time," which seemed very unlikely. Lwoff et al. (137) took the opposite view because of "the biological, structural, chemical and physiological unity of the group." Bradley (20) favored Adams' view because "it seems much more logical that the major groups of viruses had separate origins, the host evolving before its intracellular parasite." At all events, virus taxonomy is a highly controversial subject.

The various conflicting views have now been brought together in the International Committee for the Nomenclature of Viruses (ICNV) set up at the IXth International Congress for Microbiology, Moscow, 1966. The task of this committee is to produce a scheme of nomenclature which is acceptable to the majority of virologists.

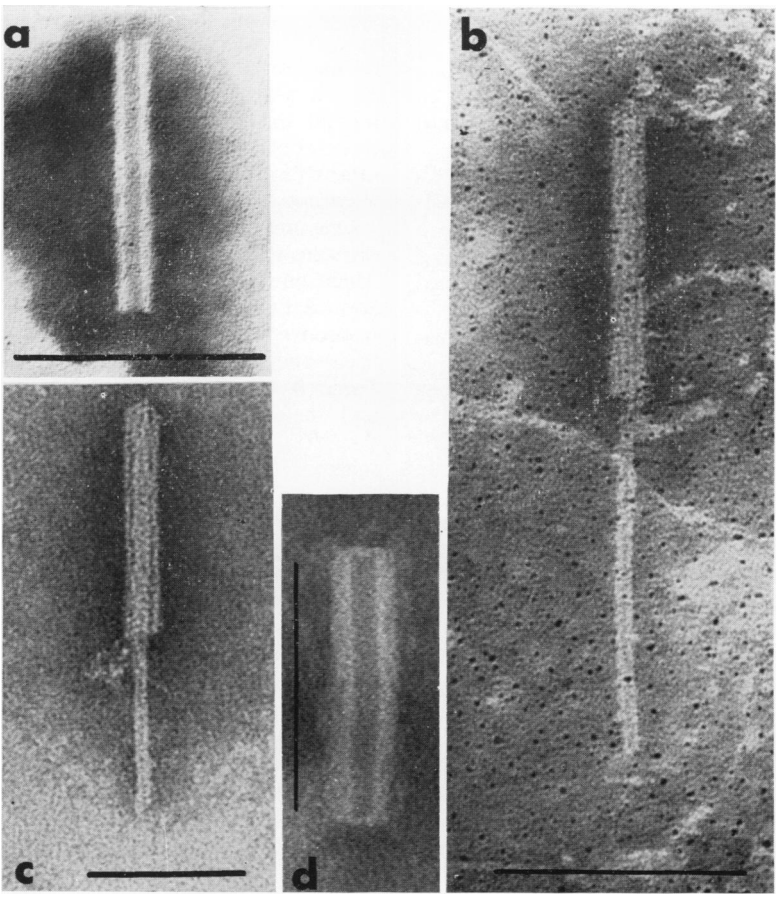


FIG. 54. *(a), (b) Rhapidosomes from Saprospira grandis, PTA, × 333,000, scales 1,000 A; with permission of J. Roy. Microscop. Soc. (20). (c) Monocin 1896X (25), PTA, × 200,000, scale 1,000 A. (d) Isolated contracted sheaths from Pseudomonas aeruginosa strain TTC, PTA, × 333,000, scale 1,000 A.*

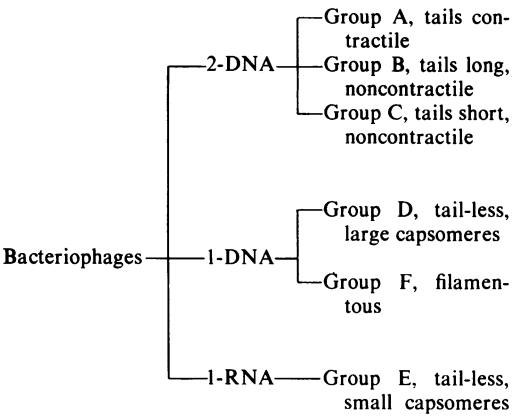
A set of rules has been approved, those relevant to this discussion being:

> "Nomenclature shall be universally applied to all viruses."
>
> "An effort will be made towards a latinized binomial nomenclature."
>
> "For pragmatic purposes the species is considered to be a collection of viruses with like characters."
>
> "The genus is a group of species with like characters."

