ELECTRON MICROSCOPE STUDIES

OF BACTERIOPHAGES

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for the degree of Ph.D.

by

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PART TWO

THE INTRACELLULAR DEVELOPMENT OF ONE BACTERIOPHAGE OF THE BACILLUS CEREUS GROUP.

PREFACE

Use of the light microscope enabled the biologist to obtain considerable information concerning the nature of living organisms: cells of characteristic morphology could be differentiated and the arrangement of these in tissues examined. This, in combination with an extensive scheme of histological techniques based on specific staining reactions, enabled further investigation of the cellular "elements" of tissues; even a few ill-defined intracellular structures could be identified. The recent application of the electron microscope to biological problems has enabled a vast extension of this approach; together with modern advances in Biochemistry, it has thrown considerable light on the intricate physiology of the living cell. The cell is no longer considered as a "bag of protoplasm" - it is now known to be made up of a number of complex structural units, often interrelated and always concerned with some important functional activity of the cell. Indeed the relationship between structure and function has been one of the striking features of recent advances in cellular physiology. In addition to elucidating the substructure of cells, the electron microscope has served to characterise their intracellular parasites, the viruses. Although the biological properties of many viruses were known before the introduction of the electron microscope, it was not until

the use of this new technique in 1939 by Ruska and his co-workers that a virus, namely tobacco mosaic virus (TMV) was observed directly.

Although TMV could be easily studied and was crystallised by Stanley as early as 1935, many plant and animal viruses were found difficult to isolate from their host cells. For this reason virologists took an increasing interest in the viral parasites of bacteria, the badteriophages, which could be easily separated from their bacterial hosts. Although they have never been crystallised, bacteriophages, or "phages" as they are generally called, were soon found to be chemically similar to other viruses (Northrop, 1938). They also possess very similar biological properties: they can be inactivated by X-rays and U.V. light and show almost every type of genetic characteristic of higher organisms (Epstein, 1953).

So far, mainly the bacteriophages of the coliform bacteria and staphylococci have been examined, and a classification on morphological grounds has been attempted (Bradley and Kay, 1960). Possibly the most important problem of all, the interrelationship between the host bacterium and its accompanying phage has received relatively little attention. A cycle of events has been proposed for two of the T phages, T_2 and T_5 of E. coli (Kellenberger,196

A great deal remains to be done, however, before a complete understanding of this problem is possible.

The present thesis describes a study of the bacteriophages of the Bacillus cereus group, and was undertaken with the aim of examining their morphology, fine structure and mechanism of intracellular multiplication. Not all of these aims were achieved, but it is hoped that what was, will contribute to the general understanding of bacteriophages, and provide methods for future investigation.

In the course of work, certain findings were made concerning the structure of the host bacterium itself. These are included since it is felt that they are of interest by themselves.

GENERAL INTRODUCTION

MICROSCOPY

The unaided human eye cannot separate fine structures closer together than 0.1 - 0.2mm. Although the light microscope, which has undergone continuous development since it was first evolved at the end of the 17th century, vastly improved on this, it became increasingly obvious that there was a limit to the useful magnification of such microscopes, beyond which increase in the size of the image gave no improvement in resolution. Such a limit is imposed not by imperfections in the instrument but by the imaging medium used. According to the Abbé equation, the resolving power of any microscope is given by the expression:-

$$d = \frac{0.61\,\lambda}{n \sin\alpha}$$

where λ - wave-length of the incident light

n - refractive index of the immersion medium

α - aperture of the objective

Hence, when white light is used as the imaging medium, the resolution limit is about 2,000Å; U.V. light gives a maximum resolving power of about 1,500Å. Further increase in the resolving power must result from a decrease in the wave-length of the imaging source. Attempts to use radiations of shorter wave-length than U.V. failed because no substances capable of focussing such shorter electro-

magnetic waves in the way that glass focusses light, and quartz U.V. were found.

A new approach came with the suggestion of Duc Louis de Broglie (1924) that small particles of matter may show both corpuscular and wave-like properties, similar to electromagnetic radiation. Confirmation of this hypothesis came in 1927, when Davisson, Germer and G.P.Thomson (1928) in a beautifully executed set of experiments, clearly demonstrated the wave nature of electrons.

Earlier, J.J. Thomson (1897) showed that beams of electrons in the form of cathode rays, could be focussed by electric and magnetic fields, and Busch (1926) envisaged their use in forming greatly magnified images of very small specimens. Further magnification was achieved by employing several electromagnetic fields or lenses in series. The electron microscope, as it is now known, began to evolve.

Resolution. At an accelerating voltage of 100 KV, the wave-length of an electron beam is of the order of 0.05Å and so from the diffraction theory, structures, or rather atoms, of this order of size should be detectable. In practise this is not found, because limitations other than diffraction come into play.

It was realised early that the electron lens, unlike the glass lens, cannot be constructed completely free from spherical and chromatic aberrations. The former, usually

expressed in terms of the spherical aberration coefficient, C_o, of the objective lens, is the more important of these two, and it, together with diffraction, determines the theoretical resolution of the system. It can be shown (Nixon, 1958) that these two are interdependent and that the theoretical resolution varies with the operating voltage according to the expression

$$d_{th} \propto V^{-\frac{1}{4}}$$

At an accelerating voltage of 100KV, this gives a theoretica instrument resolution of 1.7Å (Ruska, 1962). Even this, however, has never been achieved.

The main difficulties, in addition to those mentioned, in attaining reproducible high resolutions, include instability of the accelerating voltage, astigmatism, chromatic aberrations, and electron noise (Kay, 1961). Other losses may arise from the specimen itself. Increased thickness decreases resolution, partly due to the loss of electron energy because of inelastic collision with the object, and partly due to repeated deflections within the specimen. Specimen contamination and beam damage due to a rise in the temperature of the specimen (von Borries and Glaser, 1944) caused further reduction in resolution. Losses in resolving power of the electron microscope can be kept to a minimum by careful adjustment and alignment. Contamination can be reduced by regular cleaning of the

illuminating system and the use of double condensor.

Alternatively, from the expression:-

$$d_{th} \propto V^{-\frac{1}{4}}$$

it might appear that an increase in the operating voltage should result in an improvement in resolution. Experimental microscopes have been built operating at 220KV (Coupland, 1954), 300KV (Zworykin, Hillier and Vance, 1941) and 400KV (van Dorsten, Oosterkamp, Le Poole, 1947). Recently Dupuoy and Perrier (1962) have used 1,000KV acceleration. However elevated voltages introduce both mechanical and qualitative difficulties and these become more important as the voltage is increased. The stability of the instrument becomes more difficult to control and the design of the gun is necessarily more complex. Nevertheless the higher energy of the electron beam gives it a higher penetrating power; thicker specimens may be studied and structures previously obscured begin to appear.

Although such mechanical difficulties will probably be overcome, there is a serious drawback to the use of such systems; the contrast falls off rapidly with increase in voltage. This is of particular importance when biological material is studied, since it is inherently of low contrast. It is for this reason that expected improvement in resolution may not be fully realised;

fine structure and small particles of low contrast cannot be detected even if they are larger than the maximum resolution of the electron microscope. This leads to a consideration of "contrast" and its implications.

Contrast

In the electron microscope, the image is formed by the scattering of the incident beam by the specimen; the degree of this scattering depends on the types of atoms in the object. Thus, the intensity variations of the image arise from the relative scattering powers of the different elements of the specimen; these differences in intensity are known as "contrast".

The human eye can only distinguish as separate two points which differ in intensity by more than a certain amount; this difference, which varies for different individuals, is usually taken to be about 10% of the total intensity; hence even a particle larger than the minimum resolvable distance of the microscope will be invisible, unless it differs sufficiently in intensity from its surroundings.

Marton (1936) realised the importance of contrast differences in relation to resolution, and was the first to point out that, although the Abbé equation applies to the electron microscope, it fails to take into account the way in which the image is formed. Two years later, von

Ardenne (1938) suggested that, in many cases, resolution losses might arise directly from insufficient contrast of the specimen. This was extended by Schiff (1941) who stated that though for electron-dense materials the ultimate resolution depended on the lens aberrations, for specimens of low electron-scattering power, such as biological material, the low contrast of the specimen itself outweighed these errors and determined the maximum attainable resolution. It is clear, therefore, that in almost any study of very small particles, it is advantageous to improve on the contrast of the specimen. Small apertures, which cut out much of the scattered beam, and reduce the background intensity are often used to enhance low contrast specimens. Although the optimum size of objective aperture is generally taken as that for which the diffraction and spherical aberration effects become equal (Kay, 1961), for low contrast specimens it is often useful to insert apertures which are below this optimum size. In preventing many of the strongly scattered electrons from reaching the image plane, and contributing to the background intensity, they serve to increase the contrast. For this reason, 30μ objective apertures were used in the present study.

The amount by which the incident beam is scattered by the specimen depends both on the relative scattering powers of the constituent atoms, and on the beam energy. Lower

beam energy results in greater scattering angles and hence greater contrast. Von Borries and Ruska realised this as early as 1940, and studied the effects at 25 and 15KV. They were able to show marked improvements in contrast. At this time, however, when specimens were so much thicker than at present, the aim was greater beam penetration rather than increased contrast. Recently, with advanced specimen preparation techniques, more attention has been given to this method of improving contrast. Below 50KV Wilska (1960) noted that the resolution losses due to lens aberrations are not serious, and are more than compensated for by the increase in contrast. Working at much lower voltages, Nixon (1958), studied biological specimens at 6KV and observed that although the contrast was greatly improved there were serious difficulties to such voltages.

Indeed, low voltage operation is now known to have serious drawbacks for delicate specimens; heating effects are much more serious and specimens suffer to a greater extent from ionization and contamination. Also the intensity of illumination is greatly lowered, and this may lead to focussing errors.

It became obvious from this that there was an optimum operating voltage; one which is low enough to allow adequate contrast, but at the same time sufficiently high to minimise the difficulties mentioned above, and also to improve the theoretical resolution. Hall (1951) stated

that this voltage varied with the specimen, but was usually in the region of 50 - 100KV. In the present study of bacteriophage particles 60KV and 80KV were used. 80KV was found to give adequate contrast and a better illumination than the lower voltage, and so was used throughout.

Several methods for improving the contrast by treatment of the specimen have been developed; these include metal shadowing (Williams and Wyckoff, 1945) positive staining (Hall, Jackus, Schmidt, 1945) and negative staining (Brenner and Horne, 1959). These will be discussed later in this work (pp22, 107, 29) in relation to the present problem.

Early Research on Bacteriophages

It is surprising that, although the lytic effect of phage action must have been obvious to the bacteriologists of the 19th century, no research was done on the field until the beginning of the 20th century. The first observations on the phenomenon were published in 1915 by Twort, who described an infectious disease of bacteria, and attributed the cause to a virus, though he also stated that "it may be a minute bacterium which will grow only on living material, or a tiny amoeba which, like ordinary amoeba, thrives on living organisms". Soon after, Felix d'Herelle (1917) rediscovered the effect and renamed Twort's "lytic principle", "bacteriophage". This terminology has persisted to the present day, and indeed is very apt; it literally means an "eater of bacteria" and accurately describes the way in which bacteriophages lyse bacteria, leaving clear areas, or plaques in a uniform bacterial culture on solid medium.

The Nature of Bacteriophages

D'Herelle's prolific work stimulated the imagination of many eminent bacteriologists of the 1920's; many theories were put forward to explain the phenomenon. Of these, two gained considerable standing and, as often happens, both have survived in part to the present day. At the time, however, fierce controversy involved the supporters of each, and the two seemed irreconcilable.

The "precursor theory" stated that bacteriophages were abnormal products of metabolism present within the host cell, and caused lysis either spontaneously or as a result of some stimulus: essentially the lytic principle was considered to be a property or product of the bacterium itself. Many well-known biologists of the day, including the famous Jules Bordet, supported this view, and it became widely accepted.

The second, the "virus theory", already tentatively suggested by Twort (p.13) was advocated by d'Herelle (1926) who believed that the phage was a virus particle capable of multiplying within the host. This explained the observation that lysis of infected cells resulted in the production of more phages. Thus bacteriophages were considered similar to the plant and animal viruses but confined to the bacterial kingdom.

Neither of these theories are at present considered to completely explain the nature of phages; taken together, however, they come close to the truth. It is now generally accepted that phages may exist in several quite separate and distinct forms, just as some bacteria may exist as rod-shaped organisms or as spores. At present, it is accepted that there are three stages in the life-cycle of the bacteriophage. Phages which exist free from the host bacterial cell are known as "mature"; by themselves

they are biologically inert (Ajl, 1950) and cannot replicate Indeed in this form phages appear inanimate, showing none of the characteristics typical of living matter. However, when mature phages come in contact with and infect a bacterium, they assume the "vegetative form", and in this state are thought to bear no resemblance to mature phages (Kellenberger, 1961). It is now that multiplication and recombination take place; vegetative phages seem to have an almost unlimited ability to multiply. Finally, there are some phages, known as "temperate" phages which can exist in a third form, prophage; these may adopt a symbiotic relationship with the host cell for a considerable time after infection, without causing harmful disturbance of the host's metabolism. In the prophage state, the phages multiply as an integral genetic part of the host cell, and later generations of bacteria have the capacity to produce phages without the intervention of a mature phage. Such a bacterium is termed "lysogenic".

The Infection Process.

d'Herelle (1926) made a comprehensive study of the conditions under which phage-induced lysis took place, and noted that pH, salt concentration etc., are critical. To explain these findings and also his observations on the duration of the lytic cycle and the production of new phages resulting from lysis of the bacterium, he suggested a mechanism for infection. This was not greatly different

from that accepted today. The stages into which he divided the process were:-

(1) Adsorption of the phage onto the host cell.

(2) Penetration of the phage into its host cell.

(3) Intracellular multiplication within the bacterium.

(4) Lysis of the cell and release of more phages.

Each new phage progeny can then infect more bacteria, and initiate a new cycle. And so the process continues.

The Particulate Nature of Bacteriophages

The particulate nature of bacteriophages, suggested by d'Herelle in 1926, became generally accepted by the 1930's. However, although a good deal of work was devoted to the determination of the size of phage particles, very little was known of their nature. Indirect physical chemical methods similar to those used for plant and animal viruses (Stanley and Lauffler, 1948) were applied. The earliest attempts to determine phage sizes were based upon ultrafiltration. That viruses can pass through filters capable of retaining bacteria has been known since 1892 (Iwanowski); the method of using graded filters of known porosity, developed by Elford (1931), was subsequently employed to determine phage sizes; Elford and Andrewes, (1938) showed d'Herelle to be mistaken in believing that all phages belonged to the same species, and proved that phages from different species of bacteria

were of different sizes. To this technique, Neurath and Saum (1938) added the measurement of diffusion constants; this yielded reliable results for bacteriophage sizes in the hands of Putnam (1950) who also measured electrophoresic mobilities and sedimentation constants.

The results of various techniques as applied to phage sizes have been summarised recently by Adams (1959), and it is interesting to note that the sizes, as determined by electron microscopy, are of the same order as those determined by other methods, although they are often slightly smaller. This discrepancy is not surprising since phages are assumed to be spherical and hydrated in physical chemical measurements whereas electron microscope data are determined for dried particles (Putnam, 1950).

Thus, prior to the electron microscope era, phage particles were thought to be spherical, although Borrel (1936, unpublished) suggested, on the basis of their behaviour when passed through graded filters, that the particles might be elongated. This hypothesis was subsequently upheld by the results obtained with the electron microscope.

The Aims of the Present Thesis

This thesis describes an investigation of the bacteriophages belonging to the Bacillus cereus group of organisms. Little is known about the morphology of

of the phages of aerobic spore-forming bacteria and only one electron micrograph showing a phage associated with the protein crystal-forming Bacillus cereus strain appears to have been published, namely, a shadowed preparation showing gross morphology (Afnikian, 1960).

The aim of the present work was to investigate the detailed morphology and fine structure of four of these phages and to classify them if possible within known groups. In addition, a general study of the multiplication process was made.

These two aspects will be dealt with separately, and the thesis will be divided into two parts.

(a) Morphology and fine structure.

(b) Mechanism of intracellular growth.

PART I

The Morphology of Four Bacteriophages of The *Bacillus Cereus* Group.

INTRODUCTION

Early Electron Microscope Studies of Bacteriophages.

Whereas plant and animal viruses were known to be either spherical or rod-like, the bacteriophages were the first viruses discovered to have complex structures. Phage preparations examined in the electron microscope were seen to be comprised of two parts - a head and a tail (Pfankuch and Kausche, 1940; Ruska, 1940). This was soon confirmed by several other workers, who found that phages from different species possessed the same general shape. Since these early preparations were, however, very impure, there was some doubt as to whether the "tadpole" like objects were indeed phages. This doubt was removed by Anderson (1943) in what were probably the first quantitative experiments to be done using the electron microscope. Phage-infected cultures were subjected to sonic vibrations and the decrease in the numbers of plaques formed in such cultures was correlated with the decrease in the number of "tadpole" units.

In the first years of direct phage study, Delbruck (1946) confirmed many of the early size measurements made by indirect methods, and brought experimental proof of Elford and Andrews's Rule (1932) which stated that the size of the infective particle varies inversely as the size of the plaque it produces. These size

assessments led Wyckoff (1949) to state that "living, self-reproducing objects exist which are considerably smaller than the molecules of such well-defined proteins as the haemocyanins and erythrocruorins".

Early concepts of the shape of phages were simple: the tail was considered to be an elongated structure, and the head to be spherical. At that time, little information was available to substantiate or disprove such beliefs because of the small size and low electron-density of phage particles. The metal shadowing technique overcame some of these difficulties.

The Metal Shadowing Method.

Electron microscopy, more than most other fields of research, depends on the introduction of new specimen preparation methods, and the improvement of existing ones. One of the most important advances of this type was the metal-shadowing method of Williams and Wyckoff (1945). In essence, the method consists of allowing a stream of heavy metal atoms to fall obliquely, under vacuum, on to the specimen. In this way, structures can be made to cast "shadows"; from analysis of the shadows a three-dimensional appreciation of specimen surfaces can be made. Although Müller (1942) had used this method to calculate specimen heights, it was not until Williams and Wyckoff (1945) showed that the

correct choice of metal would give less granular shadows, that the effectiveness of the method for a wide range of specimens was realised.

The application of the technique to bacteriophages was at once obvious, and the early shadowed specimens appeared to confirm the speculations that the phage head was spherical. However, it soon became obvious that not all structure observed in such shadowed preparations was indeed fine structure of the specimen.

Artefacts

The possibility that such results were artefacts due to the collapse of the particles in the final stages of drying was first appreciated by Hillier and Baker (1946) and Anderson (1951). Since then precautions have been introduced to avoid such effects. The "Spray Droplet Method" (Backus and Williams, 1950) enables a suspension to be deposited on mounts in the form of small droplets of 2 - 5 μ diameter. Thus the drying time is much reduced, and the specimen is exposed to the unfavourable drying conditions for the minimum time. It is important that the suspending medium should be volatile, since any non-volatile material will show up in such small droplets, (Backus and Williams, 1950; Luria, Williams and Backus, 1951);

ammonium acetate is a suitable medium since it combines volatility with the reasonable buffering action required for most biological specimens.

Another technique for avoiding drying artefacts was introduced by Anderson (1951); the "Critical Point Method" consists basically in suspending the material to be studied in a liquid such as CO_2 which may be heated above its critical point and hence volatilised. Although it is more complex than the previous method, it is particularly suitable for very delicate specimens. Perhaps the method which has the widest range of application however is the freeze-drying method; this entails drying the specimen by cooling under vacuum. A combination of spray-droplet and freeze-drying has been used by Williams (1953) in his study of the T phages of E. coli and it appears from the ratio of particle-height to particle-width that the three-dimensional structure is well preserved.

The Detailed Morphology of Bacteriophages

Although bacteriophages, possessing as they do, a complex form, are distinct from plant and animal viruses, their two main units, taken separately, belong in part to both morphological groups; the head resembles the small spherical viruses, whereas the tail resembles the rod-shaped forms.

The Structure of the Head

Since the small spherical viruses can be crystallised, X-ray crystallography in addition to electron microscopy has been used in their study. The combined use of these two techniques has led to accurate determinations of their morphologies. Because of the similarities between these viruses and the phage head, and because much of what is known about their structures is now realised to be relevant to bacteriophage, the small spherical viruses will be discussed briefly.

Spherical Viruses

All viruses are composed of a central core of either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), encased in and protected by an external coat of protein. Early X-ray studies on one of the plant viruses, bushy stunt virus, showed it to have a cubic lattice (Bernal, Fankuchen and Riley, 1938) and in 1941, Bernal and Fankuchen suggested that "X-ray evidence points to a virus particle of complex structure. It is more analogous to a protein crystal than a protein molecule".

A cubic lattice was observed again with tobacco yellow virus (Bernal and Carlisle, 1948) and on the

grounds of this structural correlation between otherwise unrelated viruses Hodgkin (1949) made the important generalisation that all small spherical viruses, irrespective of species had the same basic shape. The more detailed studies of Caspar (1956) on bushy stunt virus confirmed early work and revealed the presence of numerous sub-units.

An important theoretical contribution was made by Watson and Crick (1956, 1957). They proposed that the amount of RNA or DNA present in a virus particle is insufficient to allow the coding of more than a few different types of protein molecules of limited size. If this were the case, then viruses would be expected to consist of numerous small identical sub-units rather than large complex units, and these would be packed together in a regular array, so far as possible giving each sub-unit an identical environment. Because X-ray crystallography showed that the spherical viruses possessed cubic symmetry, they suggested that the above conditions would exist if the virus particles had one of the following polyhedral forms.

Table I

Table I

Polyhedron satisfying symmetry requirements	Number of asymmetric sub-units
Tetrahedron	12
Cube ,Octahedron	24
Icosahedron	60

(After Watson and Crick, 1956)

At the same time as these advances were being made with X-ray crystallography, small viruses were being studied by means of improved techniques in the electron microscope. An angular shadow shape was observed for bushy stunt virus (Williams, 1953) and Kaesberg (1956) showed that tobacco yellow mosaic virus had a hexagonal outline. This was the first electron microscopic support of the theoretical suggestions of Watson and Crick. Within the next few years several workers brought detailed evidence of the actual forms of these polyhedra.

Further improvement of the shadowing techniques enabled deductions concerning the shape of the virus particles to be made from an examination of the shapes of the shadows cast. From these data, Valentine and Hopper (1957) and Williams and Smith (1958) suggested that adenovirus and tipula iridescent virus might be

icosahedral in shape.

Bacteriophages

Unlike the plant and animal viruses these particles cannot be crystallised, with the result that X-ray crystallography cannot be used to determine their structures; all structural evaluation must come from electron microscopy. Polyhedral phage heads were first noted by Anderson (1951) who observed that the heads of the T-even phages of E. coli were hexagonal and also suggested that the heads of T_5 phages were hexagonal. This was confirmed by Williams and Fraser (1953) in their study of the seven T phages of E. coli. Williams (1955) wrote that "it is probable that phages do not have precisely the shape of a geometric polyhedron, but are so shaped as to appear hexagonal no matter from what angle they are viewed". He concluded that this regularity in form required some degree of orientation and rigidity in the arrangement of the nucleic acid and protein. More than a decade earlier, Luria and Anderson (1942) had suggested a similar orderly arrangement; these workers have been proved correct and it is now known that the DNA is arranged in an orderly way within the head.

Detailed morphological studies for the T phages showed that the head was either a rhombic dodecahedron

or a hexagonal prism with bipyramidal ends (Anderson, Rappaport, Muscatine, 1953; Williams and Fraser, 1953). Hence it may be seen that striking similarities exist between the morphology of the phage head, and the small spherical viruses. Also Watson and Crick's theory of the relation between morphology and substructure has been upheld for bacterial viruses.

The Negative Staining Method

Although by 1959 the external form of the small viruses had been established by the combined use of the electron microscope and X-ray crystallography, no information had yet been obtained about the arrangement of sub-units within the particle. Here again major advances were made only after the introduction of new specimen preparation methods; the so-called negative staining method (Brenner and Horne, 1959; Horne and Brenner, 1959) enabled such studies to be made. The method is analogous to the use of a dye in light microscopy to show up the specimen by negative contrast. As often happens, the initial findings were the result of an accident (Hall, 1955). Although employed by Huxley (1956) to study the fine structure of tobacco mosaic virus, the wide application of the method was not realised until it was standardised by

Brenner and Horne (1959). It consists essentially of embedding an electron-transparent object in a fine grained background of electron-opaque material. The specimen then appears light against a dark background. The application of the method is two-fold; it enables material of low electron-scattering power to be studied, and, since the stain can penetrate between the sub-structures of the specimen, they too can be detected. The application of this method to the field of small viruses was immediately obvious.

Fine Structure Determinations

In their study of adenovirus, Horne, Brenner, Waterson and Wildy (1959) demonstrated how the negative staining method could be used to show up the individual virus sub-units; the arrangement of these units is clearly visible and is icosahedral. This form is probably the most suitable for small sub-units since it allows their hexagonal packing on its triangular faces, and so introduces the minimum strain.

Structural sub-units are frequently seen to be symmetric, either because of their hollow or cylindrical forms (herpes virus - Wildy, Russell and Horne, 1960) or because of their positions, lying often on the axis of symmetry of the polyhedra (e.g. turnip yellow mosaic

virus, Nixon and Gibbs, 1960). However, the basic crystallographic sub-units envisaged by Watson and Crick (1956) are by definition asymmetric, and hence these symmetric structural sub-units cannot be identical with the basic sub-units. A new terminology was introduced by Lwoff, Anderson and Jacobs (1959) to avoid confusion. In this, the complete infective particle is known as the "virion", and consists of a core of nucleic acid (genome) surrounded by a protein shell, "capsid", which is composed of a large number of structural sub-units or "capsomeres". Horne and Wildy (1961) have shown that 2, 3 or 5 "crystallographic sub-units" may combine to form one capsomere.

A review of the evidence in favour of pure cubic symmetry in ten representative species of virus has recently appeared (Horne and Wildy, 1961); since these viruses are of widely differing biological characteristics, it is reasonable to propose that most or all small spherical viruses exist in this form.

The negative staining method has also been very useful in the study of bacterial viruses, especially in establishing the hexagonal outline shown by most of the heads.

In their morphological characteristics phage heads seem to show greater variety of forms than the small spherical viruses. In some cases, the form is clearly icosahedral (Bradley and Kay, 1960; Chiozotto, Coppo, Donini, Graziosi, 1960); with other phages (Brenner, Streisinger, Horne, Champe, Barnett, Benzer, Rees, 1959) the form is still believed to be that of the bipyramidal prism originally suggested by Williams and Fraser (1953). Yet other phages seem to have an ovoid shape rather than any definite geometrical outline (Bradley and Kay, 1960).

As yet, however, knowledge of the nature of capsomeres is very limited, since in only a few cases have they been observed (Bradley and Kay, 1960, 1962; Tromans and Horne, 1961). In one instance a hollow structure has been observed (Bradley and Kay, 1960) similar to, though less distinct than, herpes virus (Wildy et al., 1960). Since the packing of capsomeres in the head has not yet been determined, the actual form of the phage head cannot be decided. It would not be surprising, however, particularly in those cases which show definite angular contours, if the capsomeres were arranged in a similar manner to that of the spherical plant and animal viruses.

The Structure of The Tail

It is also of interest to consider briefly what is known of the structure of the rod-shaped viruses, since there is now considerable evidence that very similar structures are present in the bacteriophage tail. Again, although the phage tail can be studied only with the electron microscope, the crystallinity of many plant and animal, rod-shaped viruses permits the combined use of electron microscopy and X-ray crystallography; consequently their structures have been unambiguously determined.

The Rod-Shaped Viruses

The first virus to be studied in the electron microscope was tobacco mosaic virus (TMV) (Kausche, Pfankuch, Ruska, 1939) and since these early studies, much work has been devoted to elucidating its structure. X-ray crystallography has shown that the protein sub-units are packed in a helical array, such that they have identical environments, as predicted by Watson and Crick's theory. The actual arrangement of the protein sub-units was determined by Franklin, Klug and Holmes (1957) who showed that there were just over 16 sub-units for every turn of the helix. Although TMV was studied in the electron microscope in some detail (Williams, 1952a) evidence for the helical nature was not obtained until

the negative staining method was used (Huxley, 1956); the rods were found to be hollow striated structures. It is now known that in this case the crystallographic sub-units are equivalent to the capsomeres, and that unlike the spherical viruses, the nucleic acid lies not in the hollow centre of the virus, but in a helical groove running between the protein sub-units.

This basic helical pattern is the same for most rod-shaped viruses, although the dimensions and the number of capsomeres per turn of the helix may vary (Horne and Wildy, 1961).

The Bacteriophage Tail

The negative staining method facilitated the first detailed examination of the phage tail, and it was soon realised that the tails of most phages are of more complex structure than their heads, consisting of several well-defined parts. Brenner et al., (1959) have shown that the tails of the T-even phages consist of a central core, which is probably hollow, surrounded by a sheath; this sheath clearly shows the striated appearance typical of helically arranged capsomeres. In some cases (Bradley, 1962) the tail sub-units can be detected and their arrangement in the helix exactly determined.

The tail terminates in a base-plate which has been

recorded to have a six-sided cross-section (Brenner et al., 1959) and is often seen to have a number of long fibres or tendrils extending from it; the function of these will be discussed later in this work (p.72). Anderson (1960) noted that the sheaths of the T-even phages ended near the head in a "collar" of a slightly greater diameter, this being attached to the head by a narrow neck. These findings were confirmed in 1961 by Daems, Van de Pol, Cohen, who also recorded the presence of a faint "jacket" around the sheath. Bradley (1962) found this for T_2 but not for T_4 phage and made the suggestion that the fibres are a disrupted form of the jacket.

The simplest explanation of the injection of the DNA into the bacterium is that the sheath contracts (possibly due to an alteration of the number of capsomeres per turn of the helix) and the movement of the baseplate up the tail reveals the central core of the tail, which acts as the needle in this simple "syringe" mechanism. This, however, cannot be the only mechanism involved, since several phages have been recorded (Hall, MacLean, Tessman, 1959; Anderson, 1960; Bradley and Kay, 1960; Dawson, Smillie and Norris, 1962; Mach, 1962) which possess no sheath.

The Specific Functions of Head and Tail

The best method of studying the different functions of the component parts of the bacteriophage is to separate these parts and examine them individually. Possibly the first example of this approach was described by Herriott (1951) who separated the DNA from the head by osmotic shock, and showed that the empty headed phages or "ghosts" were still able to absorb on to the host bacterium.

From this it seemed likely that the tail was the adsorption site, and this was confirmed in 1956 by Williams and Fraser when they showed that tail-less heads did not adsorb.

The long filaments of DNA extruded by fractured phage heads were photographed by the same workers (Fraser and Williams, 1953) amd the surprising length of these filaments led Williams (1957) to suggest that they were highly orientated within the head, and to confirm the earlier suggestion that the fairly rigid protein membrane was mainly responsible for the overall shape of the head (Anderson et al., 1953). This is corroborated by the fact that ghosts maintain their hexagonal outlines in negatively stained preparations (Bradley and Kay, 1960).

Particular attention has been given to the tail structures of bacteriophages, and there are two main

methods for such studies, Kellenberger and Arber (1955) observed the step-wise changes in the morphology of the T phages during the action of oxidising agents, and Williams and Fraser (1956) described the degradation products which resulted from successive freezing and thawing of these phages. The two methods gave very similar results. Disintegration occurred only in the lower part of the tail, and this indicates a higher sensitivity in this region; this agrees with the findings of Levinthal and Fisher (1952) that phages which have been allowed to adsorb on to host cells and are then mechanically sheared off have shortened tails, and substantiates the suggestion that the phage adsorbs on to the cell by interaction of the lower parts of the tail with the host cell membrane. The actual site of adsorption is now recognised to be the tail fibres, which can adsorb even when separated from the phage tail (Williams, 1957).

The contraction of the tail sheath can be made to occur artificially by the action of chemicals (Kozloff and Henderson, 1959; Kellenberger and Arber, 1955), but in most cases, the base-plate becomes detached from the tail sheath; a more natural contraction may be effected with urea (Daems et al., 1961) when the central core of the tail is exposed.

EXPERIMENTAL

Bacteriophages. Four phages isolated from bacteria of the Bacillus cereus Group were studied with respect to their morphologies. Phages A and B were isolated from Bacillus entomocidus var. entomocidus, phage C from Bacillus entomocidus var. subtoxicus, and phage D from Bacillus cereus strain 826. The details of these strains, and of the methods of isolation and propagation of the phages have been described previously (Norris, 1961).

In early experiments, the phages were suspended in distilled water, but a considerable amount of spontaneous lysis occurred under these conditions, the degree of which increased with the length of time the suspensions were kept; even storing such suspensions at 5°C. did not eliminate this effect. In order to minimise autolysis, the suspensions were spun down, and the pellet re-suspended in 2% (w/v) aqueous ammonium carbonate. This medium both maintains a suitable tonicity and also provides the volatile buffer required for the spray-droplet method (Introduction p.23). Even then phage preparations could be kept at 5°C. for only 2 - 3 days before autolysis again became pronounced. Phage B of the group was studied by means of the metal shadowing method, and all four were examined by the negative staining technique.

Specimen Supports. Films used for specimen support must have the properties of being of low electron-scattering power and of low background structure. In this study, two types were used - carbon films and formvar films.

The carbon films were prepared according to the method of Bradley (1954) in which a current of 25 amps. at 12 volts is passed through the tips of two carbon rods held in contact; this operation is carried out under as high a vacuum as possible to ensure a smooth film. The carbon rods were of spectroscopically pure Acheson graphite, and the carbon was evaporated on to the surface of a clean glass slide or freshly cleaved mica. The film was then floated on to the surface of distilled water, and picked up on standard copper mounts. Films of about 100Å thickness were prepared in this way, and were remarkably stable in the electron beam.

The formvar films were prepared in the normal manner, using a 0.5% (w/v) formvar in ethylene dichloride solution.

Shadowcasting. B phage was shadowed, the phage suspension in ammonium acetate being sprayed on to carbon or formvar-coated grids to cut down drying artefacts. Nickel + palladium shadowing and uranium shadowing were carried out in the standard manner from

a tungsten filament the working distance being 14 cm., and angles of shadowing being 15°, 20° and 25°. In the case of uranium, the metal was first scraped clean of the covering oxide layers, before being evaporated at an angle of 20°. Platinum + carbon shadowing (Bradley, 1959) gives sharp shadows of high contrast and very fine grain. The shadowing procedure was similar to that used for the preparation of carbon films; one of the rods had a central core of platinum, the working distance was 10 cm, and an intermediate aperture of 5 mm. was used in order to minimise heating effects, and ensure as far as possible a parallel beam of shadowing material. The shadowing angle was 30°.

Preparation of the Negative Stain. Throughout this work, potassium phosphotungstate (PTA) was used as a negative stain, since it gave reproducible results and facilitated the comparison of the four phages. The negative stain was prepared from a 2% (w/v) solution of phosphotungstic acid in distilled water. Undissolved material was removed by centrifugation and the solution adjusted to pH 7.4 by dropwise addition of 40% (w/v) potassium hydroxide solution.

Specimen Preparation. Two methods were used for preparing specimens for electron microscope study, the spray-droplet method and the spreading method.

For the spray-droplet method, approximately 0.5 ml. of phage suspension and phosphotungstate were mixed and sprayed by an atomiser spray gun on to carbon - or formvar-coated grids, held about 2 cm. from the nozzle. This method was found to give a good distribution of the phage particles in the negative stain, and so was used mainly throughout this work. However, although it gave reproducible results for phages B, C and D, considerable difficulty was encountered in the case of phage A due to repeated agglutination during spraying. In an attempt to avoid this, the spreading technique was employed. This method was used by Bradley and Kay (1960) and has the advantage of requiring less material than the spray-droplet method. Two to three drops of negative stain (c. 0.2 ml.) were mixed with the same volume of phage suspension, and a formvar - or carbon-coated grid was touched on the surface of the drop. Excess stain was removed by touching the grid on clean filter paper, leaving only a very thin film of staining mixture on the grid.

Difficulties were frequently encountered with both methods of negative staining due to the crystallisation of the stain and its failure to spread. The first effect was largely avoided by very thorough centrifugation of the PTA in order to remove insoluble particles.

The spreading of the negative stain can be enhanced in two ways. It has been noted (Brenner and Horne, 1959) that PTA did not spread properly over carbon films prepared in vacuum units which incorporated oil-diffusion pumps because minute oil droplets were deposited over the surface of the film. To eliminate this, all carbon films used in this work were washed with a grease solvent such as chloroform. Spreading may also be improved by ensuring that sufficient protein is present to act as a "wetting agent"; in the case of phage A, it was found that unfiltered preparations, containing cell debris, spread more readily and agglutination was reduced.

Microscopy. All specimens were examined in the Siemens E.M.I., at an operating voltage of 80 KV and magnifications of 30,000 to 40,000; 30 μ apertures were inserted to enhance the contrast.

RESULTS

Uranium Shadowing

Heavily shadowed preparations were studied in order to obtain high contrast and sharp shadows. Williams and Smith (1958) found that when the same specimen of tipula iridescent virus was shadowed from two different directions, shadows of distinctly different shapes resulted. They were able to show that such shadow shapes resembled those cast by an icosahedron when it is shadowed from different directions. The same effect may be obtained by keeping the shadowing direction constant and varying the orientation of the polyhedron; thus, because the orientations of the virus particles on the support film may be assumed to vary randomly, it is sufficient to shadow from only one direction.

The bacteriophages studied in the present work may be seen to cast shadows of two distinct shapes; one of pointed shape can be seen in Plate 1, whereas Plate 2 shows a square shaped shadow. This latter shape is better seen in Plate 3, where the outline is clearly defined. It is of interest to note that here the base plate of the bacteriophage is also casting an angular shadow, indicating that it also may possess a polyhedral shape.

Nickel and Palladium Shadowing

The shadows obtained with nickel + palladium are of much lower contrast (Plate 4) and consequently the shadow shapes are less well-defined. However, suggestions of substructures of the phage head can be detected: the head appears to consist of a central "knob" surrounded by five others in pentagonal array. In attempts to investigate this structure, phages were shadowed with carbon + platinum. This provides sharper shadows of finer grain, and is used to investigate the details of substructure.

Carbon and Platinum Shadowing

Initially, considerable difficulties were encountered using this method, due to the presence of deposits of protein impurity, which created a structureless matrix and obscured the detailed substructures of the phages. Although some phage suspensions were prepared containing less of this impurity, it was difficult to remove it completely. In such specimens, the five-sided figures with a central raised portion, and also the pointed and square shadow shapes were clearly seen. The general picture was similar to that observed with nickel + palladium shadowing.

Bacteriophage Sizes

A summary of size measurements of the phages as

determined by shadowing procedures just described is given in Table 2.

Table 2

	Length	Breadth	Height
Head	620Å	680Å	550Å
Tail	2180Å	95Å	75Å
Base Plate	89Å	130Å	195Å

General Features of Negatively Stained Preparations

The general shapes of all four phages of the Bacillus cereus group are very similar, and examples are shown in plates 5 to 8. Since the negative stain used throughout this study was potassium phosphotungstate (PTA) at pH 7.4, it can be assumed that the action is that of true negative staining, and there is no evidence of the positive staining recorded by Bradley and Kay (1962) with uranyl acetate at pH 4.2. The phage heads are of clearly defined hexagonal outline and the dimensions of all four phages, as determined by negative-staining, are given in Table 3.

Table 3

Phage	Head (Å)				Tail (Å)			
	Apex-Tail			Width	Length			Width
A	640	50	x 640	40	2,000	20	x 95	10
B	630	30	x 680	30	2,055	50	x 80	10
C	620	30	x 590	40	2,000	50	x 75	5
D	620	40	x 580	30	2,055	90	x 75	5

Points of Difference Between the Four Phages

Since these bacteriophages are immunologically related, structural similarity would be expected; such a similarity has been observed for the phages T_2, T_4, T_6 of *E. coli*. This morphological resemblance was also found for the phages of the *Bacillus cereus* group, but there are a few minor points of difference. For instance, the heads of phages C and D are often found to be partially full of DNA; also, phage A was found difficult to deal with because of its tendency to agglutinate and lyse. This was a feature not observed for any of the other phages, and it made study by negative staining more difficult. It is possible that this is due to a charge effect at the pH of the stain, and in future studies of this phage, it may be advantageous to examine the effects of varying the pH of the negative staining solution.

Fine Structure Observed in Negatively Stained Preparations

All preparations consisted of a mixture of complete phages, and those which had lost the DNA from their heads. The former appear as light against a dark background of PTA, whereas the negative stain diffuses through the head membrane of the latter, replacing the DNA. Consequently the contrast of the empty heads is much lower than that of the full heads.

Structure of Full Heads. The full heads are almost always seen to be of hexagonal outline, but occasionally, heads may be detected which are either five-sided, or which possess a very low facet at the apex (Plate 6(a)). This same plate shows the presence, in some of the full heads, of striations running across them (6 (b)). The presence of an external membrane may be seen in Plate 9, and in one instance (Plate 10) the membrane may be clearly seen to be composed of regular spherical sub-units of about 50Å diameter.

Although the micrographs of these four phages did not show any regularly arranged capsomeres corresponding to those of adenovirus (Horne, Brenner, Waterson and Wildy, 1959) Plate 9 shows a suggestion of sub-units in the heads of both phages in the field, and there is a regular arrangement over a small part of the head of the phage marked "a" in Plate 7. In this instance, the capsomeres appear to be packed to form a triangular facet which has five capsomeres on each edge.

Structure in Empty Heads

Since DNA was often spontaneously lost from the phage heads, empty heads, or ghosts, were frequently seen and the profile of the protein coat showed up in good contrast. Such ghosts, seen in Plates 6 and 7 often maintain their hexagonal outlines; also,

structures may frequently be detected within them, which are presumably obscured in full heads by DNA. Examples of such structures can be seen in plates 6(c) 8 and 9(c), where the phage tail appears to penetrate about 70Å into the head and terminate in two spherical structures. Traces of this structure can be seen in other plates of phages which have lost the contents of their heads, but to which the tails are still attached.

It is known that the phage tail may easily become detached from the head, and so the presence of phages which have lost both their DNA and also their tails is fairly common. Plate 11 shows a number of such heads, packed together in much closer array than do phages with tails; indeed one would expect the very presence of the tails would prohibit such close packing by their steric effect. This is possibly why phages have not so far been crystallised.

Then again, the appearance of heads without DNA may give an indication of the polyhedral nature of the head. In plate 12 the empty heads can occasionally be seen to take the form of two superimposed triangles, giving the general outline shown in figure 1. This effect has a bearing on the deduction of the morphology of the phage head, and will be discussed at some length later.

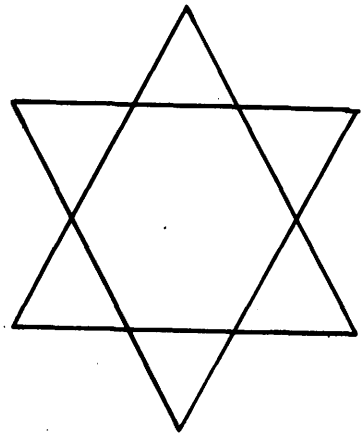


figure 1

Fine Structure of the Bacteriophage Tails

For none of the four phages was there any evidence of the contractile sheaths which have been detected in the T-even phages of E. coli. The tails of the Bacillus cereus group of phages are long, narrow and flexuous, and occasionally may be seen to be of a hollow nature (Plate 8). The tails of phages Plates 6 and 9 show suggestions of striations running normal to the axis of the tail with a periodicity of about 40Å; similar striations may be seen in the lower part of the tail of the bacteriophage in Plate 10. In addition, the sub-units may be detected; striations of periodicity 90Å, at 30° to the axis of the tail are also present.

The tails of all four phages in this group terminate in a knob, or base-plate. In some cases, this may be clearly seen to be a triangular unit with a base length of 140Å ± 5Å and height of 60Å ± 5Å. Plate 10 shows the base-plate to consist of three units radiating from the tail at about 30° to the tail axis. A similar structure may also be detected in Plate 7.

DISCUSSION

The General Morphology of The Bacteriophage Head

The shapes of all four phages in negatively stained preparations are virtually identical, and only one, the phage B was chosen to be studied by shadowing. It seems likely that shadowing data obtained for B phage apply also to the other three bacteriophages.

The angular shadows and the hexagonal outlines in PTA indicate that the phage head is polyhedral. In deducing the actual form of this polyhedron, the shapes postulated by Watson and Crick (1956) for the small spherical viruses were considered as possibilities; these are the tetrahedron, cube, octahedron, dodechedron, and icosahedron. The pyramidal prism with hexagonal ends, suggested by Williams and Fraser (1956) and confirmed by Brenner et al. (1959) for the head of phage T_2 of E. coli and the rhombic dodecahedron postulated by Valentine and Hopper (1957) as an alternative to the icosahedron for adenovirus were also considered.

The first criterion which any possible structure must satisfy is that of having a regular hexagonal outline. This immediately eliminates the tetrahedron, cube, octahedron and dodecahedron. The rhombic dodecahedron, which is not regular in its hexagonal outline, also seems unlikely. The icosahedron and bipyramidal prism both satisfy this basic requirement and so will be considered in detail.

Both these forms give the expected shadow shapes. The icosahedron, lying on a vertex, side or face, casts a square shadow; in each case if the shadowing direction is kept constant and the figure is rotated through 60°, the shadow shape becomes pointed (fig.2). An exactly similar result can be obtained from the bipyramidal prism.

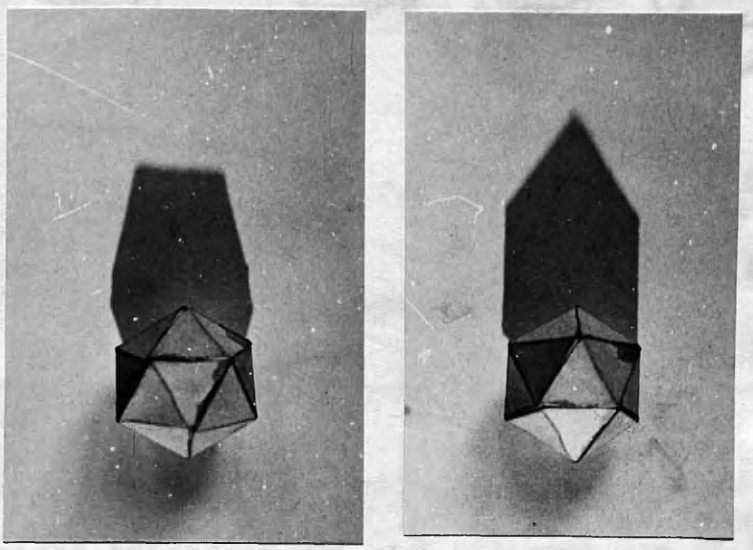


Figure 2

The form of the phage head must also be such as to yield the two superimposed triangles seen in Plate 12. This cannot be derived from the rhombic dodecahedron, and militates against this form. The icosahedron and bipyramidal prism both fulfil this requirement (Fig.3) but in a slightly different way.

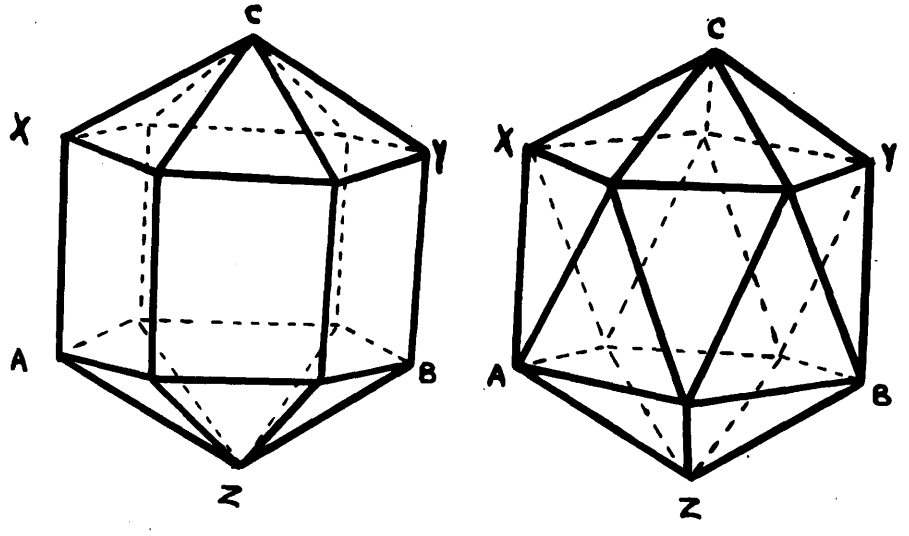


figure 3.

Whereas for the bipyramidal prism, the two superimposed triangles ABC and XYZ both lie on the same side of the polyhedron, in the case of the icosahedron, the triangle ABC lies on the lower surface, and triangle XYZ on the plane above it. Such a difference should be detectable in the electron micrographs since in the latter case, the triangles will be at different depths in the PTA and so should have slightly different electron densities. This effect is seen more clearly in figure 4.

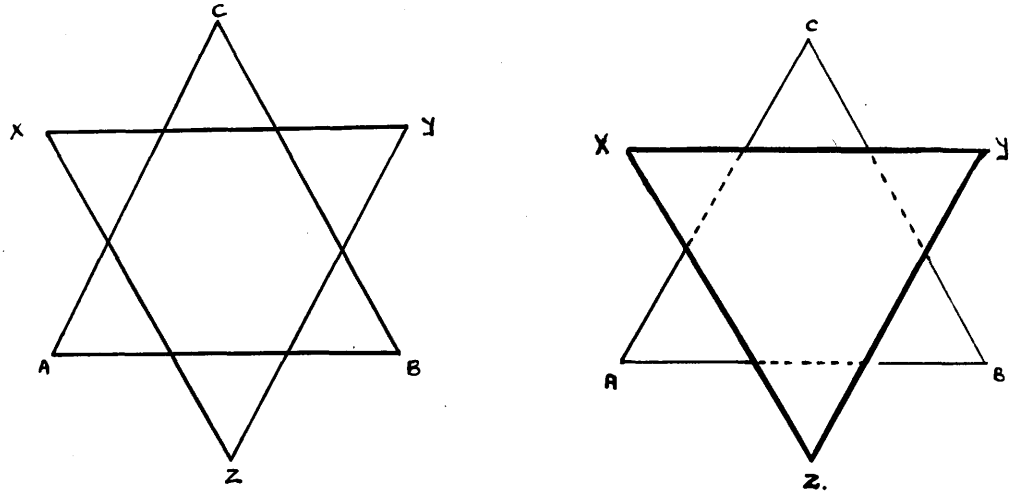


figure 4.

Figure 4(a) shows the superimposed triangles expected from the bipyramidal prism; figure 4(b) shows those of the icosahedron. As can be seen in Plate 12 such differences in intensity were observed, and from this, the icosahedron seems the more likely form. This shape has been postulated for Typhoid phage 2 and Vi phage 1 (Bradley and Kay, 1960) a phage of B. megatherium (Chiozotto et al., 1960) and phage QR (Kay and Bradley, 1962) and indeed this is the form theoretically more suited to the packing of small sub-units, since it allows the maximum hexagonal packing

on its triangular faces. At present, then, icosahedron seems the more likely, although the bipyramidal hexagonal prism has not yet been definitely disproved, and further work is required to finally resolve this problem.

Size Measurements

The size measurements made of phage B by shadowing and negative staining did not give identical results, the head size as determined by metal shadowing being slightly larger than that observed by negative staining. This is probably due to the deposit of metal over the head in the former method, resulting in over-estimation of sizes. Negative-staining, on the other hand, possibly involves some penetration of the head by the PTA, and so may result in a value less than the true one. It was also noted that the height of the head in shadowing experiments was slightly less than the diameter. This may result from slight sinking of the particles into the substrate film, or from a small amount of collapse of the phage particles during drying.

The Fine Structure of the Bacteriophage Head

Shadowed Specimens

The suggestion of "knobs" such as those seen in Plate 4, was originally recorded by Hall, MacLean and Tessmann (1959) for shadowed phage ϕ x 174;

these workers observed a central raised portion surrounded by five more knobs in pentagonal array, and suggested that the phage was composed of a series of knobs arranged at the corners of a polyhedron. Evidence in favour of this came from negatively stained phage particles (Tromans and Horne, 1961). These findings indicated that the phage may have twelve morphological sub-units at the vertices of an icosahedron. A very similar arrangement of knobs was noted by Kay and Bradley (1962) in their study of phage QR. In this case, it was stated that such a structure is "exactly consistent with an icosahedron having twelve sub-units", and it was assumed that the knobs coincided with the twelve vertices of the icosahedron.

However, in the present case, the knob structures cannot be reconciled with the PTA observations; the outlines in the negative stain are clearly hexagonal with straight sides and sharp angles. Since negative staining enables substructures to be determined in more detail than shadowing, one would expect the surface protuberances observed in shadowing to be plainly visible in PTA preparations.

It seems possible that the "knob" structure may be an artefact of the shadowing method. When a stream of metal atoms falls on a facetted figure such as an icosahedron, it would seem likely that the metal may

pile up preferentially on the faces which are most directly in its line. Faces which are obliquely placed, or protected by other protruding faces may have less metal deposited on them. Because of the small size of the facets, and the relatively large amounts of metal involved, the faces would tend to appear more spherical than triangular, and hence the knob structure will result. Both the icosahedron and bipyramidal hexagonal prism are capable of producing such appearances, and in both cases, the size distribution of the "knobs" is similar to that suggested in Plate 4.

Negatively Stained Specimens

More information as to the fine structure of the bacteriophage particle can be derived by this method, since the negative stain can penetrate between the substructures of both head and tail, showing them up in some detail.

The functions of the striations occasionally seen on the heads of the phages are not fully understood, although it seems most probable that they are features of the polyhedral form of the head.

The protein membrane is clearly visible in Plate 10 and measurements made on its thickness are in good agreement with the value of 60Å calculated for E. coli, T_2 phage (Brenner et al., 1959). Individual sub-units

can be seen around the edge of the head in Plate 10. There seems to be a small space between the capsid and its DNA contents (Plates 7 and 9) and this may be the space occupied by the elastic membrane suggested by Bradley and Kay (1960) the purpose of which will be discussed later in this work (p.72).