It can be seen that bacteriophages will therefore have to be classified within a general system and will eventually be given Latin names. At the same time, account will have to be taken of a large number of virologists who favor a vernacular name followed by a cryptogram, which would put the fundamental properties of a virus in the form of a simple code to be placed after the name (76). The present organization of the ICNV implies a certain degree of differentiation between the various groups of viruses, since subcommittees have been set up to consider those of vertebrates, invertebrates, bacteria, and plants. It might therefore be constructive to consider briefly how bacteriophages could be classified prior to their integration into a general system.

The three obvious taxonomic criteria which could divide bacteriophages into groups are nucleic acid type, morphology, and host range, the first two being generally acceptable. As has been mentioned, three types of nucleic acid are encountered in bacteriophages: 2-DNA, 1-DNA, and 1-RNA. Since these are such fundamental characters, they should be used to form three major groups, perhaps families or genera (in the context of the ICNV rules). Morphology, based on the six morphological types, would then logically follow. The resulting broad scheme is

TABLE 7. *Classification of bacteriophages*



shown in Table 7. It can be seen that morphological groups A, B, and C could, if required, be further divided on the basis of head shape, tail morphology, etc.

Although host range has been suggested as a major criterion (137), some doubts have been cast on its validity. The most obvious objection is that bacteriophages of the *Enterobacteriaceae* can infect a fairly wide range of species. At this stage, it might therefore be better to accept only those criteria which are more or less indisputably valid, namely, nucleic acid type and morphology. Serological relationships must be considered, but it is pointed out that they provide a very fine distinction between phages which appear similar in other respects, so that they would have to be used at a very low level in the bacteriophage hierarchy. The use of a coded cryptogram would be a valuable adjunct to any scheme of nomenclature, since it would enable the principal characters of a bacteriophage to be ascertained at a glance.

Until the work of the ICNV is at a more advanced stage, little more can be said. It is hoped that a proper system of nomenclature will not long be delayed since its absence is a hindrance to workers in the field.

Classification of Bacteriocins

Far too little knowledge is at present available to make any useful attempt to classify bacteriocins. The early work of Fredericq (70) on colicins is of undisputed value in facilitating communication, but to all intents and purposes it is based on host range, a criterion which is of questionable validity even more with bacteriocins than with bacteriophages. For example, several monocins are known to act on strains of both *Staphylococcus* and *Bacillus* (83). Thus, little can be done except to use the existing nomenclature until more is known. The vernacular names used for bacteriocin "families" (Table 6) are based not on the spectrum of activity, but on the producing organism. The fact that their roots lie in the specific names of the bacteria means that their host association is immediately apparent.

One possible taxonomic criterion is the natural division of bacteriocins into two basic types which could be conveniently designated as low and high molecular weight forms. Some of their respective properties, which admittedly have been studied in only a few cases, are given in Table 8. The differences in trypsin sensitivity and thermostability are somewhat speculative, though the few known examples fit into the scheme. If this division can be generally applied, it would constitute a useful basis for classification.

The position of the group of particles produced by *Bacillus* species, at present known as killers, should be clarified. The first question to be answered is whether or not they are true bacteriocins, which depends upon the definition of a bacteriocin. If this is accepted as "bactericidal particles which are unable to multiply in a sensitive indicator," then killers must be included. There is no question as to their physiological similarity to bacteriocins; the only basic morphological difference between them and bacteriocins of high molecular weight is that they are complete phagelike particles, and do not lack any components. That they contain DNA is not really a valid distinction; it seems probable that some of the incomplete phages such as pyocins contain DNA as well, though in a much smaller quantity.