The actual polyhedral nature of the phage head may be determined only by establishing the arrangement of the capsomeres, and to this date these have never been observed in the detail approaching that of many of the spherical plant and animal viruses. This may be due to the fact that the capsomeres of phages are too closely packed to allow the negative stain to penetrate between them. Bradley and Kay (1960) have suggested that the capsomeres of T_5 phage have the shape of hollow cylinders, 50Å in diameter and 100Å long. These workers used uranyl acetate at pH 4.5 as a negative stain, and suggest that the capsomeres may be visible in this medium either because some degree of positive staining has occurred or because some disorganisation of the capsomeres has resulted from the low pH, allowing PTA to penetrate between them.

In the present studies, Plate 7 shows evidence of orientation of the capsomeres of phage C, with PTA at pH 7.4; at this pH it is unlikely that this is confused

by disorganisation, and the sub-units may be clearly seen to be alligned over part of the head in triangular array. There is also a suggestion of other triangular facets, as indicated in figure 5.

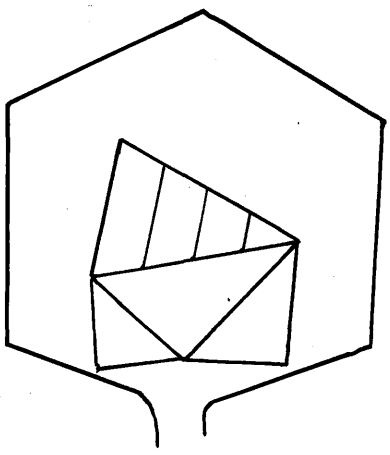


figure 5

Such an arrangement would be in agreement with the icosahedral head form. If indeed the capsomeres are very close together, the orientation of the head would be very critical if these were to be resolved. Consequently they would not often be seen.

Occasionally differently sized phage heads were observed; Plate 8 shows one smaller head in the field. The approximate volume of the small head is 10^{8}Å^{3} whereas

that of the normal head is $2.15 \times 10^{8} A^{3}$, assuming each to be spherical, for the ease of calculation. Hence it would seem that the small head has half the normal volume. Such a phenomenon has been recorded by Anderson (1960) for P phage of E. coli. but the reason for such effects is unknown.

Since PTA penetrates the empty heads, the internal structures of the phages can be established, at least in part. That these heads preserve for the most part their hexagonal outlines is evidence that the actual shape of the phage head is governed by the fairly rigid membrane, rather than by the DNA contents. This in turn upholds the theoretical suggestions of Watson and Crick (1956 and 1957) for the small plant and animal viruses. A possible indication of capsomeres may be seen in some of the empty heads. Plate 7 shows arrangements of the sub-units of two heads which are splitting and this may be seen more clearly in the photographic reversal of this plate (Plate 13). However, it is doubtful whether in these instances the capsomeres would maintain their original positions.

As previously mentioned (p. 48) partially empty heads are visible in several plates, and it is possible that these may be produced by excessive centrifugation during phage preparation, resulting in the agglutination

of the DNA into masses, for which the size and position varies. However, this seems unlikely since it is difficult to see how a given amount of DNA, already presumably packed in the most economical way, can be made to occupy a fraction of its original space. Another explanation is that it represents incomplete ejection, produced by the unnatural environment of the phages.

The Fine Structure of The Bacteriophage Tail

The tails of all four Bacillus cereus bacteriophages are long and flexuous; they are of the order of 80Å in diameter. Occasionally hollow tails were seen (Plate 8) and these were associated with empty, or partially empty heads. Bradley and Kay (1960) also noted this and suggested that in the complete bacteriophage, both the head and the tail are full of DNA; under ideal conditions no hollow tails would be found attached to full heads, since natural ejection would be expected to expel the DNA completely from both head and tail. The central channel in the tail is 20 - 25Å in good agreement with that obtained for the central core of coli T_2 phage (Brenner et al., 1959) and provides adequate space for the DNA molecule of width around 15Å (Williams, 1952(b)) to pass.

Fine structure has been observed in the tails of phages which possess contractile sheaths (Brenner et al., 1959; Bradley and Kay, 1960) but difficulty has been encountered in resolving the fine structures of the narrower tails of the phages which possess no contractile sheath. Bradley and Kay (1960) observed striations in phage tails which were negatively stained with uranyl acetate, pH 4.5, and postulated the existence of hollow sub-units. Mach (1962) showed similar banding with PTA (pH 4.0). Both, however, are open to the criticism that deformation has occurred due to the low pH (Bradley and Kay, 1960). In the present work, suggestions of striations running normal to the axis of the tail were observed (Plates 6 and 9). Plate 10 shows clear evidence of two sets of striations; those running normal to the tail axis, and another set, at an angle of about 30^{o} to the axis of the tail as shown in figure 6. This is very similar to the structure observed in the broader tail of *E. coli* T_2 phage (Bradley, 1962) and may be explained either as a series of concentric rings or in terms of a helical packing of capsomeres. Such a construction resembles that of the rod-shaped viruses, and again agrees with the theoretical deductions of Watson and Crick (1956, 1957). Such an arrangement is not visible along the whole length of the tail in Plate 10.

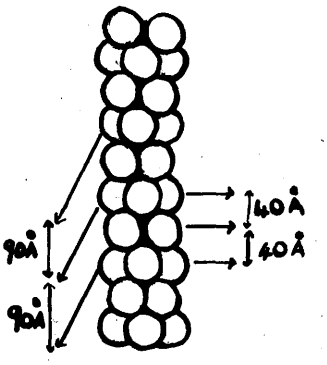


figure 6.

In order to obtain an idea of the nature of the helix, models were made of possible structures. 3 or 4 capsomeres were visible in the horizontal striations seen in negatively stained tails of E. coli phages (Bradley, 1962). A model was constructed on the basis of Bradley's findings and found to have 6 capsomeres per ring, or almost 6 capsomeres in one turn of the helix, and to be hexagonal in cross section. Such a structure would be consistent with the fact that T_2 phages are known to possess a base-plate of hexagonal cross section. It also seems likely that the tails of T_2 phages are attached to the six-fold axis of symmetry possessed by the bipyramidal hexagonal prism which is now accepted as the head shape of these phages.

In the present work 2 and 3 capsomeres were visible in the horizontal striations; a model constructed on this basis was of pentagonal cross-section. The angle of the oblique striations was measured from the model, and was found to be in quite close agreement with that observed in the tail of the phage (Plate 10).

If the tail does have such a structure, with 14 sub-units in 3 turns of the helix and a pentagonal cross section, it would be consistent with its attachment to the five-fold symmetry axis of the icosahedron, since this figure has no six-fold symmetry. Such a correlation between the symmetries of the head and tail has, as far as is known, not been previously suggested, and is as yet mainly hypothetical. The much smaller diameter of the tail of the B. cereus phage in comparison with that of the T_2 phage makes exact structural evaluation difficult and further high resolution work is necessary before definite conclusions can be reached.

Since this thesis was written, Bradley (1963) has postulated, from a slightly different basis, a similar structure for the tail of T_5 phage of E. coli.

The base plates of all four phages were frequently observed, but often appeared indistinct. In several instances, however, they had a triangular form (Plates 6, 10). Their polyhedral nature was clear from the fact that they cast angular shadows (Plate 3). Their detailed structure can be seen in Plate 14 and consists of three

units radiating from the tail; there are probably a further two or three such units below this plane. On the basis of the argument in favour of the pentagonal cross section of the phage tail, the base plate is probably also five-sided in cross section.

There was no evidence of the fibres seen by Bradley and Kay (1960) to be attached to the base plate of phage T_2, but the base plates of the phages of this study appear to terminate in the solid structure seen in Plate 14 and diagrammatically in figure 7(a).

For phage T_2 (Daems et al., 1961) an external jacket is seen around the contractile sheath and Bradley (1962) postulates that this breaks open to produce the fibres necessary for interaction of the tail with the host cell membrane, prior to injection of DNA. In the present study no sheath, jacket or tail fibres were seen, but it is possible that the fibres are contained within the structure of the base-plate; on adsorption this may rupture as postulated for the jacket of T_2 with release of the fibres (fig. 7(b)). In Plates 5 and 7 where the base plates do not take the exactly triangular form, this may already have taken place.

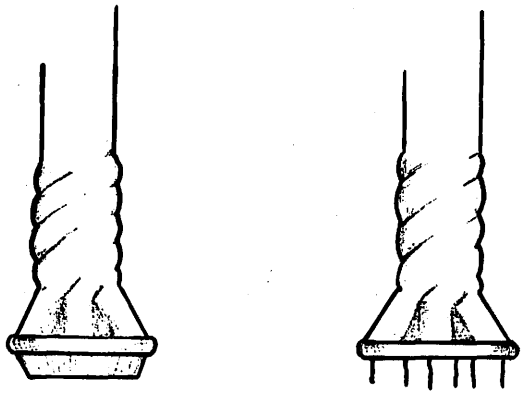


figure 7.

Figure 8 shows diagrammatically the postulated morphology of these phages.

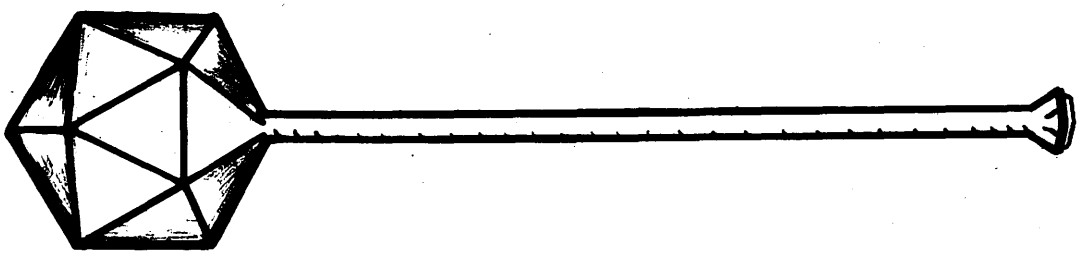


figure 8

The Classification of Bacteriophages

Although d'Herelle (1926) originally held that there was only one species of phage which was very adaptive, it soon became realised that bacteriophages, even when they attack the same species of bacterium, differ greatly in such fundamental properties as their serological specificities and morphologies. It is clearly advantageous and necessary for the detailed study of the bacterial viruses, to evolve an efficient basis for their classification; a general treatment of this topic is given by Adams (1959). Early electron microscope studies of size and shape facilitated a tentative classification on a morphological basis, and this was found to confirm previous groupings made from serological specificities. The negative staining method made possible the study of fine structures, and it became clear that although all phages consist basically of a head and tail, the complexity of their fine structures may vary greatly. On these grounds, Bradley and Kay (1960) proposed a morphological classification. These workers studied twenty-two widely differing phages, and divided them into three groups; those which have tails with contractile sheaths (such as the T-even phages) those which have no such contractile sheaths (such as T_1 and T_5 phages) and those which have very short tails (phages QR and QX174). On the basis of this classification, the four phages of the Bacillus cereus

group fall into group 2. However a general classification on this basis is not entirely satisfactory, since two phages may be morphologically similar, but differ completely in host specificity. A combination of serological and morphological classifications may eventually prove most useful.

The Possible Mechanism of Injection of DNA into the Bacterial Cell.

The first stage in the infection of the bacterium involves the adsorption of the phage tail on to the cell wall; the detailed mechanism of this process is as yet not fully understood. It has been suggested that there are two types of interaction between the phage and the host cell; Mahler and Fraser (1961) thought that the initial reaction is dependent on the formation of electrostatic bonds between the phage tail and the cell wall, whereas Garen and Kozloff (1959) in their study of T_2 phage of E. coli proposed that the formation of such bonds was a reversible process which may or may not be followed by the formation of an irreversible linkage with the host cell. When the irreversible link has been formed, the injection process must continue until completion, whereas while the interaction is still reversible the phage may be released again from the cell wall. In irreversible interaction it is thought that

the fibres at the end of the tail unwind slightly and react chemically with the surface of the cell, possibly in an enzyme-catalysed reaction.

The next stage is the contraction of the tail sheath (Garen and Kozloff, 1959) which consists of a contractile protein (Kozloff and Lute, 1959). Such a contraction possibly results from a change in the configuration of this protein, similar to that which takes place in actomyosin of muscle tissue, with a corresponding alteration in the numbers of capsomeres per turn of the helix. This contraction may force the core of the tail through the cell wall. The enzyme lysozyme (Koch and Dreyer, 1958) present in the terminal region of the phage tail, also plays a role, dissolving a small region of the cell wall; the exact relationship between the contraction of the tail and the action of lysozyme is not yet understood. At this stage, the DNA is injected into the host cell.

In those phages which have contractile sheaths, DNA injection, in addition to tail penetration may be associated with the contraction. Such a mechanism, however, cannot operate for those phages which have no contractile sheath. Here it is possible that the injection mechanism resides in the head itself. "Elastic membranes" have been observed within the head (Bradley and Kay, 1960) between the protein coat and the DNA;

Bradley and Kay suggest that this membrane may contract in response to some as yet unknown stimulus and expel the DNA. Some suggestion of these structures can be seen in Plate 6. Another structure which may be associated with the injection mechanism can be seen in Plates 6, 8 and 9 and takes the form of spherical "knobs" at the point where the tail seems to project some distance into the head. These may be concerned in the transmission of the stimulus from the tail to the head. It is possible that the DNA within the phage head is so coiled that when stimulated in some way (possibly by the "knobs") it uncoils itself like a spring thus facilitating injection. Combinations of these possible mechanisms may well be involved.

The means by which the tails of these phages which have no contractile sheath, penetrate the cell, can only as yet be speculatively explained. The fibres of the phage tails may contain considerably more lytic enzyme and therefore dissolve a larger area of the cell wall, thus allowing the penetration of the whole of the tip of the tail.

It is also possible that the nature of the cell wall of the host species controls the presence or absence of the contractile sheath.

After ejection of the DNA the protein coat and the tail remain on the cell wall and are eventually discarded.

The DNA of the phage on the other hand now disrupts the metabolism of the host cell in such a way as to divert it to the production of many more phage particles; when the full complement has been synthesised the new phages lyse the cell and are free to repeat the infection process.

The second part of this Thesis describes experiments designed to gain more information concerning the process of intracellular multiplication of bacteriophages.

PLATES

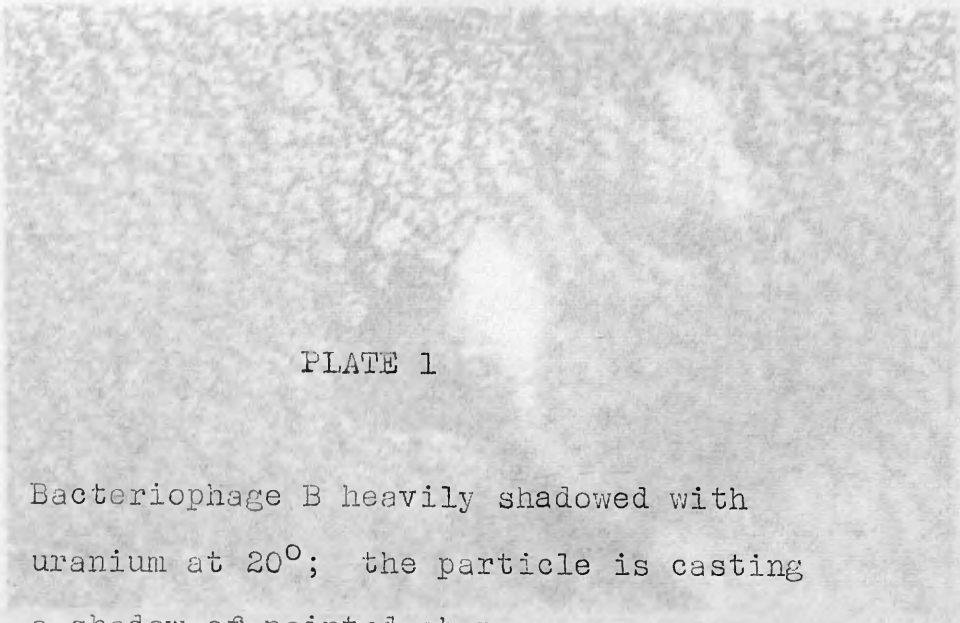


PLATE 1

Bacteriophage B heavily shadowed with uranium at 20°; the particle is casting a shadow of pointed shape.

(S/61/1326) x 315,000

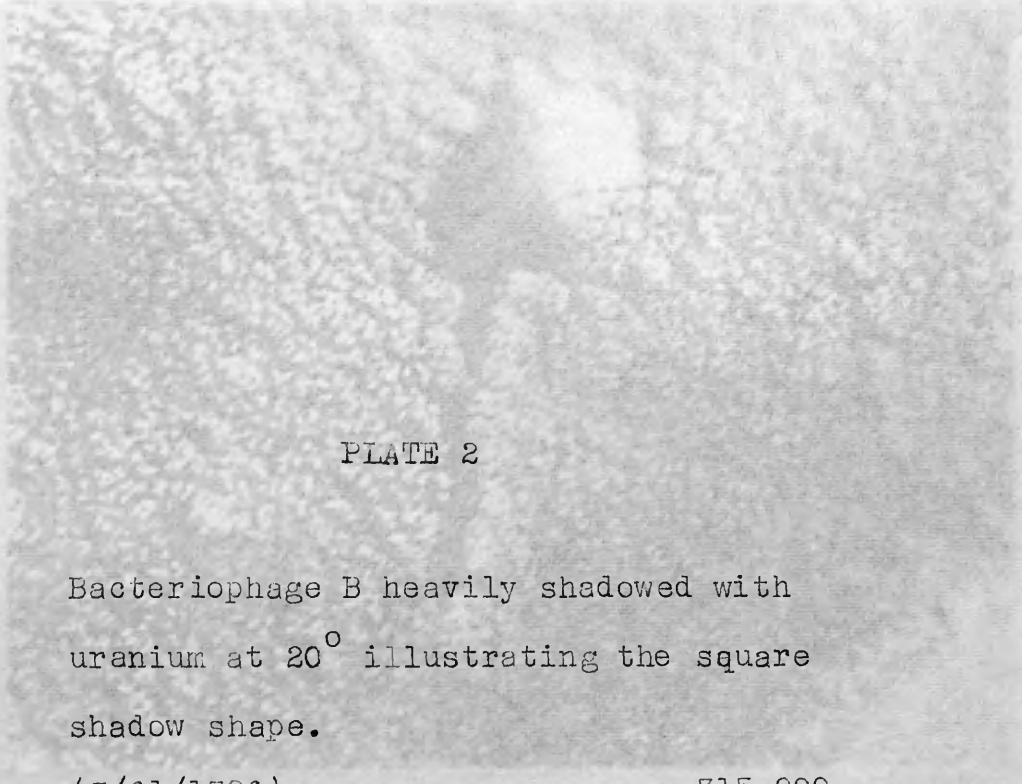


PLATE 2

Bacteriophage B heavily shadowed with uranium at 20° illustrating the square shadow shape.

(S/61/1326) x 315,000

PLATE 3

Bacteriophage B heavily shadowed with uranium at 20°, showing the square shaped shadow.

(S/61/1318) x 155,000

PLATE 4

Bacteriophage B shadowed at 20° with Ni/Pd illustrating the five-sided figure with central raised portion.

(S/61/1202) x 170,000

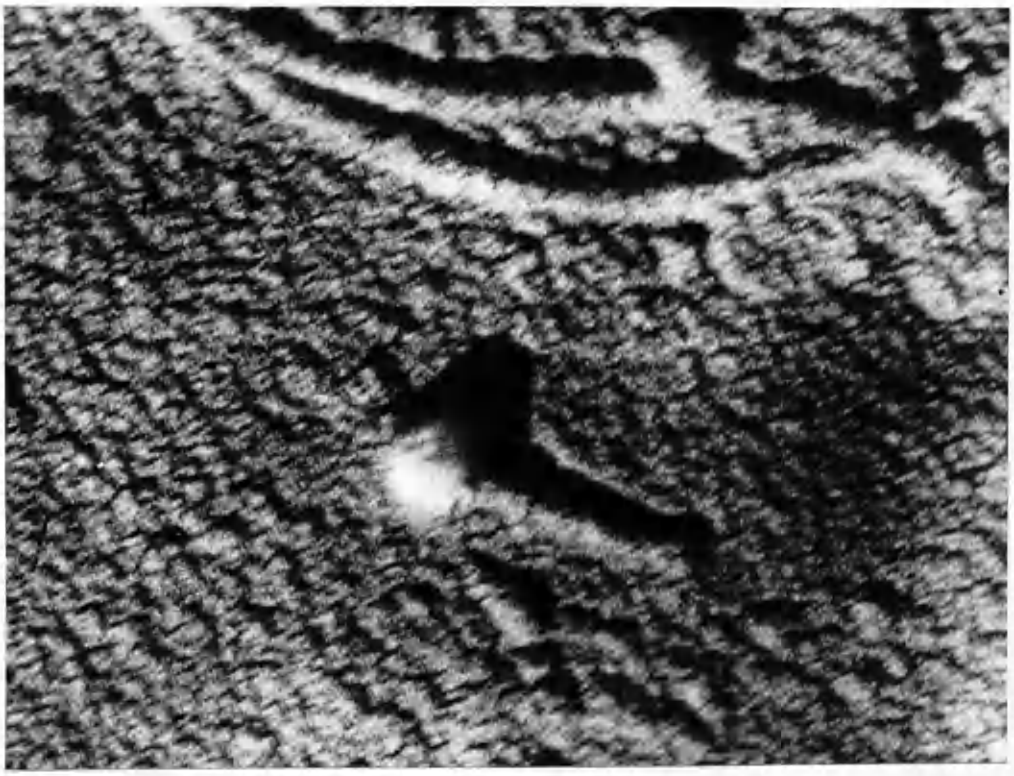


PLATE 5

Phage A negatively stained with PTA at pH 7.4.

(S/61/1504) x 314,000

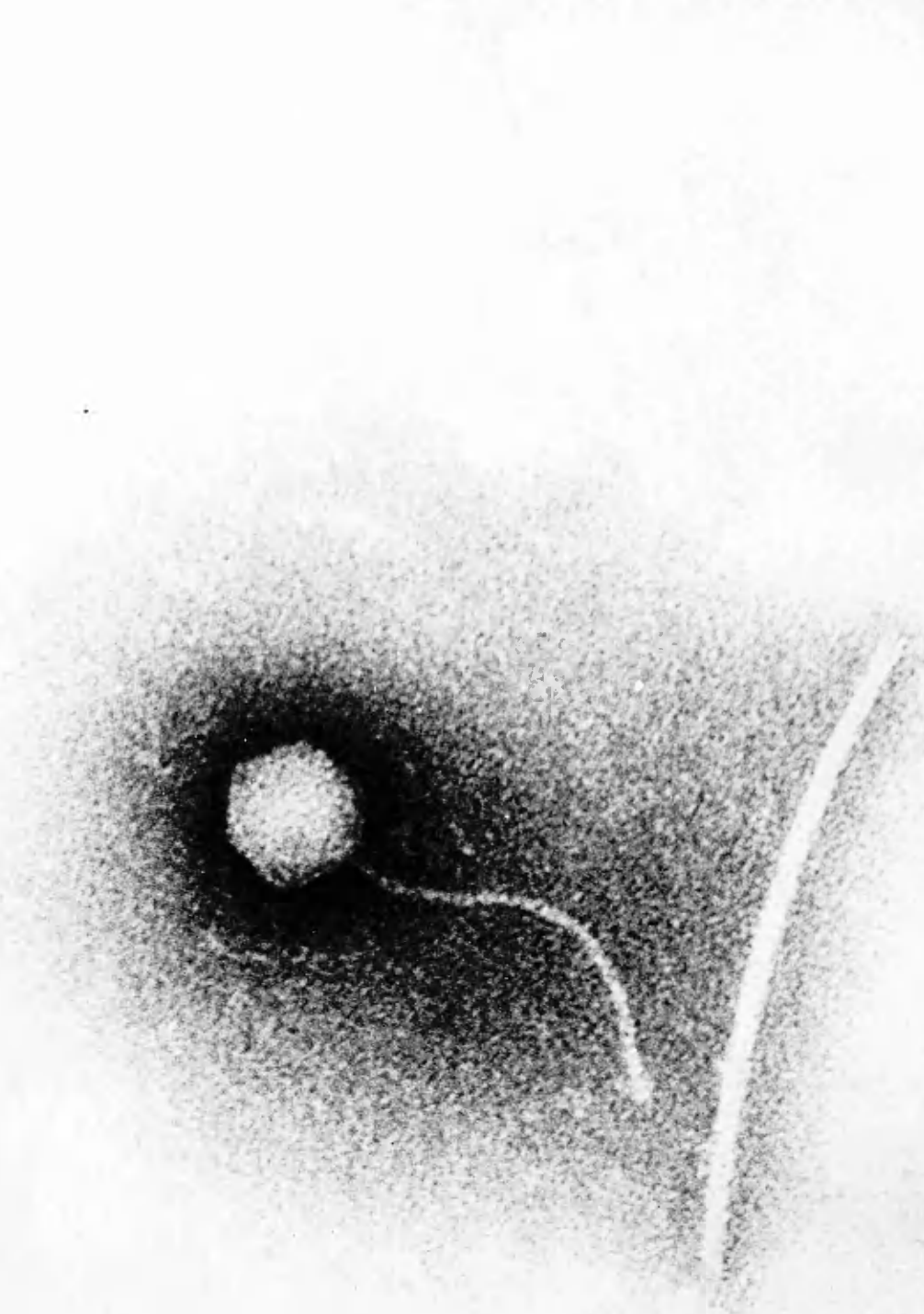
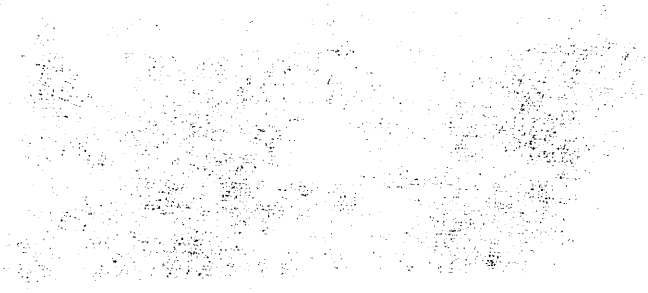


PLATE 6

B phages negatively stained with PTA, pH 7.4
Complete phages are present in this field together with ghosts and empty heads which have become separated from the tails.

(S/61/580) x 175,000



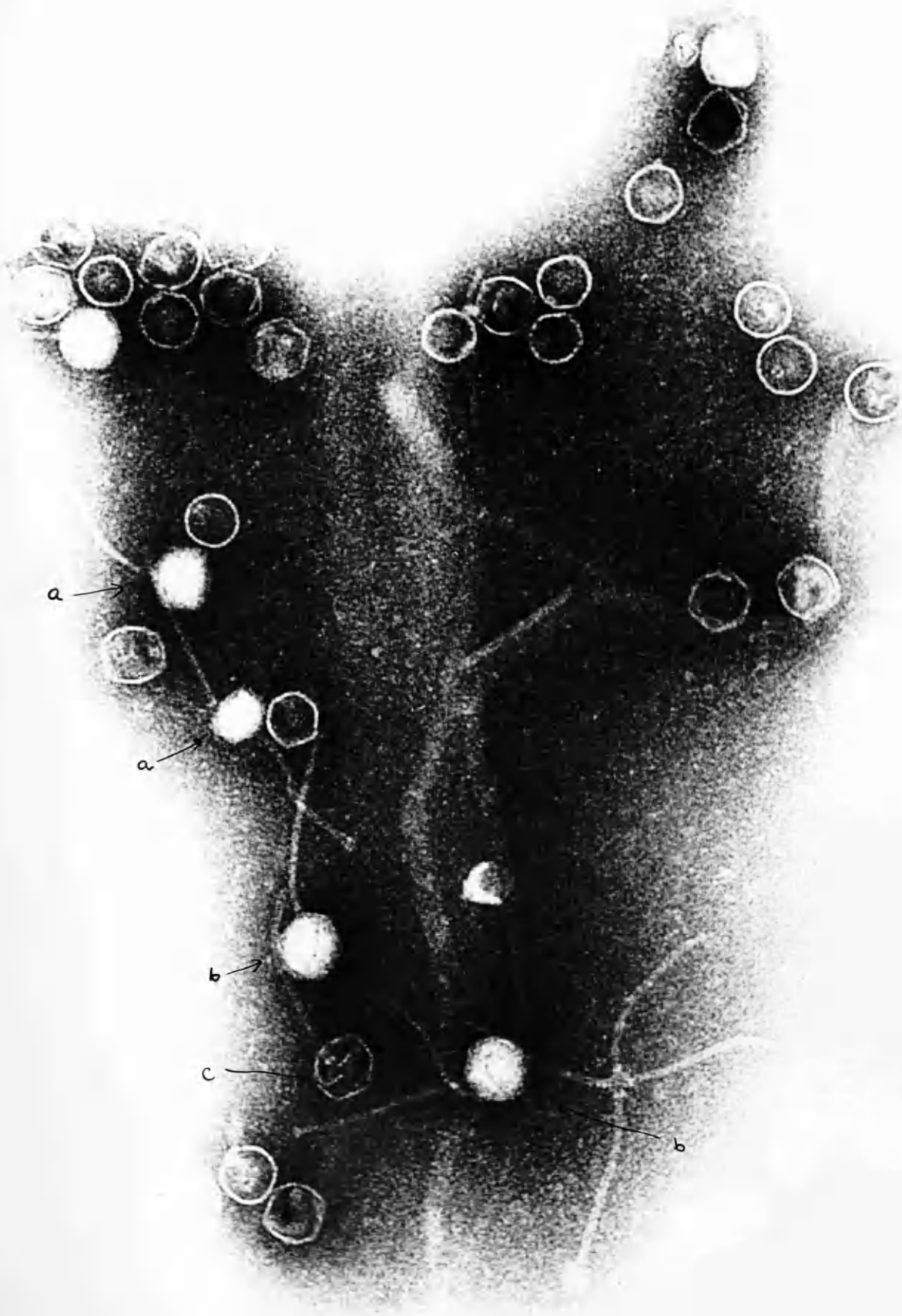

a
a
b
C
b

PLATE 7

Negatively stained C phage, showing some degree of orientation of the capsomeres of one of the heads.

(S/61/623) x 235,000

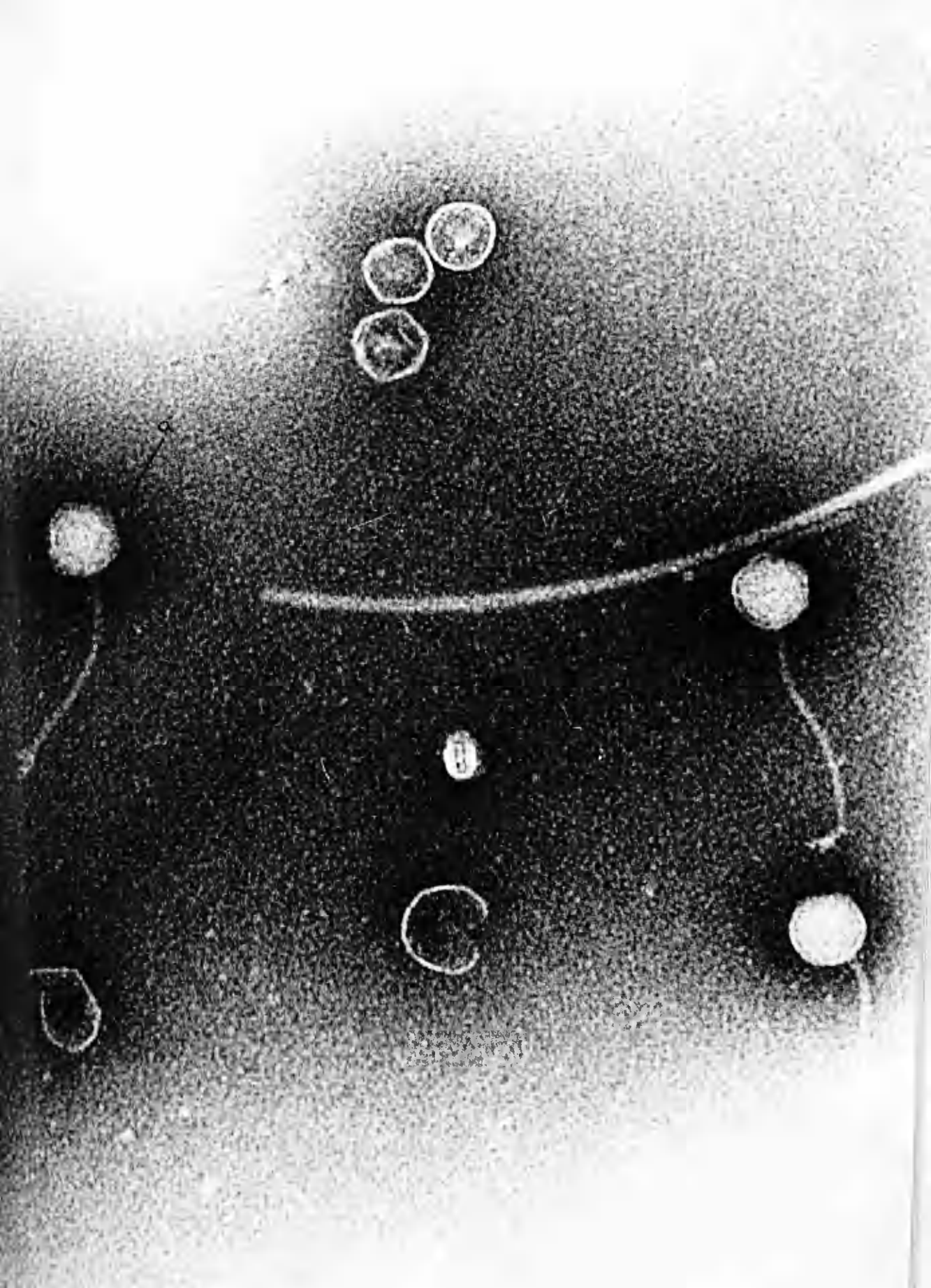


PLATE 8

D phage negatively stained with PTA pH 7.4, illustrating the hollow tail, and two head sizes.

(S/61/878) x 370,000

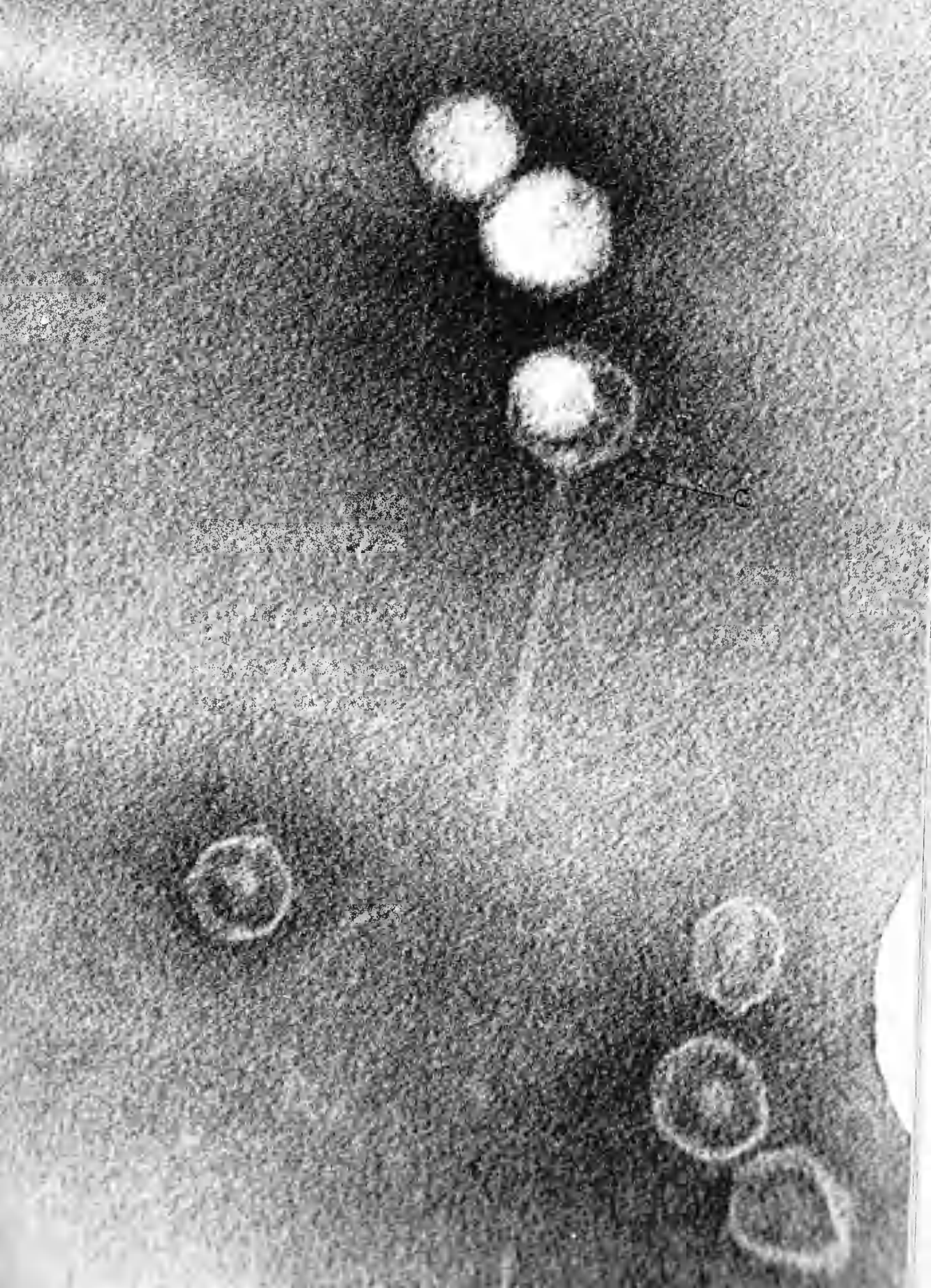

C

PLATE 9

B phage, showing an external membrane around the full heads, some suggestion of sub-units in the head, and of striations on the tails.

(S/61/578) x 260,250

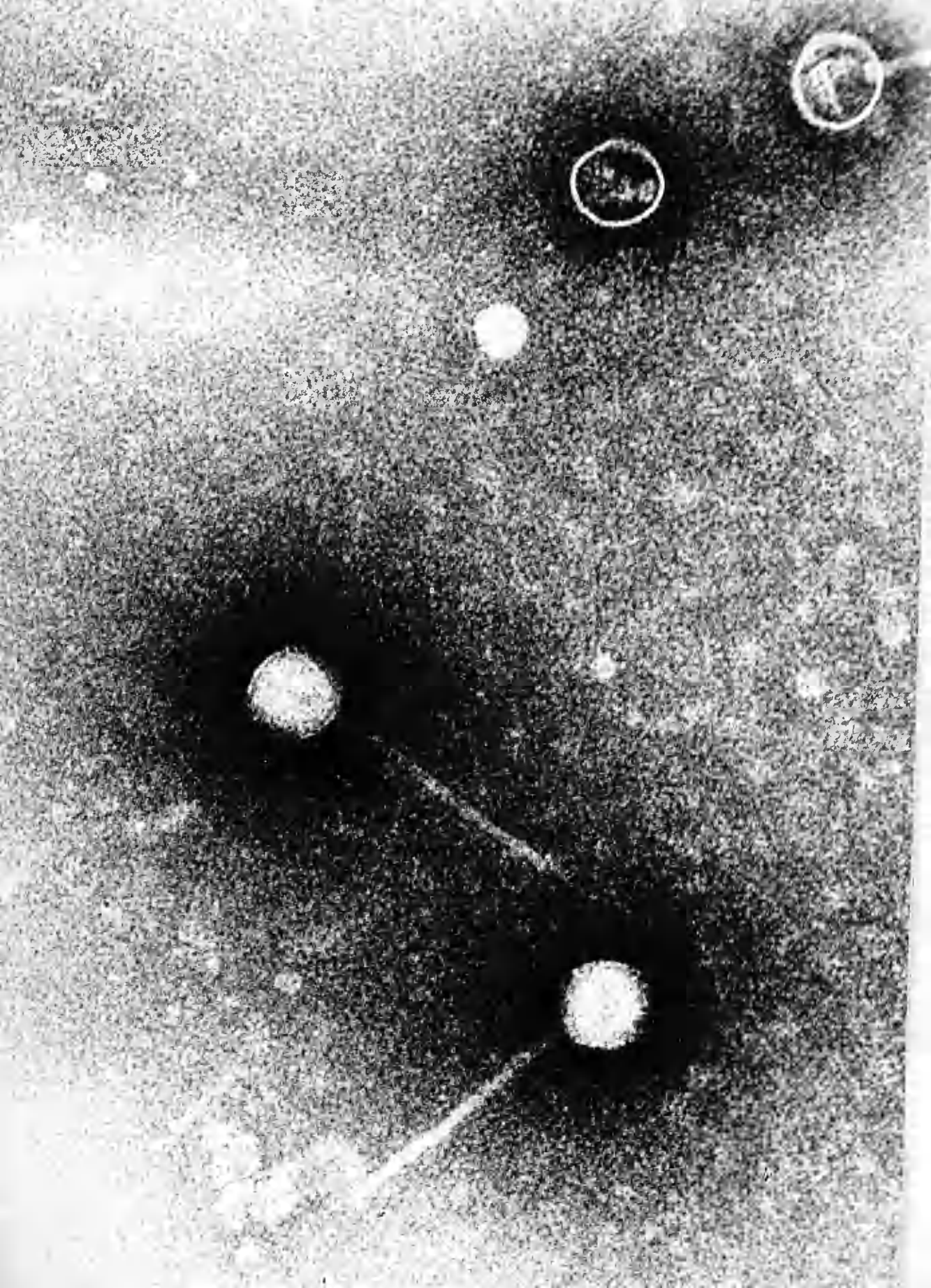


PLATE 10

B phage, illustrating sub-units around the head, and some structure in the tail.

(S/61/579) x 405,000

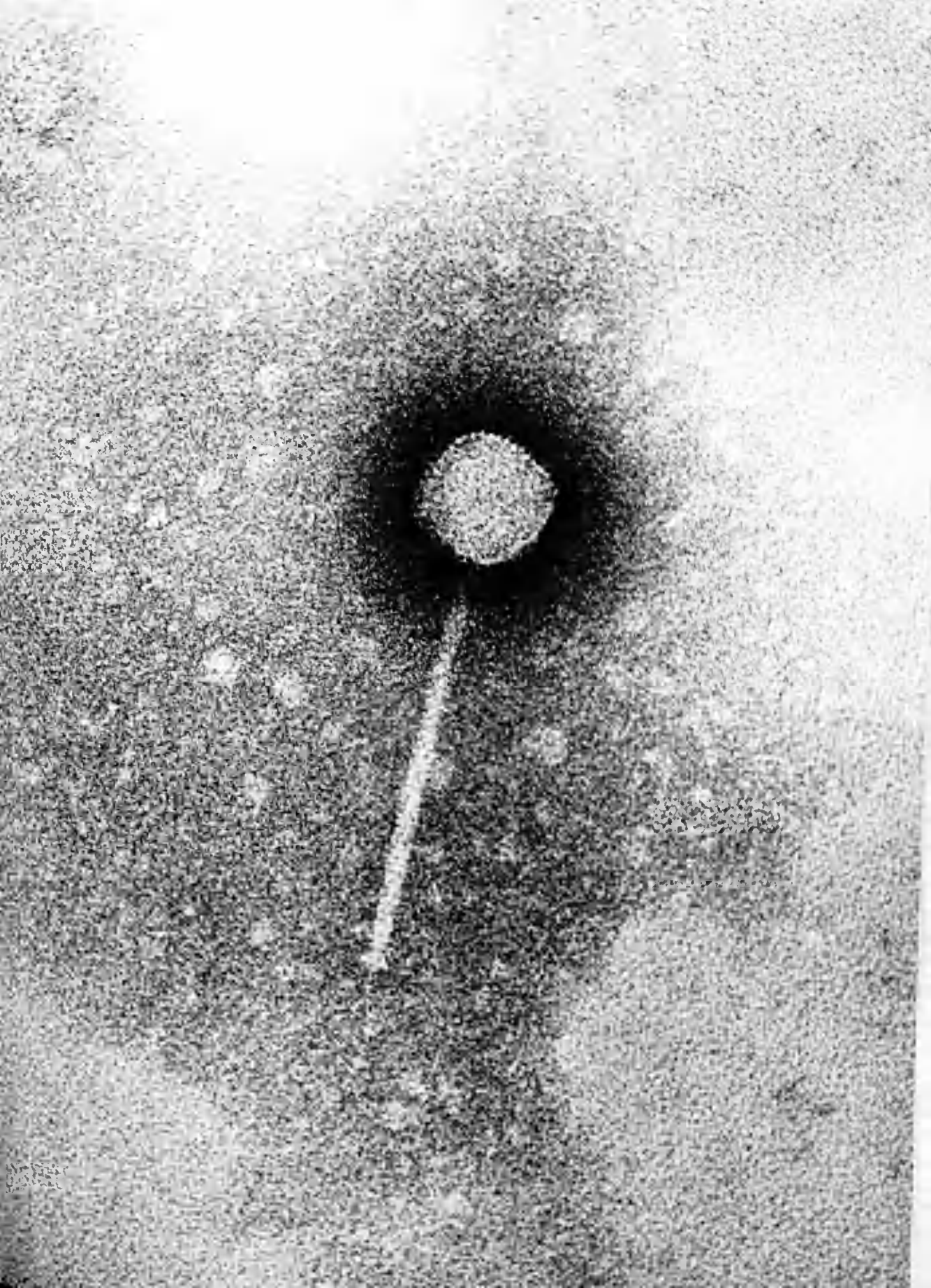


PLATE 11

Tail-less D phages, showing empty and full heads, and also partially empty heads, which contain aggregations of DNA of irregular size.

(S/61/880) x 185,000

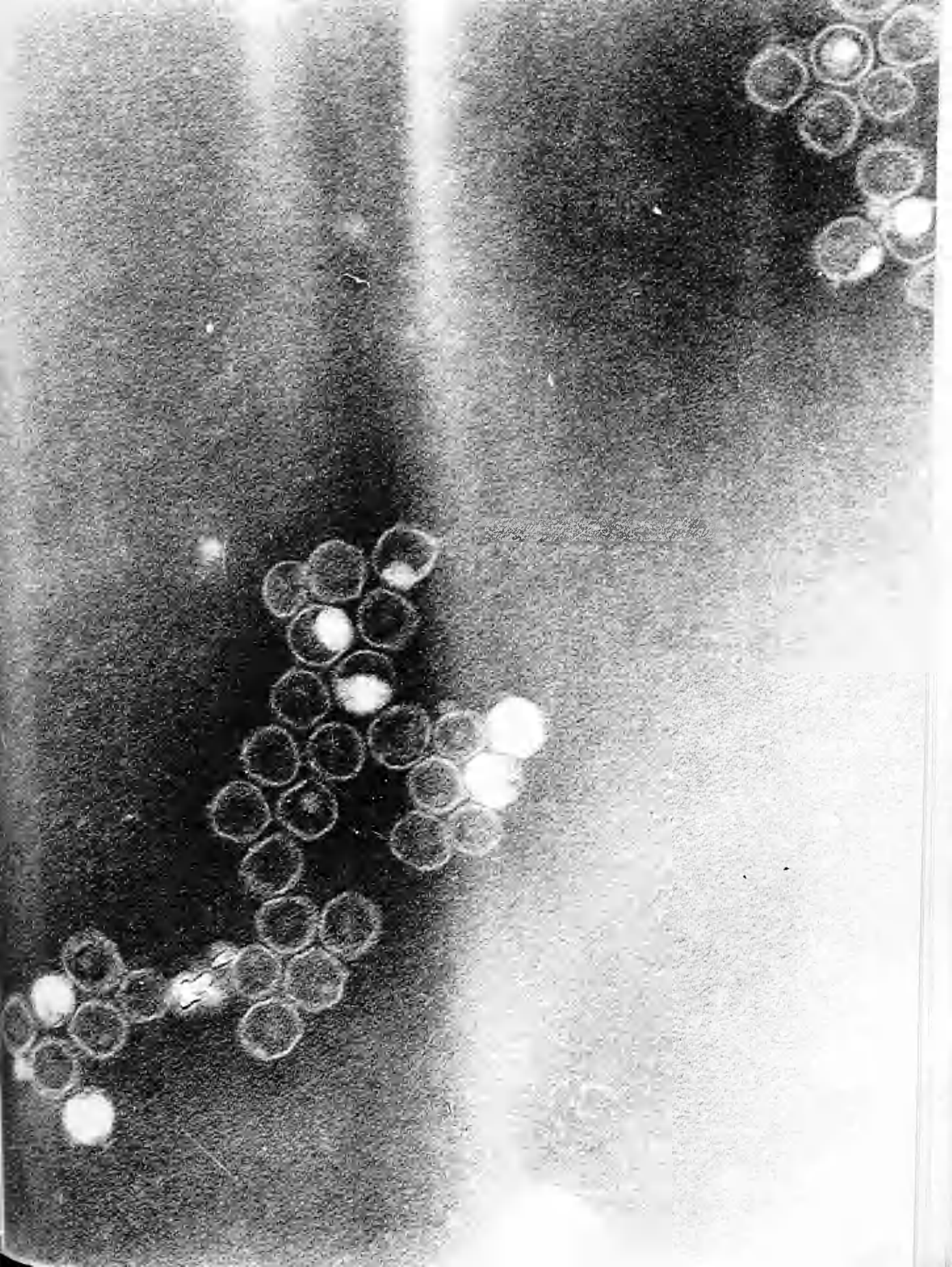


PLATE 12

Photographic enlargement of Plate 6, showing the superimposed triangle structure in two empty heads.

(S361/580) x 277,000

PLATE 13

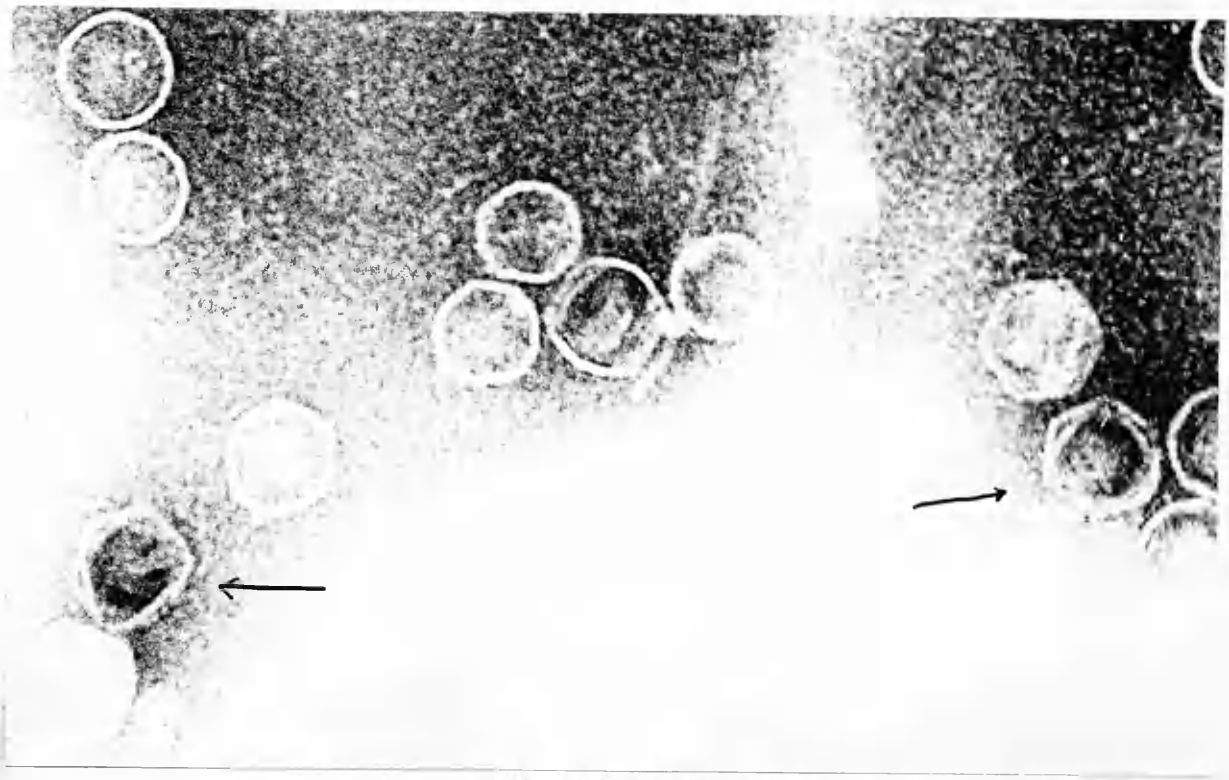
Photographic reversal of Plate 7. The substructure in the full head, and also arrangement of sub-units in the two fractured heads is visible.

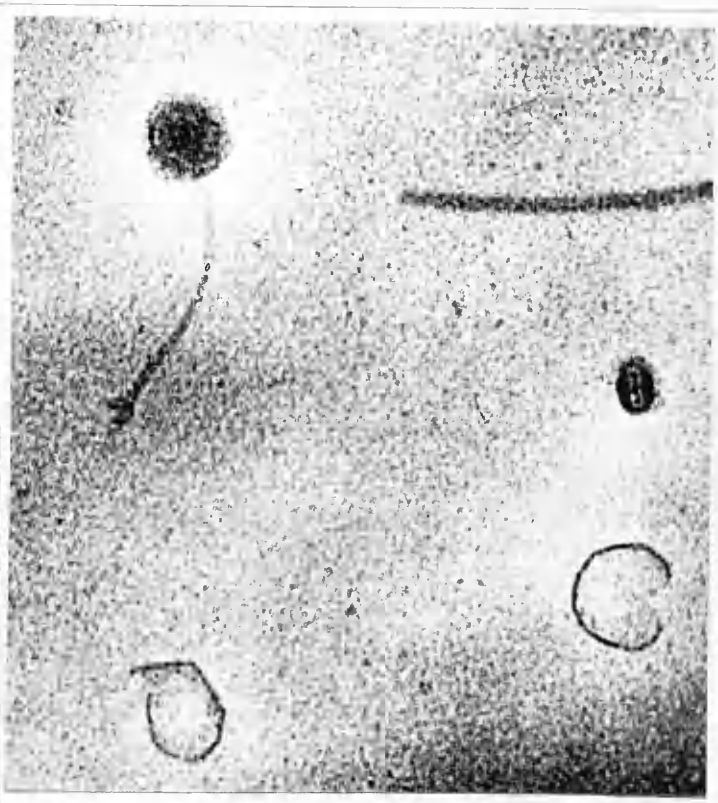
(S/61/623) x 184,500

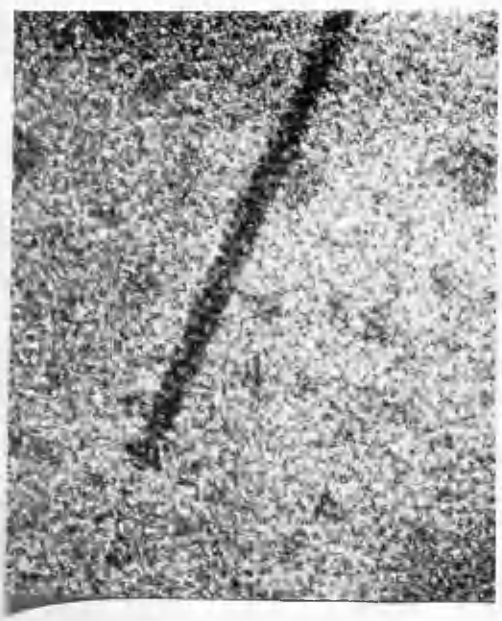
PLATE 14

Photographic reversal of Plate 10. The striations on the lower part of the tail may be clearly seen, and the structure of the base plate can be detected.