This discussion also poses a question of fundamental importance; that is, should the two different types of bactericidal particle be known under the general heading of bacteriocins? All the evidence so far suggests a viral origin for the high molecular weight types. The low molecular weight types seem to be a constituent of the bacterial cell itself, e.g., the O somatic antigens. Such a fundamental difference could constitute a valid reason for calling the two groups of entities by different names. Once again, there is insufficient evidence to give a definite answer, but this question will have to be decided in the course of time when the taxonomic position of bacteriocins is considered in relation to bacteriophages and other viruses. For the time being, at least, it is thought best to place the two groups, including the defective phages or killers, under the one heading of bacteriocins.

TABLE 8. *Properties of the two groups of bacteriocins*

Low molecular wt bacteriocins	High molecular wt bacteriocins
Not sedimentable	Sedimentable
Trypsin-sensitive	Trypsin-resistant
Thermostable	Thermolabile
Cannot be resolved in electron microscope	Visible in electron microscope as phage-like objects or components

NATURAL HABITATS OF BACTERIOPHAGES AND BACTERIOCINS

Bacteriophages and bacteriocins are, of course, found in association with the bacteria which produce them, so that both host and particle will be present simultaneously in the same environment, but there are a number of ecological factors which will affect their occurrence. For phages, efficient production depends upon the infection of rapidly growing bacteria; thus, optimal growth conditions for the host are required. With bacteriocins, the same probably applies, since they can be produced in the laboratory without induction in relatively small quantities by log-phase cells. In nature, however, one might expect certain environments to contain inducing agents formed by other organisms; mitomycin C is a bacterial product. There is also no reason why substances with a similar inducing effect should not be provided by organisms such as fungi or even algae. The general picture, therefore, is that both bacteriophages and bacteriocins are most likely to be encountered in biologically active habitats. A second ecological factor is the consistency of the habitat. Contrary to what might be expected, liquid environments may well produce fewer bactericidal particles than solid ones. In a liquid, bacteriophages, for example, would be more widely and more rapidly distributed throughout a bacterial population, resulting in the lysis of susceptible strains. In a comparatively short time, the population would be replaced by bacteria resistant to the original phage. The concentration of phages in a liquid may also be reduced by dilution.

In mud or other solid or semisolid environments, it will be the rate of diffusion which will control the exchange of bactericidal and bacteriolytic particles from the producing population to an adjacent susceptible one. High local concentrations of bacteriophages might therefore be expected, and a greater variety encountered than in a liquid. With bacteriocins, the position will be different. Since they cannot reproduce like phages, only those associated with predominant phage-resistant bacteria will be found. This means that, although the chances of finding a bacteriocinogenic strain in a given solid habitat are high, there are likely to be many different kinds for a particular species of bacterium. It can be seen that the primary role of bacteriophages and bacteriocins in nature is to affect the evolution of associated bacterial populations. The possible modes of interaction between bacteriophages and bacteria were discussed in detail by Campbell (36).

Some of these points have been confirmed by studying the natural occurrence of phages. It has been known for many years that sewage is the most prolific source of bacteriophages active on many bacterial species. However, it is in reality an artificial dispersion of human feces, and to a lesser extent soil, so that it cannot be considered as a natural environment. Soil, however, is certainly natural, and it is here that a variety of *Azotobacter* (58), *Pseudomonas*, and *Bacillus* phages and bacteriocins are found. Feces, both human and animal, provide very large numbers of phages and bacteriocinogenic bacteria, mainly belonging to the *Enterobacteriaceae*. In littoral habitats, the greatest number of phages for such organisms as marine *Pseudomonads* are found in rotting seaweed. Another good solid habitat is rotting fruit or other plant material. Here, plant pathogenic bacteria such as *Pseudomonas* spp. and *Erwinia* spp. predominate, and their associated bacteriophages and bacteriocins can usually be found.

Whereas at first sight it might seem that bacteriophages and bacteriocins are obscure entities best suited for laboratory studies, it is evident that they play a dominant part in the in the evolution of bacteria, and so indirectly have an effect on the organisms with which the bacteria are associated. A study of their ecology might therefore bring to light some important and interesting facts.

ORIGIN OF BACTERIOPHAGES AND BACTERIOCINS

The origin and evolution of bacteriophages and other viruses has been the subject of a great deal of speculation. The theories put forward were discussed by Adams (2), but their plausibility should now be reappraised in the light of more recent experimental evidence.