(S/61/579) x 437,500







PART II

THE INTRACELLULAR DEVELOPMENT OF ONE BACTERIOPHAGE OF THE *BACILLUS CEREUS* GROUP.

INTRODUCTION

Intracellular Multiplication

Pre-lytic effects in phage-infected bacteria, such as swelling and gross cytological changes, can be easily detected with the light microscope. In order to further investigate the mechanism of these processes, it is necessary to study the intracellular multiplication of bacteriophages and the detailed changes which take place within the host cell; the electron microscope initially seemed to provide an ideal means for this. Technical difficulties, however, arose in the preparation of material for such studies. There are two main methods of studying phage multiplication - by examining lysates, and by thin sectioning.

Lysates

Prior to the standardisation of techniques of microtomy, methods using lysates were employed for studies of intracellular phage propagation. Samples of phage-infected bacteria were taken at various time intervals after lysis had begun and studied by shadowing; this gave a kinetic picture of infection. Alternatively premature lysis was induced in one of several ways, the most common of which were intense sonic vibrations (Anderson and Doermann, 1952), osmotic shock (Anderson, 1950) or lysis from without (Doermann, 1952).

Cyanide was often added to "freeze" the metabolism.

Early findings seemed to pose questions rather than answer them. Luria, Delbruck and Anderson (1943) recorded the lacerated appearance of the cell wall after phage-induced lysis, but found the infection process difficult to interpret. They thought that the complete phage particle entered the cell, and concluded that since the cell was always seen to be surrounded by phages, the entry of one phage particle so altered the cell wall as to block the entry of further particles. This ingenious hypothesis is now known to be incorrect and to be due to the limitations of the methods of these workers, which gave only a two dimensional concept, and prevented them from distinguishing between full and empty phage heads.

The infection process of E. coli T_4 phage was studied by Wyckoff (1948), who noted that the bacterial protoplasm lost its uniform nature after infection, becoming granular in appearance. Similar changes were observed by Levinthal and Fisher (1952) who stated that "...as each new aspect of the problem has been investigated, it becomes more apparent that the virus could not be considered an independent self-reproducing entity growing in the host cell. Rather the host-cell complex which is formed after the cell is attacked acts as a unit to

produce new phages." These workers appreciated that the disappearance of infectivity after adsorption on to the host cell membrane is associated with the complete break-down of the phages necessary for the formation of such a complex; indeed a somewhat similar concept of the host-cell complex is now regarded as an integral part of phage infection and multiplication (Kellenberger, 1961).

Kinetic studies were also being made on phage + bacteria systems, and the latent period, or the time during which the phages grow inside the cell was determined for several systems. Doermann (1952) found that for T_4 phage of E. coli, no phages, neither newly-formed nor the original infecting particles were detectable during the first half of the latent period and that the numbers of phages increased thereafter at a rate proportional to the increase of the intracellular DNA. This close relationship between DNA formation and phage multiplication is now regarded as an essential feature of the process (Kellenberger, 1961).

Premature lysates were also studied in an attempt to identify the precursors of the mature phages. Among the most commonly observed and controversial structures noted under these conditions are the "doughnuts" which are empty phage heads in the shape of a crumpled disk with a central depression; these

units were recorded as early as 1949 by Wyckoff and good micrographs were also published by Levinthal and Fisher (1952) and De Mars, Luria, Fisher and Levinthal (1953). That the doughnuts were indeed empty head membranes of the same size and shape as the phage head was shown by the use of the critical point method (Anderson, Rappaport and Muscatine, 1953). The rôle of these doughnuts in phage growth was a question for conjecture. Originally it was believed that they were direct precursors, later to be filled with DNA to form mature phage particles. Evidence for this came mainly from kinetic experiments (Levinthal and Fisher, 1952); the number of doughnuts increased prior to the increase in the number of phages. Herčik (1955) proposed a scheme for phage development based on the globules or granules seen to be produced in the cell protoplasm (Wyckoff, 1949); these globules were thought to aggregate to form doughnuts which in turn formed solid particles, and the tails were thought to result from a similar aggregation of globules. Earlier De Mars et al. (1953) concluded "that the doughnuts represent an incomplete stage of maturation of phage particles, although their properties in the extracellular state may not be fully representative of those of the native intracellular particles." This distinction between

the extracellular and intracellular particles is the basis of the modern concepts of the rôle of the doughnuts. That these structures may represent fragile, newly-formed heads which have been broken during the artificial lysis has been suggested by Kellenberger and Séchaud (1957). These workers also noted tail-like structures in preparations of T-even phages, and proposed that, again, although they may be precursors, it is more likely that these units result from the disintegration of newly formed, fragile phage particles.

Thin Sectioning

Although some information on phage multiplication had been derived from a study of shadowed sections (Noda and Wyckoff, 1952) the first sections to show detail in the cell cytoplasm and its changes during phage formation were those of Maaløe, Birch-Anderson and Sjöstrand (1954). These workers encountered considerable difficulties in the preparation of bacterial material suitable for thin-sectioning, and indeed up to this date, surprisingly few studies have been made using this technique.

Fixation in a medium containing amino acids, followed by subsequent washing of the specimen with a uranyl acetate or lanthanum nitrate solution before dehydration is advocated by Kellenberger, Ryter and Séchaud (1958); such treatment gives a reproducible

preservation of the bacterial cell structure. As a result, Kellenberger and his co-workers were able to make detailed studies of intracellular phage growth. Their initial findings indicated that the process might be more complex than the previous studies on lysates had suggested. Thin sectioning methods did not reveal the phage-related structures observed in lysates which were previously assumed to be precursors of the complete phage particles. Extensive studies of the DNA of the bacterial nucleoids by Kellenberger, Séchaud and Ryter (1959) showed their break-up after phage-infection, and these workers postulated a new mechanism for phage formation.

Not a great deal of information is available on the processes involved in phage multiplication and only a few systems have been studied as yet; a summary of the currently accepted mechanism is given below, but for a detailed account, andreferences to the relevant biochemical work, the reader is referred to the reviews by Kellenberger (1961) and Mahler and Fraser (1961).

As with the morphological studies, the T-even phages of E. coli were studied first, and the mechanism given below is based on the study of T_2 phage. It is known that although no immediate synthesis of nucleic acid takes place after phage infection, profound changes

take place in the metabolism of the bacterial cell, and protein is synthesised. The cell then becomes distorted and swollen probably as a result of the break-up of the bacterial nucleoids. Inhibition of nucleoid disintegration by chloramphenicol, a substance known to prevent protein synthesis, indicates that these changes result from the initial synthesis of protein. Under normal conditions, the bacterial nuclear substance, originally a mass of fibrillar material, becomes increasingly delocalised; vacuoles filled with fibrous material, begin to appear at the edges of the cell. It is now fairly certain that this fibrous material is in fact phage-induced DNA, or "vegetative phage". The occurrence of such pools of DNA at the cell margin suggests that each infecting phage particle may result in its own DNA pool; these expand and finally merge, the nuclear material becoming widely dispersed throughout the cell instead of localised in a central region. This vegetative form of phage reproduction is the state in which all multiplication, replication and recombination takes place, and represents the intermediate "DNA $_{\text{complex}}$" which is distinct from both the DNA of the normal bacterial nucleoids, and that of the complete phage particle; it is reminiscent of the "intermediate complex" of Levinthal and Fisher (1952). The actual molecular form of the DNA in the pool is as yet unknown - it may exist as one single molecule or as

an assembly of smaller molecules.

The DNA pool of the infected cell continues to expand until the first phage-like bodies appear; it then ceases to increase in size. In the vegetative state, the DNA is believed to exist as a dilute plasma, and the formation of the phage-like particles results from a concentration of this DNA by about 15 times. Such a concentration must involve an increase in "order" and thus a considerable decrease in entropy, consequently it must be accompanied by a gain in entropy by the host cell. A gain in entropy by living systems is considered to be part of the disorganisation ultimately resulting in death. What motivates this concentration is as yet unknown, but it has been suggested that linkages may form between the DNA and protein molecules. Support for this suggestion comes from the observation that addition of chloramphenical prevents the formation of phage-like bodies; instead the DNA pool increases in size until it occupies almost the entire bacterial cell. Removal of chloramphenicol results in the rapid formation of large numbers of the condensates.

Such precursor particles do not survive lysis, and so are not identical with the "doughnuts" previously assumed to be direct phage precursors. These result from lysates of a later stage in the lytic cycle.

Rather than being forerunners of the phage particles, they seem to result from the disintegration of the fragile phage particles which have as yet an incompletely formed protein coat. Rupture of these would result in loss of the DNA leaving the empty membranes so often referred to in the early literature. The tail-like structures also found in lysates may be accounted for by a similar explanation; fragile newly formed phages with tails attached being broken down into their component parts, the head and the tail. The actual point in the cycle at which the tail becomes attached remains doubtful. It seems unlikely that a separate pool of tails is formed and that these completely formed units then combine with the fully formed heads; it is more probable that at some point in the development of the phage, the tail grows out from the head. To date, however, tails have never been detected on intracellular phages.

In short, in the mechanism of multiplication of T_2 phage of E. coli, the following precursors seem likely; the condensate, which does not survive in the lysates, the fragile head without a tail, the fragile head with a "growing" tail. A suggested scheme of development of this phage is shown in figure 1.

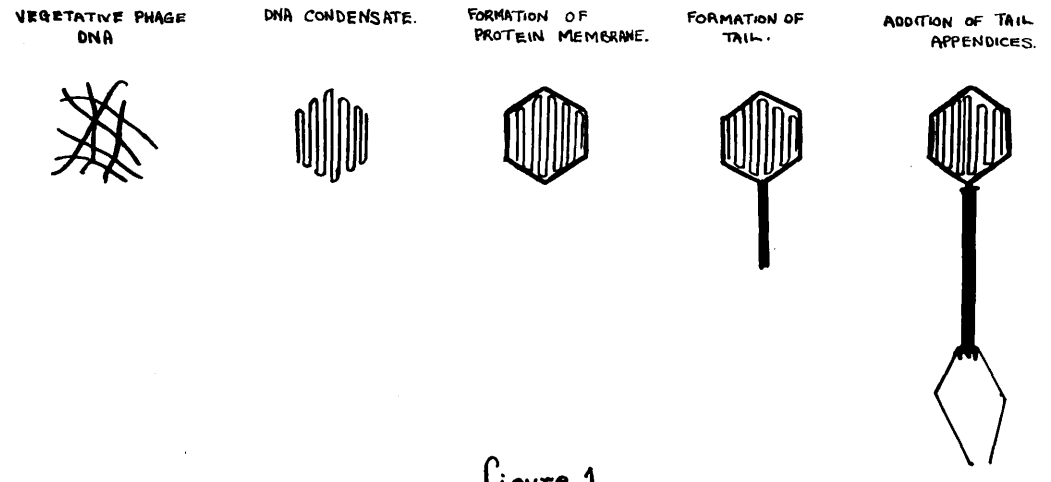


figure 1.

Some variations from this basic scheme have been observed for other phages. For instance, during infection by phage T_5 of E. coli the DNA becomes completely distributed through the cytoplasm of the cell (Kellenberger, 1961) and no discrete masses can be seen. On the other hand λ phage of E. coli causes little change in the cytoplasm, suggesting that the phage DNA becomes associated with the bacterial nucleoids in this case (Kellenberger, 1961) and that subsequent development takes place without delocalisation. A scheme of development of phages within mycobacteria, essentially very similar to that observed for T_2 phage of E. coli has been shown by Takeya, Koike, Mori and Toda (1961).

From this brief analysis it can be appreciated that very few systems other than T_2 have been investigated

by thin sectioning. In the hope of further elucidating the multiplication process and providing a comparison with the mechanisms already suggested, it was decided to study the intracellular growth of one of the phages of the B. cereus group.

Section cutting

Fixation

The early light microscopists had recognised that osmium tetroxide was an excellent preserver of cytological detail, so it is not surprising to find that this substance was used for the early sections for electron microscopy, (Claude and Fullam, 1946). It is now a very widely used fixative. Pease and Baker (1948) used osmium tetroxide to preserve their tissue when they cut the first reproducible thin sections, and other workers followed their example. However, it was soon evident that these results, although vastly superior to the other fixatives employed for light microscopy, were rather unreliable, and it was not until Palade (1952) made a detailed study of the effect of pH on the fixation process that the method was standardised; satisfactory fixation occurs at slightly alkaline pH, and "Palade's Fixative", consisting of 2% osmium tetroxide in an acetate - veronal buffer, is still widely used today.

This standard fixative has been modified slightly in order to make the tonicity more suitable for different tissues, and this was effected by Rohdin (1954) and Zetterquist (1956) by the addition of sodium and potassium salts in the proportions in which

they are found in mammalian tissue. A second method is the addition of sucrose to increase the tonicity (Caulfield, 1957) and this fixation is especially applicable to botanical material. A further modification is the Kellenberger standard fixative (Kellenberger, Ryter, Séchaud, 1958); calcium and magnesium ions are added to the buffered osmium solution, and the specimens are washed with uranyl-acetate or lanthanum nitrate solution. Such a fixation procedure gives good preservation of bacteria and other micro-organisms.

The actual mechanism of fixation by osmium tetroxide is still not fully understood. Some workers believe that the micrographs represent purely osmium deposits, whereas others hold that osmium staining does not occur at all, and that fixation alone takes place (Valentine, 1961).

Although it gives highly satisfactory and reproducible results for most materials, buffered osmium has some disadvantages. It is thought to be poisonous on inhalation and it may "leach" out parts of the tissue. Claude (1962) claims that this latter effect may be greatly reduced by fixing with unbuffered osmium at 4°C.

Luft (1956) introduced buffered potassium permangate as an alternative to osmium tetroxide. A 2% (w/v)

$KMnO_4$ solution is buffered to pH 7.4 with the same buffer as that used in Palade's fixative, and although it is not a general purpose buffer, it has certain important applications. It is a strong oxidising agent, and micrographs show that all except the lipoproteins react; the result is that the cell membranes show up in very high contrast, and so this fixative is useful when used in conjunction with the epoxy or polyester resins which preserve fine structure, but provide low contrast. Examples of its application to membrane structures are provided by the study of myelin sheaths (Robertson, 1957).

The mode of action of this fixative is unusual (Valentine, 1961). Bradbury and Meek (1960) suggested that no fixation occurred at all, and that this took place in the dehydrating alcohols. The low molecular weight of potassium permanganate would not favour high contrast, but detailed study of sections fixed in this way show that there is a high concentration of particles of less than 100Å diameter along the membranes, and it seems probable that these result from the interaction of the fixative with the alcohols during dehydration (Valentine, 1961).

Apart from the two fixatives mentioned above, that most commonly used is formalin. Alone, this does not preserve specimen structures against

polymerisation damage or electron bombardment. It has, however, the important advantage of very rapid penetration through the specimens, and when formalin fixation is followed by staining with buffered osmium, good preservation is obtained. Attempts are also being made to evolve a fixative which preserves both enzymic activity and cell structures, and satisfactory results have been obtained with glutaraldehyde and acrolein (Sabatini, Bensch and Barnett, 1962). As with formalin, it is advantageous to stain with osmium tetroxide after fixation with these materials.

Embedding

The greater resolution and magnification of the electron microscope showed that the embedding media used for light microscopy frequently resulted in damage of the specimen fine structure. The ability to cut reproducible thin sections (Pease and Baker, 1948) emphasised the importance of a suitable embedding medium. Although mixtures of the standard material, paraffin wax, with such substances as collodion (Pease and Baker, 1948) were to some extent successful, the major advance came with the introduction of the methacrylate monomers (Newman, Borysko and Swerdlow, 1949). These have many desirable features, for instance the hardness of the final block may be controlled by selection of a

suitable mixture of methacrylate monomers, the non-viscous nature of these monomers facilitates rapid and complete infiltration of the specimen, so giving a homogenous block, and the sections are of satisfactory contrast in the electron microscope. For some years the methacrylates were universally employed as embedding media, but it gradually became evident that they were not ideal for all specimens. Perhaps their most important defect is that they undergo a very considerable volume contraction on polymerisation and this can cause significant damage to delicate specimens. To overcome this difficulty, Borysko (1956) used a partially polymerised syrup as the embedding material and completed the polymerisation at a higher temperature. However, in addition methacrylates are unstable in the electron beam; they melt, causing specimen collapse, and then volatilise, contaminating the illuminating system of the microscope and thereby impairing the resolution. This difficulty is not easily dealt with.

Other embedding media which do not possess these inherent difficulties have been developed as substitutes for the methacrylates. Among these are the epoxy resins, introduced by Maalφe and Birch-Anderson (1956) of which the most suitable is Araldite (Glauert, Rogers and

Glauert, 1956). The polyester resins (Kellenberger, Schwab and Ryter, 1956) and especially Vestopal W (Ryter and Kellenberger, 1958) are also suitable media. Such resins contract very little on polymerisation and are extremely stable in the electron beam. On the other hand their extremely viscous nature makes specimen impregnation and block homogeneity difficult, and the sections are of very low contrast, almost always requiring post-staining. It seems unlikely that an embedding medium which combines the advantages of methacrylate with those of Araldite and Vestopal will be developed, since the higher viscosity determines the low contraction, and the stability in the beam decrees low contrast.

Recent advances have been made in the attempts to find a water soluble resin; this would have the obvious advantage that dehydration and embedding may be combined, complete dehydration being unnecessary. A preliminary report on such substances is given by Glauert (Kay, 1961).

Microtomy

Most specimens are too thick to be examined directly in the electron microscope and so must be treated in one of two ways. If their surface structures are to be studied, replica techniques may be used;

if information on their internal structures is required, the specimens must be sectioned. The latter method is that most widely used in the study of biological material. Although sections of thickness suitable for light microscopy were routinely cut by histologists, great difficulty was encountered in attaining the thinness necessary for electron microscopy.

Biological material was first cut for the electron microscope by von Ardenne (1939) who used the standard microtomes to cut wedge-shaped sections; the thin parts of which provided small areas suitable for electron microscopy; since most of the section is too thick, this method is clearly not very satisfactory. O'Brien and McKinley (1943) claimed that very high knife-speeds were essential for extremely thin sections and indeed these workers, using knife-speeds of 10,000 r.p.m. or over, succeeded in cutting the first sections of uniform thickness.

The first workers to realise that slight modification to the standard light microscopy microtomes could give machines capable of reproducibly thin sections were Pease and Baker (1948). They pointed out the influence of the embedding medium on the ease of section cutting and also showed that for very thin sections, the size of the block face became critical.

The second major advance was the technique of floating newly cut sections on to a fluid surface prior to their collection on to specimen mounts. This method was extensively used by Gettner and Hillier (1950).

There are two main types of ultra-microtome based on the two different methods of controlling specimen advance. The first employs mechanical advance, similar to that used for light microscopy although much finer; the Porter-Blüm and Huxley microtomes are the modern examples of this type of instrument. The second type uses thermal expansion, where the advance is controlled by the rate of heating of the arm holding the specimen; its modern counterpart is the LKB ultratome.

Knives

The first sections for electron microscopy were cut with the steel knives used in ordinary microtomes, but these rapidly became blunt and constantly had to be resharpened. Glass knives (Latta and Hartmann, 1950) were a significant advance in the field of microtomy, and have now completely superseded metal knives. They are easily made, and since they are cheap, they may be discarded when they no longer cut satisfactorily. Provided that care is taken in the selection of a good cutting edge, these knives can cut

effectively down to the lower limit of the microtome used. The more costly diamond knives, introduced by Fernandez-Moran (1956) have several attractive features. The cutting edge is long-lasting in comparison with that of the glass knife, and they can be easily cleaned by careful rubbing with a small piece of soft wood. However, although hard materials such as metals and bone are much more readily cut by means of the diamond knife, for most biological materials the cheaper glass knife is perfectly adequate.

Post-Staining

That treatment of low contrast specimens with certain chemicals, known as "electron stains" leads to an increase in contrast was appreciated early, when Hall, Jackus and Schmidt (1945) used phosphotungstic acid as a stain for collagen fibres. It was generally assumed that specimens could be stained only prior to embedding with such fixatives as osmium tetroxide and permanganate, or after fixation in the dehydrating alcohols (Jackus, 1956; Huxley, 1957). Thus it was a major advance when Gibbons and Bradfield (1956) demonstrated that sections could be stained simply and effectively by floating the newly cut section on the surface of the required stain.

The post-staining of sections has various advantages over staining prior to sectioning; the staining does not result in derangement of the fine structure and a comparison of different stains can readily be made since consecutive sections are very similar. It is important to note that staining should always be carried out before the material is examined in the electron microscope; although electron bombardement does not cause macromolecular changes, there is an extensive breaking and reforming of chemical bonds, and so the effects of staining may appear different (Valentine, 1961).

A general stain is of great usefulness in increasing the contrast of the section as a whole, and showing up fine structures otherwise invisible. The important stains in general use were studied by Watson (1958) and include phosphotungstic acid, uranyl acetate and ammonium molybdate. Aqueous solutions of such materials can penetrate embedding materials, but it is frequently found advantageous to dissolve the staining materials in a "softening agent" such as alcohol for Araldite sections, or to heat the staining solution (Brody, 1959).

Beer (1962) in a theoretical treatment of electron stains, suggests various approaches to achieving selective staining; a few such stains have been recorded (Swift and Rasch, 1958; Kendall and Barnard, 1963). A scheme for section staining has been compiled by Mercer (1963). However, further work is still required in this field to give a system of staining analogous to that available in light microscopy.

EXPERIMENTAL

Phage Infection

In the early stages of this work, several methods were used to prepare specimens of infected bacteria which were suitable for fixation and embedding. The best of these methods was then used during the remainder of the study.

The first method involved growing B. entomocidus var. entomocidus on solid agar, the surface of which was then flooded with a concentrated phage suspension (titre about 10^9). A layer of agar was placed on top of this, to "sandwich" the cells, and so prevent them from being dislodged and lost during the stages of fixation and embedding. In the second method, cells were grown in a solid agar medium and the phages were inoculated into the agar. In both cases, plaques were seen to develop within the agar, and these were cut out. The small cubes of agar containing cells at various stages of infection were cut into pieces of a suitable size for fixation (1 to 2 mm. in length).

The third method used was that of adding the infecting phage suspension to a suspension of bacterial cells. After incubation at 37°C. in order to allow infection to proceed, samples were withdrawn at intervals and studied with the light microscope. The cells became very distended in appearance when lysis was about to occur (40 minutes incubation). At this point, osmium

tetroxide at pH 7.3 prepared according to the Zetterquist method was added and the resulting mixture spun down. The pellet so obtained was then processed for electron microscopy. Alternatively, cultures were flooded with the infecting phage suspension; after sampling to determine the beginning of lysis, the infected bacteria were washed off and fixed with osmium as before. This method gave the most satisfactory results, and so was used for most of the material described in the present thesis. By these means, cultures of different ages were infected and studied by thin sectioning techniques.

Uninfected cells were prepared by rinsing off the bacteria from agar and treating the suspension exactly as described previously.

Fixation

Although osmium tetroxide was mainly employed in this study, material was also fixed with potassium permanganate in the initial experiments.

Osmium Fixation

The fixative used was Zetterquist's isotonic buffered osmium tetroxide at pH 7.4. Specimens consisting of small blocks of agar were fixed for 2 to 3 hours, and subsequently washed with continuous changes of distilled water for about 30 minutes. Material consisting of a pellet of partially fixed cells was

resuspended in fresh fixative and fixed for a further 40 minutes. Here the fixation time required is much shorter, since all the cells are in direct contact with the fixative. The cells were then washed with several changes of distilled water.

Permanganate Fixation

This fixative was prepared according to the method of Luft (1956) and was used only for those specimens prepared in agar blocks. The material was fixed for 1 to 2 hours, then washed with several changes of distilled water until there was no pink discolouration of the washing fluid. The oxidative action of this fixative resulted in considerable loss of material, and was not therefore used in later experiments.

Dehydration

Because a water-soluble embedding material was not used in this work, complete dehydration of the specimens was essential. This was carried out by using a series of alcohols of graded concentrations. The time sequence used is given below.

50% alcohol/water	20 minutes.
70% alcohol/water	20 minutes.
90% alcohol/water	20 minutes.
absolute alcohol	60 minutes with two changes.

When pellets were used, the material was suspended in the various alcohols, and spun down at each stage.

Embedding

Methacrylate Embedding

In the initial experiments, butyl methacrylate, containing 5% methyl methacrylate and 1% benzoyl peroxide accelerator was used as an embedding material. The impregnation sequence used is given below:-

50% alcohol/butyl methacrylate	60 minutes.
butyl methacrylate	60 minutes.
butyl methacrylate + 5% methyl methacrylate	60 minutes.
butyl methacrylate + 5% methyl methacrylate + 1% benzoyl peroxide.	60 minutes.

The specimens were then placed in gelatin capsules containing the final impregnation mixture, and hardened at 50°C. for 30 hours.

Araldite Embedding

The araldite mixture was prepared by the method of Glauert and Glauert (1958) the following proportions being used:-

Casting resin M	10.0 ml.
Hardener 964 B	10.0 ml.
Dibutyl phthalate	1.0 ml.
Accelerator 946 C	0.4 ml.

The mixture was first prepared without the accelerator and the specimens were soaked in 50% alcohol/resin for 2 hours and then for 18 hours in the resin

without accelerator, the resin being changed twice. All the soaking was carried out at 48°C. so that the viscosity of the resin was decreased, and the impregnation facilitated. The material was then soaked for a further 2 to 3 hours in a freshly prepared resin, which contained accelerator. Specimens were placed in gelatin capsules containing the final resin mixture, and these blocks were hardened at 52°C. for 2 to 3 days. In the case of the pellet, difficulties were encountered in spinning down and indeed this proved impossible after the 50% alcohol/resin stage. Great care had thus to be taken to avoid breaking up the pellet too much, but even so, a considerable amount of material was lost.

The hardened blocks were trimmed to give a pyramid of specimen of about 0.2 mm. side suitable for sectioning.

Section Cutting

For routine sectioning the Porter-Blüm microtome was used; serial sections were cut on the LKB Ultratome. Glass knives were employed in both cases.

Preparation of knives

For the Porter-Blüm microtome, glass in the form of 12" long strips of ½" thick glass was carefully washed free of dirt and grease, and thereafter the cutting edge of the glass was not touched. The strip was scored fairly deeply and broken as shown in figure 2 (a).

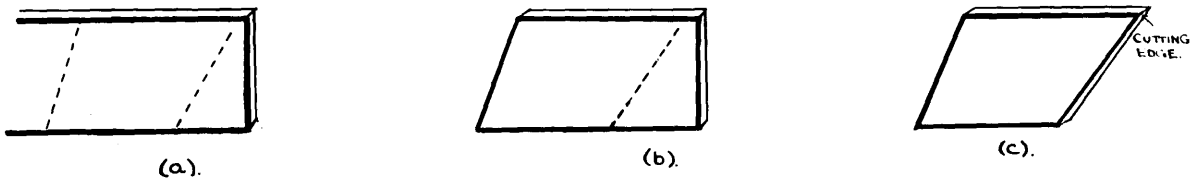


figure 2.

The rhombs so formed were again scored, this time evenly and lightly; the score was stopped about 0.2 mm. before the cutting edge, and the glass was broken along this line using two pairs of pliers. The break travels freely along the unscored part of the glass, and the edge so obtained was less impaired by serrations than that prepared by scoring right to the cutting edge.

For the LKB ultratome, the knives were prepared from glass of $\frac{3}{8}$" thick, and a slightly different method was used for breaking them. The glass was scored as shown in figure 3, the score being stopped 1 - 2 mm. from the edge. The score line was then placed along a firm straight bench edge and pressure was applied

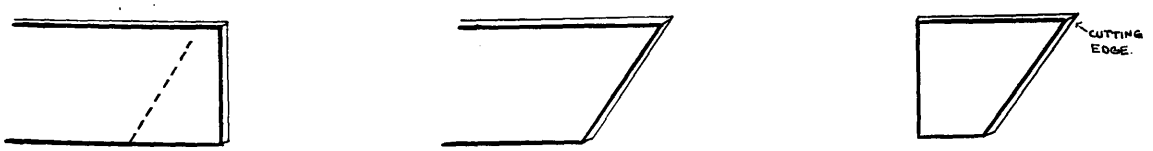


figure 3

with the hand to a point farthest from the cutting edge. The break was then made by pulling horizontally with one pair of pliers, which were bound with adhesive tape to reduce splintering and ensure an even pressure. The knife was broken off to give a square end as shown in the diagram.

This method gave reproducible results for the $\frac{3}{8}$" glass. However, the thicker glass was difficult to break in this way and often gave inferior knives; the original method was found preferable in this case.

A suitable knife was selected and waxed into a trough with dental wax. For the thicker glass, a metal boat was used; for the $\frac{3}{8}$" glass, a strip of black "Scotch Tape" was adfixed to the glass and held in position with wax melted on the outside.

Sectioning with the Porter-Blüm Microtome

This instrument functions on the mechanical advance principle, the advance being by fractions of the pitch of a screw thread, with the smallest advance an indicated 250Å. Controlled by this mechanism is the advancement of a cantilever arm at the end of which is a chuck holding the specimen. The knife was adjusted to be a few degrees from vertical and the trough was filled with a 10% acetone solution. This solution has a low surface tension, thus making the contact angle between

the glass and the water very small. The sections cut in this way were flattened by exposure to chloroform vapour and sections of interference colours of silver or grey, corresponding to a thickness of below 800Å, were collected on to formvar-coated grids.

Serial Sectioning on the LKB Ultratome

In order to cut serial sections of constant thickness, a very constant advance and cutting speed is necessary. Manually operated microtomes are not adequate because slight changes of speed during the cutting stroke result in changes of section thickness. For this reason, the serial sections in this study were cut on the ultratome. This instrument employs thermal expansion of the specimen arm to control specimen advance, and the cutting stroke is operated by a constant speed motor drive - the actual speed of the sectioning strokes may be varied to suit the material being cut.

The block face was trimmed to a "trapezium" shape which facilitated ribboning of the sections. The knife was originally set at 4° to the vertical. The conditions required for serial sectioning proved much more critical than those for routine single sections, and several difficulties arose.

Firstly the cutting edge of the knife, being smaller than that used in the Porter-Blüm, necessitated a very much smaller block face. Large block faces resulted

in sections which were too thick, block face wetting, missing a stroke or uneven cutting. Secondly the angle of the knife was found to be very important; for the araldite blocks it had to be adjusted to 1° to 2° to the vertical before consecutive sections could be obtained. Even these adjustments did not produce entirely satisfactory results, and the cutting remained uneven. Some sections were seen to possess uneven interference colours and this suggested that the block might be inhomogeneous, possibly due to incomplete impregnation in the highly viscous resin. In an attempt to remedy this, the blocks were "cured" at 60°C. for 3 days; these treated blocks gave better sections, and up to 20 sections of even thickness were cut consecutively. As before, chloroform vapour was used to flatten the sections, which were then collected on formvar coated Sjöstrand grids.

Post staining

Araldite-embedded sections provide very low contrast and so all the sections in this study were stained before examination in the electron microscope. The grids were floated face-downwards on the surface of the staining solution so that the section is in contact with the stain. The solutions and the times for which they were applied are given in the following table.

Stain (in 50% alcohol)	Time
uranyl acetate (5%)	45 mins.- 1 hour.
phosphotungatic acid (2%)	10 min. - 30 min.
Lanthanum nitrate (Satd.)	10 minutes.
Sodium uranate (Satd.)	1 hour - 2 hours.
Ammonium molybdate (10%)	2 hours.
potassium permanganate (2%)	10 minutes.

Uranyl acetate was used generally in this study, and 5% uranyl acetate stain, applied for 45 mins. - 1 hour was the standard staining condition, although some workers advocate saturated aqueous solution for 2 hours or 1% in absolute alcohol for 5 mins. (Birbeck and Mercer, 1961). For the material in this study, the 5% solution in 50% alcohol for 45 - 60 mins. was found to give a more even stain.

RESULTS

Fixation and Embedding

As already mentioned (p.92) bacterial cells are frequently difficult to fix and embed, since they are easily damaged. The criterion of good fixation for such material is that the cell membranes should be continuous, and adhere closely to the cytoplasm. Buffered osmium, used in early experiments proved satisfactory and was used throughout; there was no shrinkage of the cell contents away from the membrane, and the fine structure of this and also that of the cell cytoplasm was well preserved (Plate 1). The fibrous nature of the nucleoids was also maintained (Plate 2) although a partial agglutination sometimes occurred.

Of the two embedding materials used, only araldite proved suitable. Methacrylate "explosion" caused very extensive damage to the cells, and although development of a method using prepolymerised methacrylate might have overcome this difficulty, it was decided to use araldite throughout this work, since this is stable in the electron beam, and preserves the fine structure of the material. The low contrast of such sections necessitated post staining of all sections, as little information could be derived from the unstained material. Uranyl acetate was found to give good general staining, and all sections were stained with this substance unless specifically stated otherwise.

Uninfected Cells

Plates 1 and 2 have been selected from many as typical examples of sections of uninfected bacteria. The structure of the normal bacterial cell appears to be essentially simple in comparison with, for instance, mammalian tissue. The bacterium consists of three main parts - the external membrane, the cytoplasm and the nucleus. The fine structure of the cell membrane may be seen in Plate 1. It is generally thought to consist of two parts; the higher electron density outer membrane, which is the actual cell wall (C.W.) and the lower contrast plasma membrane (P.M.). Plates 1 and 2 show the cell wall to be one layer of width 140Å ± 30Å. The plasma membrane is separated from the cell wall by a low contrast layer or space of about 60Å width and is bounded by dense layers of about 30Å thickness.

This system of membranes bounds the cytoplasm. Within the cytoplasm numerous particles were seen known as bacterial ribosomes. These are of fairly high electron density and do not appear to be regularly arranged, but are spread uniformly over the cytoplasm. The nucleus of the bacterium occupies a roughly central area, and consists of fibrous material, generally assumed to be the DNA, embedded in an electron transparent material.

The Various Stages in the Intracellular Growth of Bacteriophages.

An 18 hour culture of B. entomocidus var. entomocidus was harvested and mixed with phage suspension as described. After 40 minutes, the cells showed alterations in phase contrast microscopy which are normally associated with phage lysis. Buffered osmium tetroxide was now added, and specimens were prepared for microscopy (p.112).

Such specimens contained cells at various stages in the process of phage infection. Some contained fully formed phage particles while others showed only limited delocalisation of the nuclear material. Typical examples of different stages in intracellular growth have been selected, and are presented below in their possible sequence. Plates 3 to 8 describe the progressive break-up of nuclear material and the formation of the phage particles; plates 9 to 11 illustrate various characteristics of the fully formed particles.

Growth of The Phages

In Plate 3, very little change can be seen in the structure of the cell; the nuclear material is however broken into large masses. A more extensive break up can be seen in Plates 4 and 5. In cells showing such changes, no phages or phage-like particles were seen.

On the other hand, diffuse, hollow phage-like bodies of 450Å ± 30Å are visible in cells where the nuclear material is almost completely delocalised (Plate 6). In some sections there are particles of greater electron density (Plates 7 and 8); these are generally circular in outline, but some angularly shaped particles are also present.

Fully Formed Phage Particles

Plates 9 to 11 show particles which seem to be fully formed phages, and the following is an account of their characteristics.

The phages are commonly circular and 560Å ± 30Å in diameter; they are usually found to occur in the small areas of nuclear material, rather than in the cytoplasm (Plate 10). Their increase in size over the less electron dense particles seen in Plate 6 is probably accounted for by the presence of the membrane of lower electron density seen in Plates 8 and 10. This measures about 40Å and may correspond to the protein coat of the mature extracellular phages. The fully developed phages differ from the partially formed particles in their general appearance as well as their electron density and size. The immature particles are characteristically hollow (Plate 6) whereas the complete

phages are almost completely "solid", possessing only a very small central area (Plate 8) or "slit" (Plate 10) of material of similar contrast to the external membrane.

As mentioned, the intracellular phages are generally circular although angular contours are occasionally visible. Plate 9 shows particles of five-sided (marked A) and six-sided (B) appearance. The ellipsoidal particles visible in Plate 7 are probably artefacts, resulting from slight compression during sectioning.

Phage tails have not previously been observed in sections of infected bacteria. However, Plate 11 shows a cell which contains a large number of phages, some of which (indicated A) appear to show tails. It is possible, however, that this is a misinterpretation due to confusion of the particles with the fibrous matrix of nuclear material in which they lie.

Lattice Structures

The cells in which the nuclear delocalisation was considerable and phage particles are visible, lattice structures were often seen. Typical examples are given in Plates 12 and 13. These were never seen in uninfected cells, or in those in which the infection process was in its early stages.

The lattice arrangements consist of small circular units of very low electron density and 410Å diameter, surrounded by a membrane of 50Å width and of slightly higher electron density. The most striking feature of these lattices is their extremely highly ordered arrangement. In a typical section, there are at least 60 sub-units packed closely together making up the lattice.

From single sections, it was impossible to say whether or not the network was two dimensional or three-dimensional; indeed Kellenberger, Ryter and Séchaud (1958) observed a fairly high degree of arrangement of phage particles in two directions only. However, the intensity differences (seen in Plate 14) between adjacent units in the lattice was strongly suggestive of a three-dimensional network. Proof of this could only be brought by cutting serial sections.

Serial Sections

Although attempts were made to cut serial sections on the Porter-Blüm microtome, it was not until the LKB microtome with its advantages mentioned previously (p.118) became available fairly late in the course of this study that this could be done effectively.

The sections were cut at instrument advance 500Å and interference colour grey; at this thickness, which is of the same order of size as the lattice sub-units,

successive units, if they are spheres, should appear in not more than three successive sections, and so an indication of their arrangement may be gained. It was, however, found very difficult to study this material in serial sections, since it proved unexpectedly difficult to identify one bacterial cell among many others in serial sections. This had to be achieved by comparing carefully both the size and the shape of the cell under study, and also the general appearance of the surrounding cells, in consecutive sections. After many series of sections were examined, some degree of success was attained. Typical results are described below.

Series I (Plates 15 - 20) shows a series of six consecutive sections; the size of the bacterial cell varies little over the series, suggesting that approximately the centre of the cell is being cut. It can be seen that although the phages do not appear in more than two consecutive sections, the lattice is present in all six sections, and has approximately the same number of sub-units in all six. This indicates that the array is three dimensional, and occupies quite a large portion of the cell cytoplasm.

Series II (Plates 21 - 23) shows by the variation of the cell size, a cell cut near the edge. The first section (Plate 21) shows the presence of phages, but

there is no evidence of lattice sub-units; the second (Plate 22) shows 6 sub-units whereas there is a fairly large number in plate 23. It would seem that the sections have cut through the edge of the cell and also the edge of the lattice. The significance of this is not fully understood, but is discussed later.

Colateral Findings

In the process of this work, certain observations were made, which, although they are not directly associated with the process of phage multiplication, were thought to be of interest by themselves. They are included in the following section.

Cell Division

Bacteria multiply by simple binary fission; a cell in the process of such division is shown in Plate 24. The nuclear material of the cell is being split to form the nucleoids of the two "daughter" cells. After this, a membrane forms, thus separating the two cells. The mechanism of formation of this has not been unambiguously determined. Serial sectioning provided information on this process of membrane growth and series III (Plates 25 - 27) shows a typical cell in the process of division. It can be seen that although Plate 25 shows a cell, completely divided by the invaginating membrane, the division is incomplete in the next section

(Plate 26) and is in its early stages in Plate 27. Since these sections are not more than 600Å thick, this considerable difference in the amount of cell membrane laid down occurred in a distance of 1800Å. Further investigations of the formation of the cell membrane are included in the section dealing with stains other than uranyl acetate. The relation of these findings to the mechanism of cell division is discussed later.

Spore Formation

The early experiments involved infection of bacteria which were at a fairly late stage in their growth cycle; although no phages were observed, it was thought of interest to include some observations on spore structure as found in these cells. In this case, permanganate fixation was used in addition to osmium fixation, and there are certain obvious differences. Plates 28 and 29 show sporulating cells which are osmium fixed. Plate 30 shows a permanganate fixed bacterium. In this case, the bacterial ribosomes, always visible in osmium fixed sections, are absent, but the contrast of the membranes of the spore is greatly increased.

Plate 28 shows two spores at different stages of development. From this section, it appears that the bacterial spore is of an extremely complex nature

consisting of a central region or "core", surrounded by a series of at least nine membranes. The overall structure of the spores of these organisms is very similar to those of B. cereus var. alesti (Young and Fitz-James, 1962).

The organism marked A in Plate 28 contains a spore which is apparently fairly well developed, and its structure can largely be identified with that recorded by Young and Fitz-James, 1962). The central area or core seems to consist of a fairly electron transparent material, possibly nuclear protein (N) and a very high concentration of electron dense material, the spore core (S.C.) which appears to be a highly concentrated mass of bacterial ribosomes. This is surrounded by an inner membrane (I.M.) which may be double and is enclosed by the cortex wall (C.W.). The layer of low electron density which surrounds this is the cortex (C), and this is enclosed in a membrane (O.M.) of similar contrast to the inner membrane. The external layer, spore coat (S.C.) is of a comparable electron density to the nuclear material within the core. Finally, there is a layer of poorly defined structure, which probably represents the exosporium.

The cell marked B in Plate 28 shows a spore which consists only of one fairly electron dense membrane, and

presumably is at an early stage of development.

Plate 29 shows a transverse section of a further two sporulating cells and these show the same type of structure. Cell C has a series of membranes essentially similar to that described for cell A of Plate 28, although in this case the cortex has a greater diameter. By contrast, cell D in Plate 29 shows a spore in the early stages of growth, a dense layer similar to that described for cell B (Plate 28) being enclosed between two layers of lower electron density.

The cell shown in Plate 30 is permanganate fixed and is of a different general appearance. The cell cytoplasm is uniform, and does not show any evidence of the ribosomes so obvious in osmium fixed cells. The spore shows about five membranes, the outer of which is of high contrast, and resembles the outer membrane seen in Plates 28 and 29. However, this is an incompletely formed spore, and so the exact identification of the various layers is difficult.

Stains

Although the plates already shown indicate that uranyl acetate in alcoholic solution is a good general purpose stain, adequately increasing the overall contrast after 1 hour's staining, it has one serious disadvantage; it is not very stable. Frequently a fine precipitate was found to form on the section,

obscuring the fine detail of the specimen. The addition of two or three drops of 50% acetic acid, minimised this effect. Also, an "ageing" effect was noted; a fresh stain reacts much more rapidly and efficiently than one which has been kept even for a day or two.

Of the other stains used, phosphotungstic acid was found to be a good stain for cell membranes, although it does not increase the contrast of the cell cytoplasm. Plate 31 shows an example of a PTA-stained bacterium which is in the process of cell division; the invaginating membrane appears to have forked ends. However, PTA in alcohol proved fairly corrosive, and for staining times over 15 minutes, tended to dissolve the specimens.

Sodium uranate proved an effective all-purpose stain, and was less troubled by the precipitation shown by uranyl acetate. It does, however, act more slowly and the sections must be stained for 2 hours (Plate 32).

Ammonium molybdate increased the general contrast of the cell to some extent, and especially that of the cell membrane (Plate 33). However, the contrast enhancement is not so great as that of uranyl acetate. Lanthanum nitrate (Plate 34) gave results very similar to those of ammonium molybdate, Potassium permanganate (Plate 35) gave increased contrast, but only in certain areas. However, on examination, the staining was found to be very granular.

DISCUSSION

The Structure of the Bacterial Cell

Before discussing the possible sequence of events in intracellular phage growth, it is interesting to discuss some of the structural features of uninfected cells which were noted in the course of this work.

There are generally accepted to be two main components of the bacterial cell membrane; the cell wall and the plasma membrane. The outer layer, the cell wall, is largely responsible for enabling the bacterial cell to withstand fairly wide differences in tonicity. Here it is interesting to contrast the rigid bacterial cell membrane with the elastic membranes of tissue cells. In Gram positive organisms, including those of the *B. cereus* group, the cell wall is thought to consist mainly of mucopeptides (Salton, 1962) which stain fairly heavily with osmium tetroxide, giving the typical high contrast outer layer.

In this work under the standard conditions of osmium fixation and post-staining with uranyl acetate, the cell wall of the vegetative cells of *B. entomocidus* var. *entomocidus* appears to consist of one layer of thickness around 140Å. This differs from the structure proposed for *B. subtilis* by Glauert, Brieger and Allen (1961) who recorded the presence of two dense layers each of about 40Å thickness, separated by a slightly less dense layer of 120 to 170Å thickness. Apart

from this difference, the structure of the cell membrane of B. entomocidus var. entomocidus is very similar to that of B. subtilis (Glauert et al., 1961).

The inner layer of the cell membrane, known as the plasma membrane, adheres closely to the cell cytoplasm and is separated from the cell wall by a narrow layer of low contrast, or a space. The dimensions of the space noted in this study are again very similar to that recorded for B. subtilis (Glauert, et al., 1961).

The plasma membrane is believed to be semi-permeable and thus controls the diffusion of ions from the environment to the interior of the cell. In ultra thin sections this appears as an osmiumphilic layer located between the cell wall and the cytoplasm. When the cutting direction is normal to the cell membrane, this inner layer itself may be seen to be of complex structure, consisting of a series of layers (Plates 1 and 2): a low contrast layer of about 60Å thickness is surrounded by two high contrast layers of about 30Å thickness. The central layer is now believed to be lipid (Hughes, 1962). The dimensions of the layers of the plasma membranes are very similar to those recorded by Glauert et al. (1961) although the inner layer (L_2) is considerably thicker

than that of B. subtilis. It is of interest to note that this inner layer is absent from B. cereus (Murray, 1957) and it is unexpected that B. entomocidus should differ from B. cereus in this respect. It may be that the inner layer seen in Plates 1 and 2 merely represents the edge of the cytoplasm, as suggested for B. subtilis (Glauert et al., 1961); however, the fairly high electron density of the layer and its common occurrence seem to make this rather unlikely.

The membrane structure recorded under the standard conditions of this study is shown diagrammatically in figure 4.

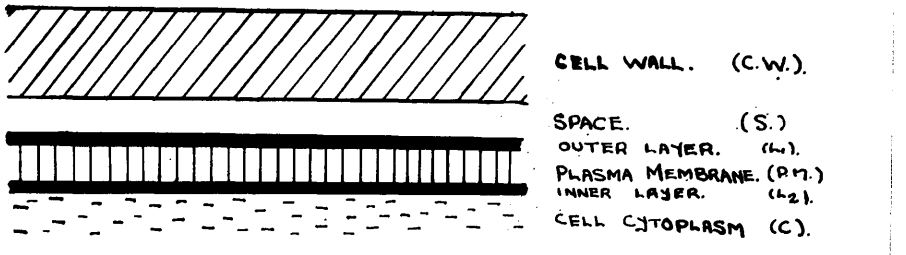


Figure 4

It must be remembered however that the appearance of the bacterial cell membrane varies considerably with the methods used for its study. The space which separates the cell wall from the plasma membrane may

vary greatly and may give an indication of the standard of the fixation procedure; when there is a very wide space between the cell wall and the plasma membrane, then considerable shrinkage of the cell contents may be considered to have occurred, due either to the fixation or the dehydration techniques. Plate 6 shows some enlargement of this space, but such cytoplasm shrinkage was not common. Indeed, the space may be entirely an artefact of the method of study and it is possible that it is completely absent in the untreated cell.

The appearance of the cell wall also seems to depend on the post-stain used. Although under the standard conditions of uranyl acetate staining, the cell wall of B. entomocidus consists of only one layer, post-staining with potassium permanganate shows some evidence of two dense layers of about 30Å surrounding the cell wall, similar to that suggested by Glauert et al. (1961) (Plate 35). The rather granular nature of this stain, however, makes interpretation of this fine structure difficult. Finally the apparent dimensions and structure of the various components of the cell membrane depend also on the direction at which membrane is cut. The lower part of the cell in Plate 14 shows a very wide cell membrane in which the plasma membrane is invisible; presumably this has resulted from the

section passing tangentially through the cell wall.

The appearance of the bacterial cell membrane may also vary slightly with the age of the cell, and whether or not it is sporulating. It seems then that it is difficult to draw absolute conclusions as to its actual structure. It is important to note, however, that no apparent difference was noted between the structure of the uninfected and phage-infected cell.

Phage Infection

Immediately after infection of B. entomocidus var. entomocidus by its specific phage, little change takes place in the bacterial nucleoids (Plate 3); the general appearance of such cells closely resembles the uninfected cells. Although it is possible that these are cells which are resistant to phage infection, it seems more probable that this stage corresponds to the period of initial protein formation thought to be necessary for the subsequent break-up of thenuclear material of phage-infected cells (Kellenberger, 1961).

The nucleoids then begin to break down into smaller masses (Plate 4). This break-up, thought to result from the initial protein synthesis by the phage, is not a normal function of the cell; although the nucleoids of the normal cell are of a branching nature

they are almost completely confined to a central area within the cell. Even in dividing cells, the nuclear material which is in the process of splitting, does not become delocalised into small masses. Plate 4 shows the early stages of this phage-induced nuclear breakdown where the large central mass is beginning to split into smaller parts.

The process continues fairly rapidly; Kellenberger (1961) has shown that the breakdown of the nucleoids of E. coli induced by T_2 phages, has largely occurred 10 minutes after infection. Plate 5 shows the nuclear material existing in small pools throughout the cell. These smaller DNA pools closely resemble the original nucleoids in appearance. In this system, however, there is no evidence of the development of pools around the margins of the cell, which have been observed for T_2 phage/E. coli systems (Kellenberger, Sechaud and Ryter, 1959). These workers suggest that such marginal pools indicate that each infecting phage produces its own DNA pool, and that these later merge to give the large pool of vegetative phage DNA which is formed prior to the development of phages. The absence of such marginal vacuoles in both this study and also that of λ phage (Kellenberger, 1961) indicates that this is not a general feature of intracellular phage multiplication. It

would appear that if T_2 phages form their own separate pools, the DNA which was injected by the extracellular phage remains near the site of injection. In systems which do not show marginal vacuoles, the injected DNA may migrate from the site of injection before the DNA pools begin to form; such an effect may hence be related to the mechanism of injection. Indeed it is interesting that neither "B" phage of B. entomocidus var. entomocidus nor λ phage of E. coli produce marginal pools or possess a contractile tail sheath. On the other hand an injection mechanism involving contraction of the tail sheath would seem likely to be the more "powerful", the DNA being more likely to migrate from the site of injection. Until more systems have been studied and more information is available on the injection process it cannot be said with certainty that the mode of development of pools is related to the morphology of the mature phage tail.

Nuclear delocalisation continues until the DNA is almost completely dispersed through the cell cytoplasm. The extent of this dispersal is more pronounced than that shown by T_2 phage in E. coli (Kellenberger et al., 1959; Kellenberger, 1961) but less so than that produced by λ phage (Kellenberger, 1961). At this stage the phage-like bodies begin to appear. These take the form of hollow low contrast rings forming from the highly dispersed nuclear substance (Plate 6) and

probably correspond to the early stages in the concentration of DNA from the very dilute pool to the highly orientated DNA of the phage head. The frequency of the occurrence of these DNA "concentrates" may be related to the extent of the dispersal of the DNA through the cytoplasm; the more dilute the DNA, the lower the frequency of these low contrast particles (Kellenberger, 1961). On this basis, the occurrence of the phage-like bodies in this system should be intermediate between that of T_2 and T_5 phages of E. coli (Kellenberger, 1961).

The extent of the concentration of DNA within these particles may be assessed by their electron density; the fully formed units are of high electron scattering power, as seen in Plate 8. Also it is obvious that the particles become increasingly more "solid" in appearance in comparison with the early stages which are hollow. However, the apparently fully formed phages still show some evidence of a hollow centre (Plates 7, 8, 9) and the significance of this will be discussed later.

The phages can be seen to develop randomly throughout the cell, and do not appear to be confined to regions near the centre or along the edges of the bacillus. No evidence was found, in the large number of sections examined, of the two-dimensional arrangement of particles which is thought to be of fairly common occurrence for T_2 phage in E. coli (Kellenberger et al., 1959).

The final stages of phage development are thought to involve the formation of the protein coat around the DNA concentration. Such a coat would necessarily be of very low contrast and consequently difficult to detect against the cell cytoplasm. Kellenberger (1961) states that it can only be observed when the plane of the sectioning is perpendicular to the membrane. Such a situation would be rather rare on the basis of the bipyramidal head form generally accepted for T_2 phage (Part 1, p.32). However, the icosahedron, approaching as it does a sphere, should satisfy this condition more frequently; hence, if this is indeed the form of the head of B phage (Part 1, p.57) the head membrane should be detected fairly frequently in fully formed phages. In fact, it can be clearly seen in several sections (Plates 8, 9, 10). Such partially completed particles with head membranes are thought to give rise to the doughnuts on artificial lysis (Kellenberger, 1961).