As Adams stated, the various ideas fall into two basic categories, the "creation" and "degeneration" hypotheses. The first suggests that viruses are descended from a very primitive life-form which existed before organized cells (165); this is not considered very likely. The

"degeneration" theory considers that viruses evolved from complex organisms by a degenerative process in which they lost all the protoplasm unnecessary for an intracellular parasite. Again this idea seems unlikely, though one might argue that the *Bdellovibrio* represents an intermediate step in a degenerative process. The only attractive feature about the theory is the indication that the host evolved before its viral parasite.

In 1953, Lwoff (135) proposed a theory for the origin of bacteriophages which suggested that a series of mutations caused part of the bacterial genome to attain a degree of autonomy, allowing it to exist outside the cell. To protect it from extracellular conditions, and to permit its re-entry into another cell, it was necessary for it to synthesize a protein coat before leaving its original host. Proceeding from this, Adams (2) suggested the possibility that phages might have evolved as a means of genetic transfer from one cell to another. These ideas are much more attractive to many biologists than the "creation" and "degeneration" hypotheses.

Campbell (36) discussed the evolution of bacteriophages in detail. He considered the possibility that phages are "transient offshoots of something not in itself a virus," but partly rejects this because of their abundance and structural complexity. These two facts suggest a long evolutionary history of phages as such. This eliminates "transient" from the above statement; phages could still have a nonviral origin. Campbell (36) favors the suggestion of Adams (2) mentioned above that phages might represent a form designed to effect bacterial recombination. The fact that phages can incorporate bacterial genes and transmit them to other bacteria supports this idea.

If, therefore, one accepts the plausible idea that bacteriophages originated from the bacterial chromosome, one would expect to find some link between the various extrachromosomal elements (F factors, temperate phages, etc.), as suggested by Subbiah et al. (204). They proposed an evolutionary spectrum in which bacteriocins might occupy one end and virulent phages the other. Consideration of the characteristics of extrachromosomal elements is required to evaluate this possibility.

The sex factor or F episome is that which is most closely associated with bacteriophages and their activity (male specificity of some phages), and which cannot be considered a bactericidal agent itself. It is a length of DNA which can exist separate from the host cell chromosome. It acts like a virus in that it "infects" other cells. To do this, it passes along an intercellular link, believed by some to be the F-pilus, though it cannot exist completely separated from the cell. Because of its lack of complete autonomy, it could be placed first in an evolutionary spectrum.

Bacteriocins might be considered to come next, but here one may run into difficulties because of the diversity of forms and their properties. There are the killer particles with their DNA, the phagelike components which may contain nucleic acid, and the low molecular weight O somatic antigen forms with no nucleic acid. The *Bacillus* killer particles are generally considered to be defective temperate phages, implying that they have degenerated or devolved from temperate phages proper. The reason for this supposition is that their DNA is incapable of having any effect on the genetic activity of a sensitive cell, always assuming that it is injected, which is far from certain. Seaman et al. (189) considered killers to be poorly evolved phages, indicating an incomplete evolutionary step rather than a devolutionary one. The near homology of killer DNA with host DNA suggests the parcelling up of a piece of the bacterial chromosome in a protein shell, the first step of Lwoff's theory (135).

Although the same sort of arguments could apply to phagelike components, a devolutionary origin seems much more probable. In the case of pyocin tails, for example, an episome seems unlikely to code for a selection and penetration assembly before providing itself with a receptacle (the head). The logical explanation is that a temperate phage genome lost its ability to code for a head. This idea is supported by the studies of Epstein et al. (63), who showed that T4 mutants could produce phage particles with components missing, or mixtures of a few kinds of phage components. The position of the low molecular weight bacteriocins is obviously more difficult. If, as seems certain, they are the O somatic antigens of their host cells, then the location of the lytic principle is presumably within the cell wall. The episome or plasmid giving rise to them (presumably coding for them in the same way that the F factor codes for the F-pili) does nevertheless exist inside the cytoplasm or nucleoplasm. The implication is, therefore, that small bacteriocins are quite different from large phagelike ones, and hence might occupy a different position in an evolutionary scale. Indeed, there seems to be some analogy between them and F factors: the "autonomous" proteins of both are associated with the cell wall. Could, then, the plasmid of the small bacteriocin be the forerunner of, or a side evolution from, the F factor? This question cannot be answered and the position of the bacteriocin group as a whole remains totally obscure. Temperate phages are,