Finally, the phage heads acquire tails. As stated earlier, the mechanism of this is not fully understood; there seem to be three possibilities. Firstly, the tails may be formed during phage lysis. Secondly, they may be produced in a separate DNA pool, and then, as fully formed units, they may be added on to the heads. Thirdly, they may "grow" out from the heads at some stage in their development. The first suggestion is

unlikely since Kellenberger and Séchaud (1957) have detected tail related structures on artificial lysis. Kellenberger (1961) has produced considerable evidence against the second suggestion, based on counts of the tail-related structures and doughnuts in lysates. Therefore it seems that the last mechanism is the most probable. However, it is difficult to determine the exact mode of tail formation, since it is difficult to detect tails in sectioned cells for two reasons. Firstly, they are narrow, and of very low contrast. Secondly, they must lie in the plane of the section before they can be seen. This would at once decrease the expected incidence of these structures.

Any mechanism of tail formation must explain why, if the head is perfectly symmetrical, there is only one tail; what makes one corner of a symmetrical figure different from the others. This may be explained by the way in which the DNA is packed within the head. It is generally accepted that the DNA of the phage head exists in the form of long strands, and so there are probably only a few DNA molecules of considerable length packed in a very orderly manner; if the ends of the molecules lie at set positions in this orderly array, this may set up a point or points around the edge of the symmetrical figure which are distinct from all other points. Such differences may control the manner

in which the protein sub-units are laid down around the DNA. From this packing of protein capsomeres, the tail may develop, and so it seems most probable that the packing of the DNA and also the formation of the protein coat are complete or almost complete before the tail begins to be formed.

The DNA of the heads which show protein coats is not completely "solid", but has a small core, or slit of material, possibly protein, of similar electron density to the external membrane (Plate 10, A and B). This appears to extend from the centre to the edge of the particle, but the angle of sectioning will determine its apparent shape. The protein coat may build around this slit and consequently may be incomplete. This would provide a site for tail growth, and the tail may grow from this "slit". A suggestion of such a phenomenon is visible in Plate 10 (B), where material of low electron scattering power extends some distance from the head. It has already been suggested (Part 1, p. 50) that the tail proceeds some distance into the head; this mechanism of tail formation would seem to support this finding. The postulated mechanism can be seen diagrammatically in figure 5.

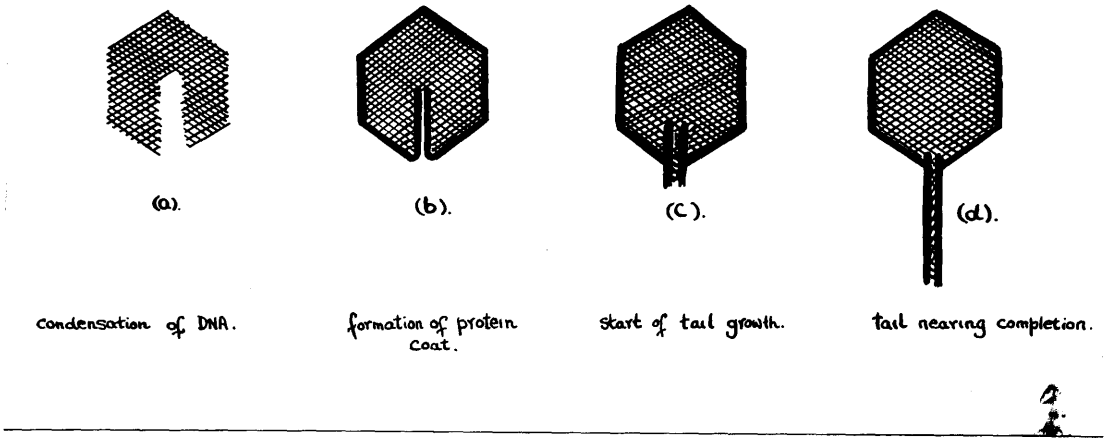


Figure 5

The Intracellular Phage Particles

The phage particles can be seen to differ quite significantly in electron density. This may be accounted for by varying degrees of concentration of the DNA within the particle. However, it is also possible that this effect is associated with the method of study. If the particle is 550Å in diameter, and the section thickness is 500Å, then clearly the phage particles may appear in two or three sections as shown in figure 6.

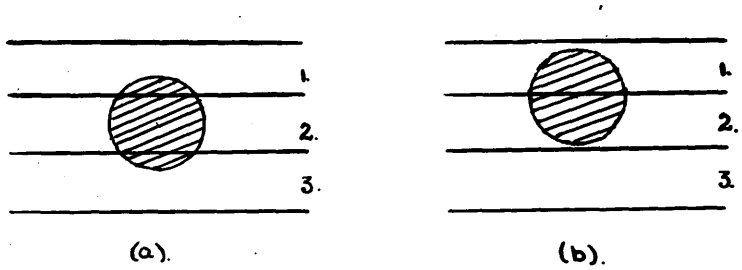


Figure 6.

In figure 6 (a) the particle is almost completely within section 2, and hence this would result in a circle of high electron density. Sections 1 and 3 contain a small part of the phage particle, and would appear on study as circles of lower electron density, since most of the section thickness will be occupied by the lower contrast cell cytoplasm. The particle in figure 6 (b) would show two circles in sections 1 and 2 of about equal electron density. Such an actual variation in contrast in successive sections is visible in plates 15 and 16 (marked C) and plates 22 and 23 (marked D). However, it has not proved possible to detect the same particle in three sections, since the contrast of the two small parts of phages would be extremely low.

There is a significant difference in size between the complete intracellular phage particles, at 550Å and the size of the mature extracellular phages (620Å) as determined by the negative staining method described in part 1 of this thesis. This discepancy is not altogether unexpected since the embedding procedure involves complete dehydration, and the possibility of slight shrinkage is introduced. However, this shrinkage cannot be considerable since there is no visible shrinkage of the cell cytoplasm from the membrane. Similar

discrepancies have been recorded by Horne (1962) for other biological substances studied by these two methods.

Plates 8 and 9 show clear angular shape of the intracellular particles. Kellenberger (1961) showed intracellular T_2 phages to be of hexagonal outline and the preservation of sharp contours in the present study is further evidence that the fixation procedure provides adequate preservation of fine structure. However, most of the phages which were found to possess membranes were noted to be round in outline, this may explain the ease with which the membrane is detected; if the angular form of the head is not fully preserved, the orientation of the head membrane with respect to the sectioning direction will not be so critical. It has been stated (Valentine, 1961) that intracellular virus particles often appear spherical in osmium fixed material, whereas permanganate fixation shows angular outlines. It would be of interest to compare the effects of the two fixatives in this system.

Cell Lysis

The final stage of the phage multiplication is the lysis of the cell. It is known that a cell on the point of lysis contains a large number of virus particles; the actual number of these particles is the "burst number" of the organism, i.e., the number of phages liberated on lysis. Unfortunately the actual burst

number of B. entomocidus var. entomocidus is unknown. Plate 11 shows a cell containing a large number of phage particles, and this may well be on the point of lysis.

The mechanism by which the cell lyses is as yet unknown. It may be a mechanical effect, due to the numbers of phage particles within the cell, or it may result from enzymic action. Plate 36 shows a transverse section of a cell which contains several fully formed phages showing membranes and which seems to be in the process of lysis. Filaments, closely resembling the DNA noted by Williams (1952) are extruded from the cell. However, the rather severe processes of fixation and embedding would have been expected to have caused such a cell to disintegrate completely. It seems more probable that the cell was on the point of phage-induced lysis, but that the treatment of the viscous resin actually caused lysis.

Lattices

A type of inclusion, commonly observed, was the lattices seen in Plates 12, 13 and 14. A similar type of organelle has been recorded by Van Iterson (1961) who found vesicles of 250 to 300Å in B. subtilis. These were of about the same electron density as those observed in this work, but they are of a much less

ordered array, and apparently associated with the growth of the cell membrane and cell division. Peripheral inclusions have also been frequently associated with cell growth, (Chapman, Hillier, 1953; Glauert et al., 1961).

However, the location of the clusters of the vesicles found in the B. cereus organisms appears to be random in the cell, and not closely associated specifically with the cell membrane. Also, although their electron density is similar to those of B. subtilis, their arrangement is very orderly and there is no evidence of the membrane seen around the vesicles noted by Van Iterson (1961).

The function of these vesicles is not yet clear; only speculative suggestions can be offered at present. Similar, though not identical, hexagonal arrays have been reported as being part of the cell wall, and it was considered possible that if the cell wall of B. cereus had been sectioned parallel to the surface of the cell, similar patterns would be expected. However, this would require that the lattice were only two dimensional, whereas serial sectioning revealed that the lattice was indeed three dimensional. Another possibility was that the lattices represent an array of "bubbles" or holes in the cytoplasm. This was

discounted on the grounds that such a structure would be deformed on sectioning.

Since B. cereus belongs to a crystal forming group of bacteria, and since the lattice was regular enough to be accepted as crystalline, it seemed possible that it might be associated with crystal formation. Comparison of the appearance of the crystals recorded for these organisms by Norris and Watson (1960) however, revealed clear differences in appearance and dimensions. Also, crystal formation is a characteristic of old cultures of B. cereus, and the organismsin the present study were examined at an early phase of growth.

The most striking finding was that lattice structures were observed only in phage infected cells in which the nuclear material was extensively delocalised and in which phage-like bodies were present. It may well be that they are directly related to the intracellular growth of phages. Two possibilities seemed to exist; (a) they were a transient phase of phage growth, or (b) they represent end products of the disrupted metabolism of the infected cell. In view of their similarity in size to intracellular phage particles possibility (a) at first seemed the more attractive. However, the structure possesses the staining properties of neither DNA nor protein, although it is possible that use of

"Kellenberger's" fixation procedure would have led to some degree of staining. On present evidence it is not possible to discount the possibility that they represent the "prophage state" (p.15). On the other hand, in its lack of staining properties it resembles the plasma membrane and may therefore be similar in chemical composition; the lattice structures may be mucopolysaccharide laid down as a result of disruption of the normal metabolism of the cell.

In addition, the mode of formation of these structures is also not clear. It seems unlikely that they develop by a slow accumulation since, if this were so, one would expect to find single particles or small clusters. Instead, all available evidence points to the existence of large clusters, and it is considered the "crystallisation" as a whole from some preformed matrix is more likely. Plate 37 shows an area, completely free of ribosomes and a similar area, marked "B" in Plate 15 was found near an array of particles. Such a matrix was commonly found surrounding, or close to lattices in other sections and the material between vesicles has a similar appearance. It may be therefore that it is from this that the "crystals" are formed.

The sub-units always appear circular in cross-section, suggesting that they are spherical. If they

were cylindrical then in longitudinal section this would have shown up. Indeed in the many sections studied this was never observed.

In an attempt to further characterise these lattices, it was decided to attempt to determine the spatial distribution of sub-units. It was considered most likely that packing was hexagonal, because this is generally accepted as the most economic way of packing small spheres and because each sub-unit was frequently found to be surrounded by 6 similar units. Centre to centre distances were determined in order to test this; if indeed the system was hexagonal then distances "a" and "b" in the following figure should be identical.

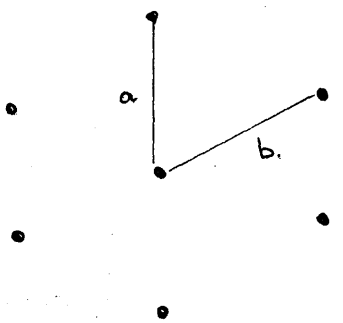


Figure 7

In many sections this condition was satisfied; the exceptions may have been due to slight compression during cutting.

In some instances a considerable degree of variation in intensity of sub-units was noted; this

was the first indication that packing may be three dimensional. In attempts to settle this serial sections were made which revealed that the same lattice appeared in at least 6 sections, thus proving three dimensional packing.

Attempts to superimpose serial sections and gain information concerning the mode of packing in three dimensions have so far proved unsuccessful, possibly because of slight compression of sections. Also, the outline of the cell differs slightly in successive sections. An error of even 50Å due to superimposition would alter the whole arrangement.

Cell Division

During cell division, the nuclear material divides equally between the two daughter cells, and the cell wall grows in, or invaginates to separate the two halves. These then split apart, producing two complete cells.

The mode by which this invagination occurs is still in doubt. The earliest thin sections of bacteria which showed adequate detail were those of B. cereus of Chapman and Hillier (1953) and these workers noted the presence of light areas, or "peripheral bodies", which occurred just in front of the ingrowing membrane, and suggested that these may be directly associated with the synthesis of cell wall material. Suggestions of such peripheral bodies are seen in Plates 21 and 22,

but no clear evidence was found. It should be remembered however that the cells shown in these plates are phage-infected, and this is known to disrupt the normal metabolism of the cell; division is probably the most important function of the cell, and it seems possible that this process is interrupted when the cell is infected. Thus the synthesis of the peripheral bodies may be blocked; cells apparently in the process of division (Plates 12, 21, 22, 23) may have been in this state when they were infected. Plates 21 to 23 suggest that the first stage in the process is a "thickening" of the cell wall, the plasma membrane pushing further into the cell cytoplasm at the point of division. As this process proceeds, the cell wall follows the plasma membrane, and indeed may be synthesised within the layers of plasma membrane. Plate 24 shows this process in its initial stages.

A study of cell division in serial sections shows that it is misleading to make deductions on the process from single sections, since the appearance of these clearly depends upon the part of the cell which is being sectioned. Series 3 (Plates 25 to 27) shows three consecutive sections through a dividing cell. In the first plate, the division seems complete, the membrane running right across the cell, and the nuclear material existing in two quite separate masses in the two cells.

It is noticeable, however, that these masses do lie fairly near to the dividing membrane, rather than being centrally situated. However, Plate 26 shows that at a distance of 500Å further through the cell, the membrane is not continuous and that the nuclear material is not fully segregated, but lies between the two membranes. Plate 27 shows the cell in the early stages in division, and a considerable amount of nuclear material lies between the invaginating membranes. This suggests that such a cell as that in Plate 26, or that marked "A" in Plates 15, 16, 17 etc., cannot be unambiguously interpreted as having completely divided since sections further through the cell may show incomplete membrane formation.

It also appears that membrane growth starts annularly from the edges inwards at all points on the circumference, and that the movement or division of the nucleoids is not complete when this begins. There are then two possibilities: either the movement of the nuclear material is stimulated by the ingrowth of the cell membrane, possibly because the plasma membrane contains material capable of initiating such nuclear movement, or the division of the nucleoids initiates the invagination of the plasma membrane. This latter suggestion seems the more acceptable, since all genetic information is thought to be associated with the

nuclear material and so it would be reasonable for this to "trigger off" the process of division.

There remains the problem of how the invaginating membranes eventually join together forming a single continuous wall. Plate 31 which shows a cell stained with phosphotungstic acid may provide a clue to this. In both cases, the invaginating membrane appears to have a forked end; it is possible that these represent the growth points of the membrane; on meeting these may easily become linked by means of an interaction with the "V" shaped ends. This plate also shows one side of the invaginating membrane to have extended much further across the cell than the other side. This would be unexpected if all membranes are assumed to proceed evenly from all points round the cell wall, initiated by the nuclear movements. However, it seems probable that this effect is an artefact resulting from the angle at which the section is cut through the wall.

Once the cells are completely divided, there are two possibilities; either they may remain in contact, and continue to divide, forming long chains of bacterial cells, or they can separate, and exist as independent units. The latter situation is found for the bacteria of the B. cereus group, and it has been suggested (Lominski, Cameron, Wyllie, 1958) that the separation is brought about by the action of an enzyme which

digests some substance "cementing" the cells together.

Spore Structures

No phages were detected in cells showing spores. It is as yet impossible to say however if this is due to sampling during the latent period when the phages are invisible (this period may be longer during sporulation); then again the sporulation process may make the cells more resistant to phage infections. The fundamental changes involved in spore formation make it difficult to determine whether any of the nuclear changes associated with phage infection have taken place. However, if phage infection has indeed occurred, it has clearly not interfered with sporulation.

The spore is the state in which the bacillus stores all the necessities for the regeneration of the complete cell, for its life and multiplication. In this form, the bacillus can withstand environmental conditions such as excessive heat or dryness, which would certainly result in the destruction of the normal cell; in suitable media, the normal cell can be completely regenerated from the spore.

Plates 28, 29, 30 show spores in various stages of formation. That in cell A in Plate 28 has a complex series of membranes which appear to be different in composition, surrounding the central core of the spore;

cell B shows only a single membrane. It would seem that the spore of cell B is in the early stages of formation, whereas that of cell A is nearing completion. This is borne out by the general appearance of the core and the cell cytoplasm. The cytoplasm of cell B is very similar to that of the normal cell (Plate 1). The number of ribosomes of the cytoplasm may be slightly less than, and that of the spore slightly greater than that of the normal cell. However, cell A shows a cytoplasm greatly depleted in ribosomes, and the content of the spore shows a very high concentration of ribosomes, confined to quite definite areas in the core. These areas are of high electron density, and resemble in shape, the "chromatinic areas" observed by Robinson (1953). The areas which are free of ribosome in the spore are filled with a low electron density substance, which probably represents nuclear material; this shows alignment in various places. Although the ribosomes are definitely restricted to certain areas in the spore, there is no visible membrane separating them from the nuclear material. Spore formation, therefore, involves the accumulation of ribosomes and nuclear material within the spore. This would provide the genetic information and means of protein synthesis required to regenerate the complete cell.

The nature of the membranes surrounding the core

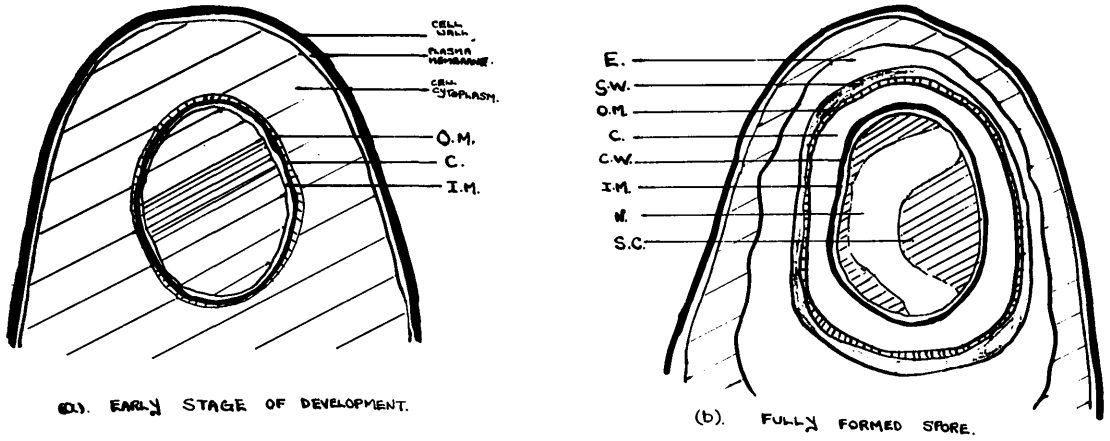
are known to vary with the organism being studied. Those of B. entomocidus var. entomocidus studied in the present work were found to be similar to those of B. cereus (Young and Fitz-James, 1962) and closely resembled those of B. subtilis (Tokuyasu and Yamada, 1959) although the lamellar structure observed by these workers was not seen.

The cortex, the most important layer, is thought to contain piccolinic acid, a substance which is believed to be concerned with regeneration and heat resistance. It is known to vary in width throughout spore formation (Young and Fitz-James, 1962) developing from a thin, electron dense layer in the early stages to an electron transparent area in the fully formed spore. Cell B (Plate 28) appears to contain a spore in the early stages of development. In turn, the spore in cell D (Plate 29) seems slightly more advanced, while those in cells A and C (Plates 28 and 29) are probably at a later stage. The width of the cortex in cell A is comparable with that of a related organism B. alesti (Norris and Watson, 1960). By contrast with the spore of B. subtilis (Tokuyasu and Yamada, 1959) that of B. entomocidus shows a suggestion of substructure. However, this may also depend on the stage of development. No direct evidence was obtained, in the present study, in favour of the suggestion of Fitz-James (1960) that

invagination of the plasma membrane is involved in formation of spore membranes. It was noted, however, that the low contrast membranes surrounding the spore in cell B were similar to plasma membrane in appearance and invagination may merely have been missed.

Together with the highly concentrated, dehydrated contents of the spore core, the complex system of membranes may be concerned with the ability of the spore to withstand extremes of environmental conditions, such as high temperature which is a characteristic of this phase of the life history of sporulating organisms. Several thinner layers may provide greater resistance than a single thick layer. Alternatively, at least some, if not all, of these membranes may be concerned with reformation of the cell wall in regeneration.

Use of a permanganate fixation procedure enabled the nature of the system of membranes to be further clarified although the ribosomes cannot be seen. Plate 30 shows a spore which has 3 or 4 complete membranes and is surrounded by a membrane of high electron density which is as yet incomplete, or broken on sectioning. This does not appear to be the external membrane, as there is a suggestion of other membranes being laid down outside it, but it may be responsible for the greater part of the resistance of the spore.



(a). EARLY STAGE OF DEVELOPMENT.

(b). FULLY FORMED SPORE.

DIAGRAM OF SPORE STRUCTURE.

E ... EXOSPORIUM.
S.W ... SPORE WALL.
O.M. ... OUTER MEMBRANE.
C. ... CORTEX.
C.W. ... CORTEX WALL.
I.M. ... INNER MEMBRANE.
N. ... NUCLEAR SITE.
S.C. ... SPORE CORE.

Figure 8

Figure 8 shows the suggested structure of the spores of B. entomocidus var. entomocidus. The fully developed spore is depicted in figure 8 (a), showing the complete system of membranes; the early stages of spore formation, showing the early cortex development is shown in figure 8 (b).

Post Staining

Almost all staining was carried out on osmium-fixed material which gives a well standardised result. It provides a general background stain, although it is thought to stabilise phospholipid membranes selectively. Further staining in section adds to this osmium staining to produce high contrast.

It was commonly observed that increased thickness resulted in better contrast. Thicker sections reached a higher contrast in a much shorter time than thin sections, and indeed this is known to be the case for light microscope staining. As a result thin sections do not yield as much information as one might expect.

Uranyl acetate, the stain most widely used in this work, proved a good general stain, and appeared to be specific for nucleic acids, as can be seen from the high contrast of the phage particles, and the ribosomes. This is in keeping with Watson's (1958) finding that it is a general purpose stain. Mercer (1963) has shown for tissue sections that uranyl acetate when used in conjunction with osmium fixation stains DNA and RNA extremely well, and leads to some increase in contrast of material containing amino acids which was previously of low contrast. Since the cell walls of Gram positive organisms, such as those of the B. cereus group are thought to be largely composed of mucopeptides or amino acid complexes, it would seem that the cell wall material should be of

significantly lower intensity than the phage particles; indeed this was observed (e.g. Plates 7, 8, 9).

The staining mechanism probably involves one of the complex ions which results from the hydrolysis of the salt. For instance it is known that the anion $UO_2(C_2H_3O_2)_3^-$ is formed, and this is facilitated by the addition of a few drops of acetic acid (p.133). This anion may itself stain, or it may in turn decompose to form the commonly recorded UO_2^{2+} according to the equation

$$UO_2(C_2H_3O_2)_2 \xrightarrow{C_2H_4O_2} UO_2(C_2H_3O_2)_3^- \longrightarrow UO_2^{2+} + 3C_2H_3O_2^-$$

The solution of some copper from the grid, forming a copper uranyl complex may well enhance the staining effect.

Similar results were observed for sodium uranate, although the staining was much slower. Thus it seems that the uranyl ion is the effective staining unit; the uranyl acetate salt probably dissociates more readily than sodium uranate.

Perhaps the first selective stain to be used was phosphotungstic acid, which was employed by Hall, Jackus and Schmidt (1945) in their studies of collagen. It is known that the effectiveness of the stain is determined by its pH, and Bendettini and Bertolini (1963) claim that staining takes place only at pH 3 or below. In this work the stain was used at pH 2 to 3.

This low pH has the effect of making the stain rather corrosive.

It is now thought that PTA may have quite specific binding sites for proteins. On this basis, it would be expected that the mucopeptides of the cell wall material should be of high contrast due to its high content of amino acids. This is indeed observed. Plate 31 shows surprisingly little staining of the ribosomes of the cytoplasm, and this is difficult to explain. The time of staining may be responsible in this case; for instance, it is known that some structures stain quickly while more general staining is achieved only on longer exposure to the staining fluid.

Ammonium molybdate was found to stain the cell cytoplasm generally, and to low contrast; the phage particles are of relatively low contrast also. This is in agreement with Watson (1958) who recorded this stain to be of low specificity. It does, however, appear to stain cell wall material effectively.

Similar results were obtained with lanthanum nitrate, except that the contrast of the cell wall material is similar to that of the cytoplasm.

Finally, potassium permanganate appears also to be non-specific, although the general contrast is higher than in the previous two cases.

In conclusion then, it seems that uranyl acetate and sodium uranate are the most promising stains used in this work. The membrane contrast is selectively increased by PTA, at the expense of the detail of the cytoplasm, whereas ammonium molybdate increases the membrane contrast and also preserves to some extent the contrast of the cytoplasm. However, the detail of the membrane structure revealed by uranyl acetate is greater than with any of the other stains; for this work, this stain proves almost ideal, since it seems to act generally but also gives good staining of DNA, of which phages are composed.

PLATES

PLATE 1

This is a section of uninfected B. entomocidus var. entomocidus, in which the cell structure is shown. Stained with 5% uranyl acetate in 50% alcohol.

(S/62/1747) x 120,000

C.W. - cell wall.
S. - space between cell wall and plasma membrane.
P.M. - cytoplasmic membrane, or plasma membrane.
L_1 - outer layer of plasma membrane.
L_2 - inner layer of plasma membrane
N. - fibrous nuclear material.
C. - cell cytoplasm.