TABLE 9. *Comparison of F-pili and filamentous phages*

Character	F-pili	Bacteriophage
Morphology	Long flexible tube	Long flexible tube
Diameter	95 A (130)	70 A
Length	Variable	8,500 A
Composition	Protein	Protein
Function	May have role in phage RNA and DNA and bacterial DNA transfer	Phage DNA transfer
Adsorption	To receptors in cell wall	To tips of F-pili
Serological relationship	RNA phage infection via F-pili not blocked by antifilamentous phage serum	Filamentous phage inactivated by homologous antiserum
Method of production by bacteria	"Extrusion" through cell wall	"Extrusion" through cell wall

however, much more likely to represent an evolutionary step. They can exist, like F factors, as episomes, or autonomously in a virulent vegetative form, thus constituting a possible link between the F factor and the virulent phages.

It is often considered that virulent phages represent an evolutionary step above the temperate phages. They may have lost their ability to exist in the prophage or episomal state, a somewhat unnecessary property. This is supposedly supported by the fact that many virulent phages cannot induce stable lysogeny in a sensitive cell. However, such evidence is rather negative because there is a strong possibility that a bacterial strain which could be lysogenized either has not been encountered or has become extinct. At all events, the vegetative state of an active bacteriophage appears to be the present end of the evolutionary scale. In summary, some sort of evolutionary link may well exist between F factors and virulent phages although the intermediate steps contain a good deal of uncertainty.

Unfortunately, there is no direct experimental evidence to wholly substantiate the fundamental concepts of the theory, and indeed it is difficult to visualize the sort of evidence which would be convincing. There is, however, one type of bacteriophage whose ancestry might conceivably be traced back directly to the F factor, and that is the filamentous type. The following purely hypothetical sequence of events suggests the evolutionary links between the two entities: (i) a portion of the bacterial chromosome becomes detached to form an episome; (ii) it acquires the ability to code for the synthesis of F-pili; (iii) these become used for intercellular DNA transfer; (iv) the pilus eventually becomes autonomous and hence a filamentous phage.

The credibility or otherwise of the sequence is best considered by comparing the characteristics of F-pili and filamentous phages. These are summarized in Table 9 and it can be seen that the two entities are far from identical. They certainly bear a close morphological resemblance to one another, though there is no unit length for the pilus as there is for the phage. The lack of serological relationship observed by Bradley (*unpublished data*) is hardly surprising, since it would not take many mutations to cause the small chemical changes required to produce different antigenic properties. Perhaps the most significant similarity is that both filaments are extruded by the bacterium, the only difference being that the F-pilus remains attached to the host cell while the phage becomes detached and autonomous. Obviously, these comparisons are inconclusive, but it must be remembered that the F-pilus episome, which may have been the original ancestor of the filamentous phage, is likely to be different from those under study today and that used in this comparison; indeed, it is very probably extinct. While this demonstration proves little or nothing, it is included here as an example of a not impossible train of evolutionary events which points the way to the sort of research which could be carried out to establish a definite link in the F factor-to-bacteriophage spectrum. For example, if a bacterium bearing an F factor could be mutated by some means and made to produce a virulent filamentous phage where previously there was none, then there would be a much firmer basis for such speculations and more experimental material with which to work.

The extremely important problem of the origin of bacteriophages bears a direct relation to that of other viruses, and indeed to life in general. The solution will be obtained only by the continued examination of bacteriophages and related particles by a large number of techniques over a long period of time. It is evident from the fore-

going that bacteriophage and bacteriocin research is, by accident or design, providing a great deal of the sort of information required to discover the origin of bacteriophages.

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