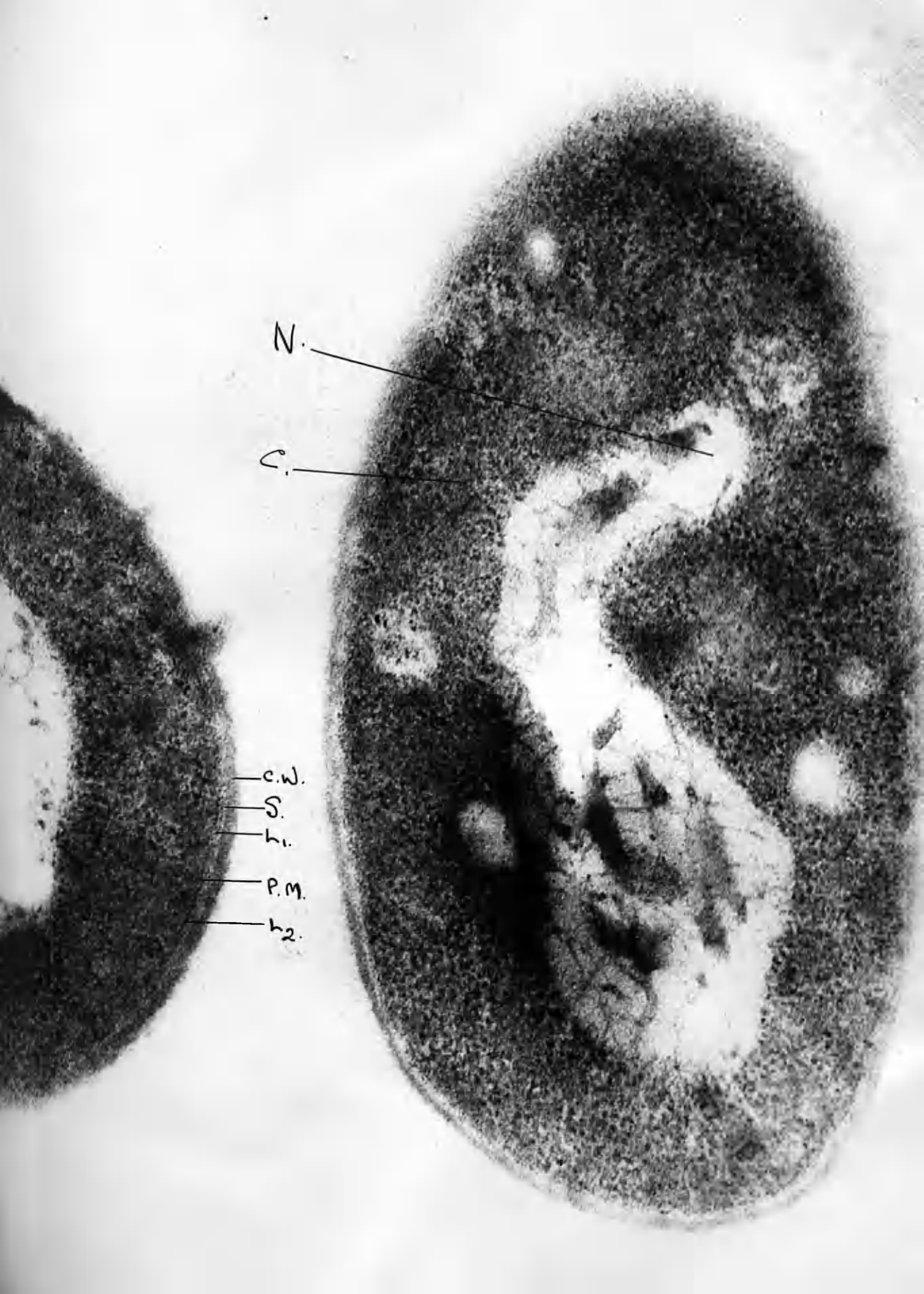

N.
C.
C.W.
S.
h1.
P.M.
h2.

PLATE 2

Uninfected cell showing localisation of nuclear material. Stained with 5% uranyl acetate in 50% alcohol.

(NS/62/75) x 137,500

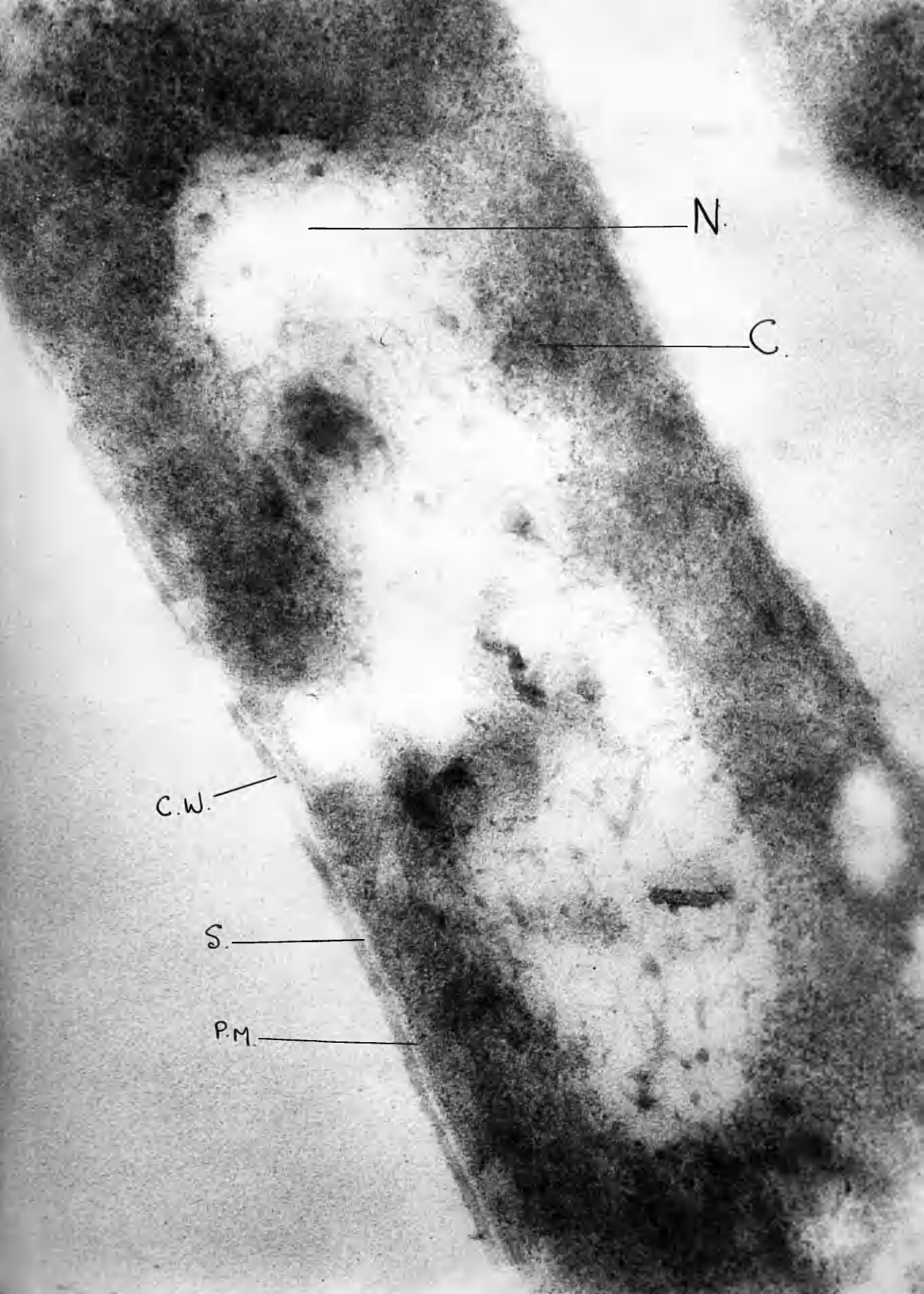

N.
C.
C.W.
S.
P.M.

PLATE 3

Infected cell showing the nuclear material still localised.

(S/62/1847) x 108,300

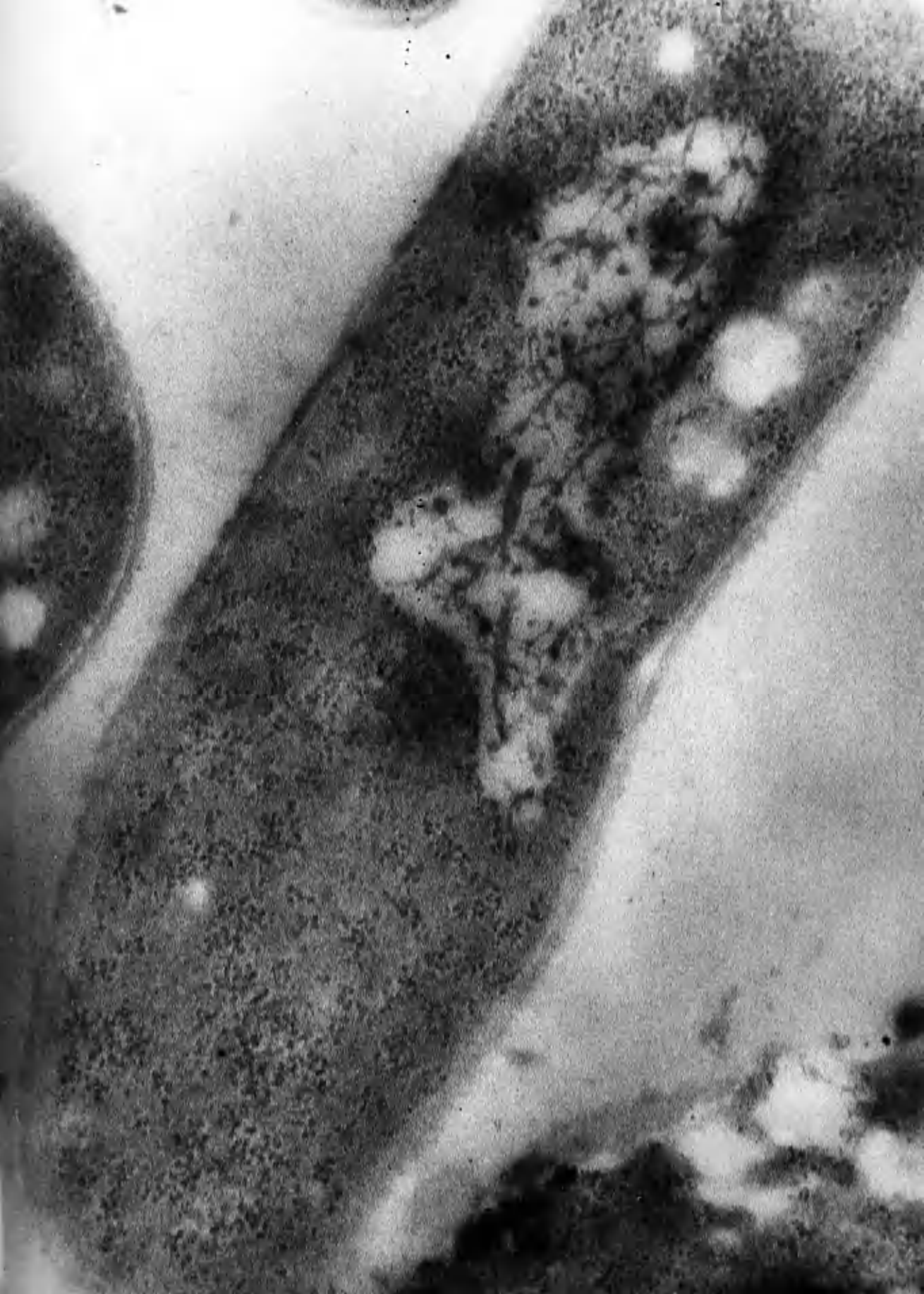


PLATE 4

Initial break-up of nucleoids into smaller masses.

(S/62/1678) x 108,000

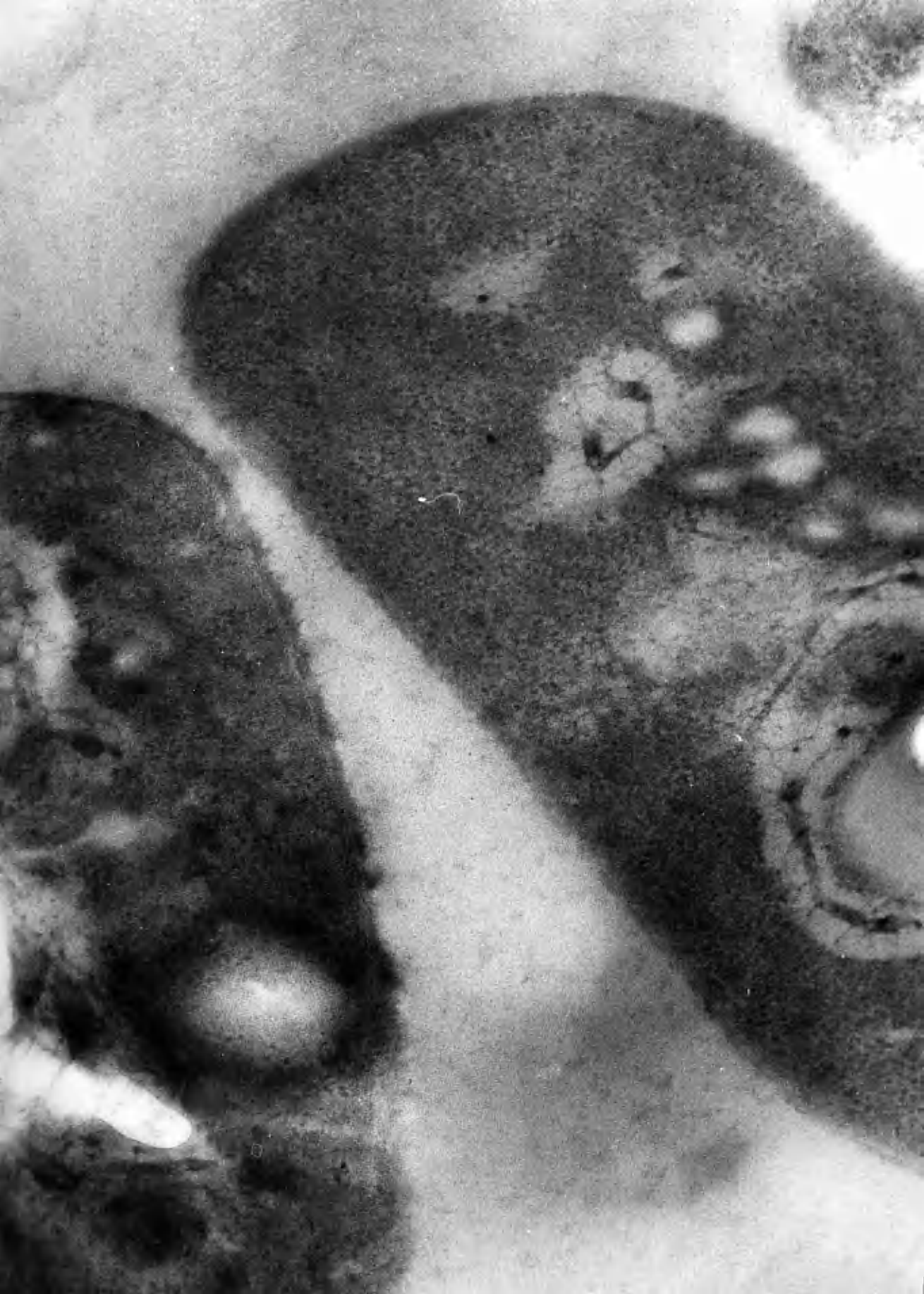
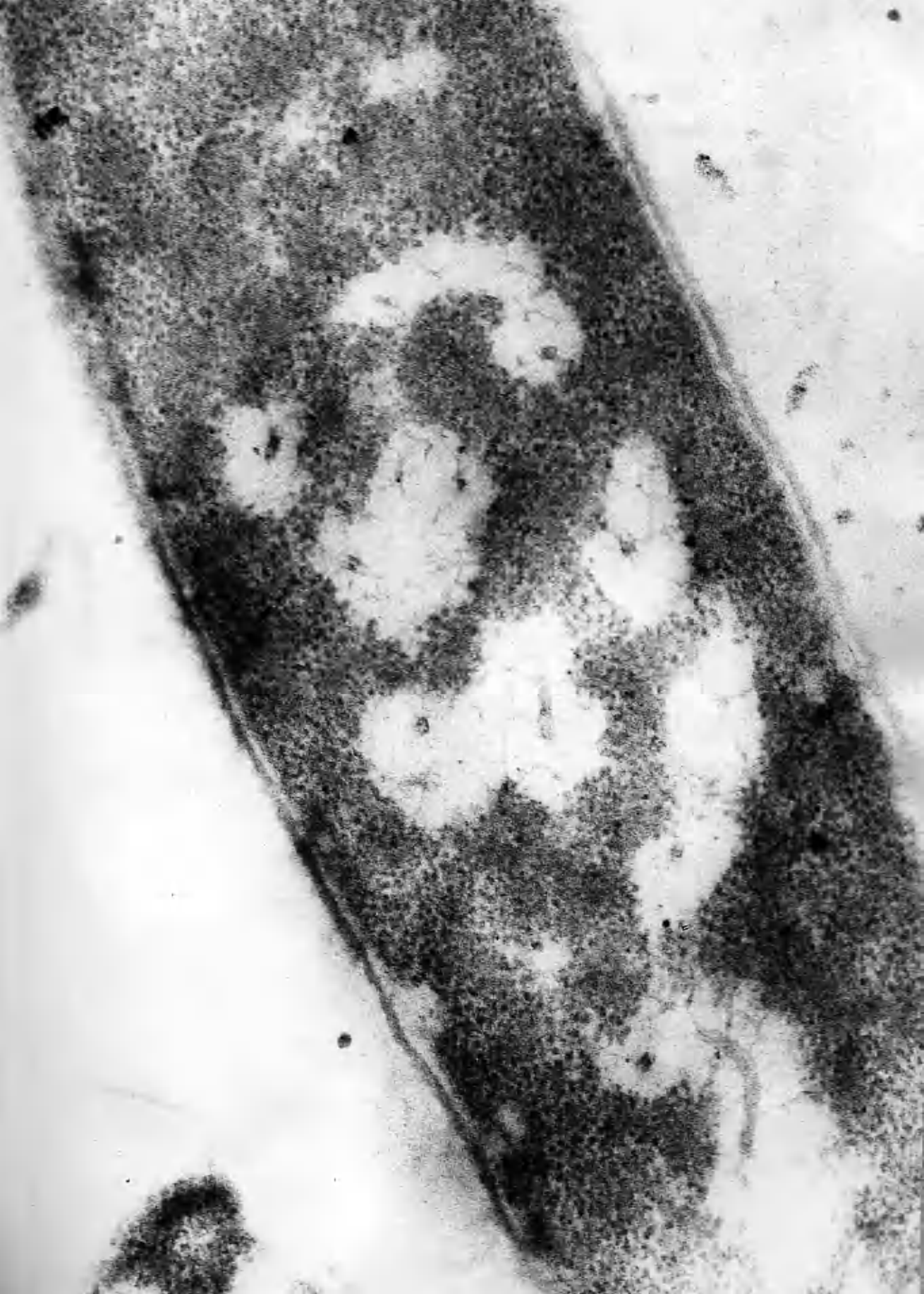


PLATE 5

Pronounced delocalisation of nuclear material.

(S/62/2459) x 118,000



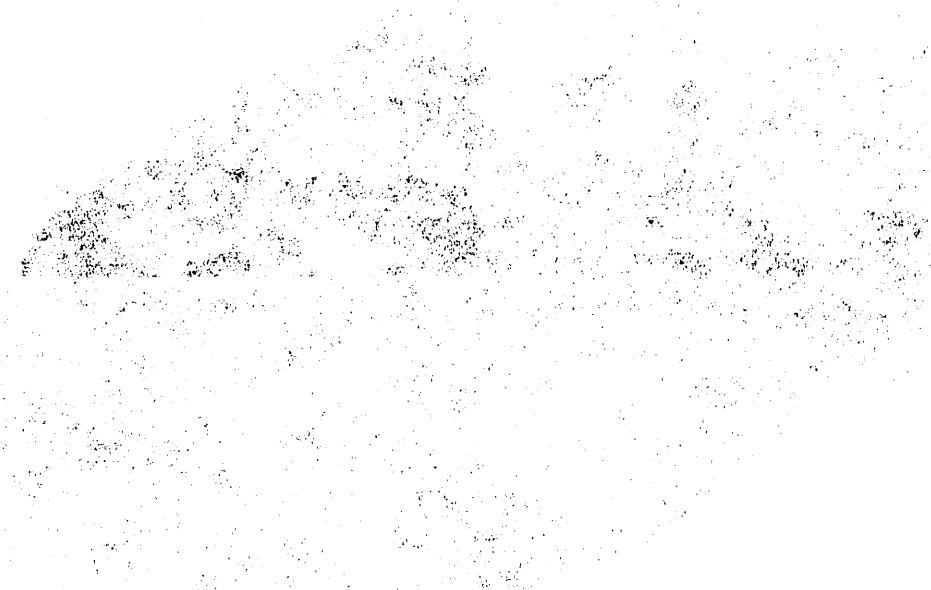


PLATE 6

Appearance of phage-like bodies (marked A)

(NS/63/200) x 102,300

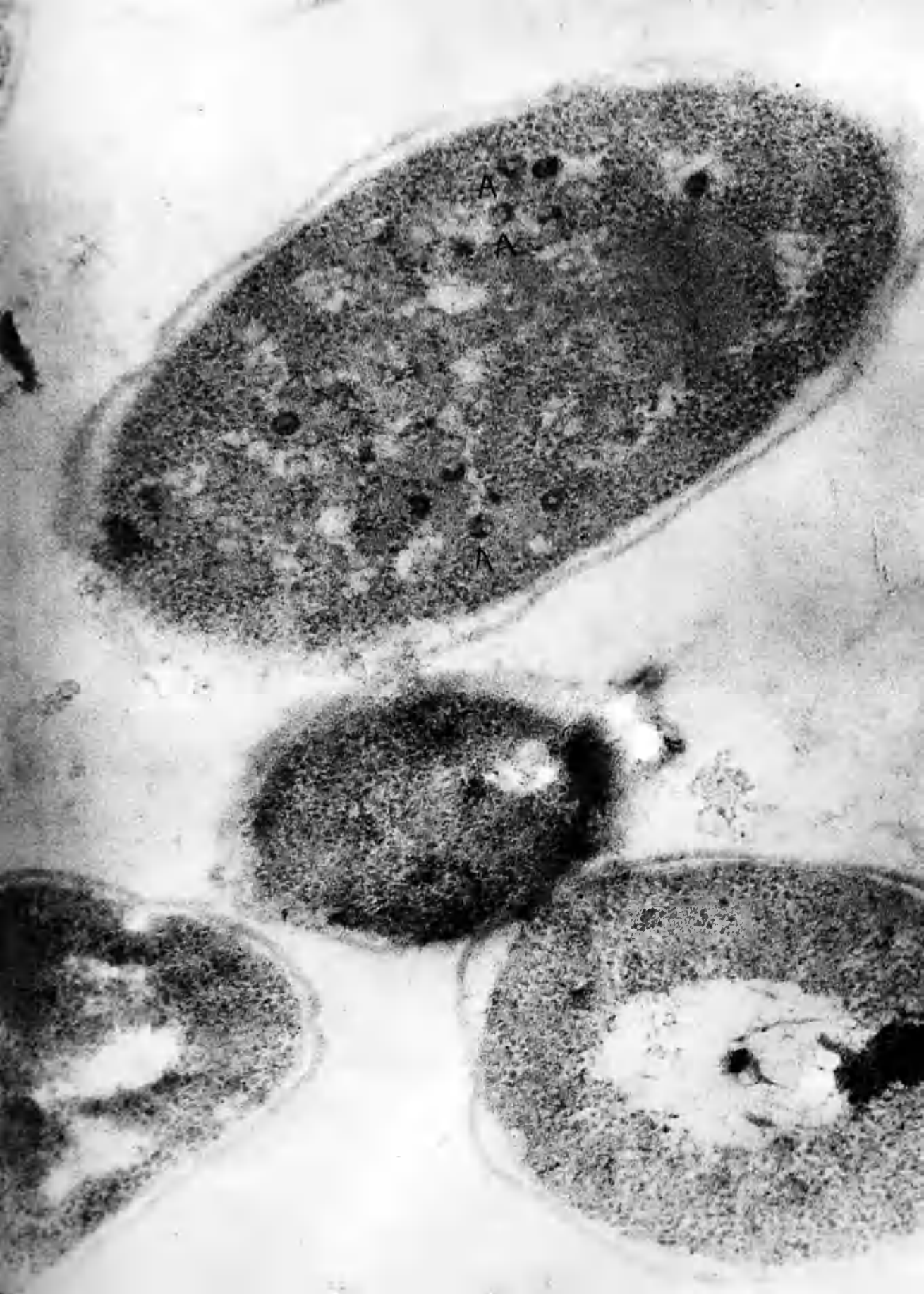

A
A
A

PLATE 7

Development of phage within the cell.

(S/62/706) x 124,000

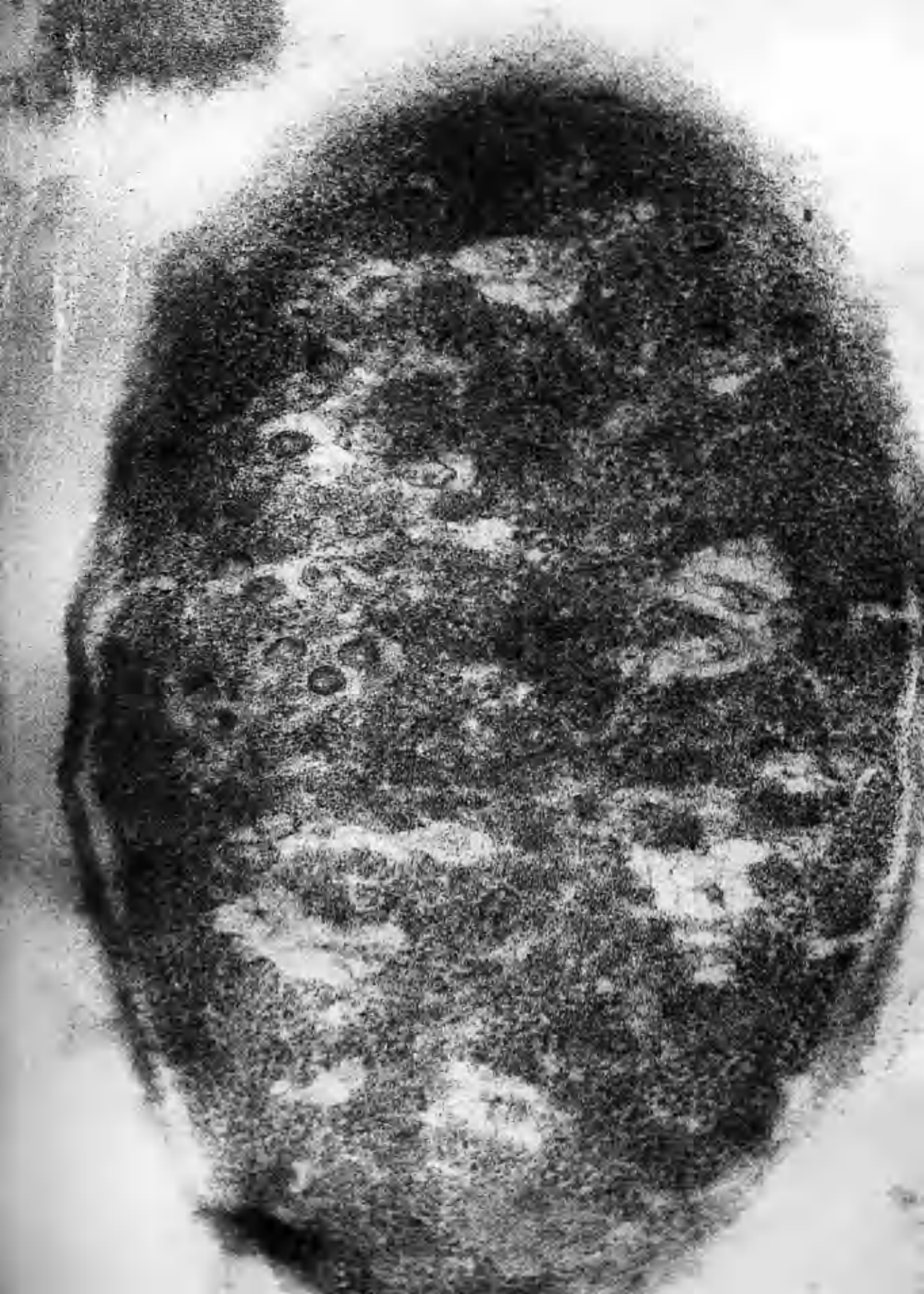


PLATE 8

Fully formed phages, which in some cases show external membranes.

(S/62/2362) x 126,500

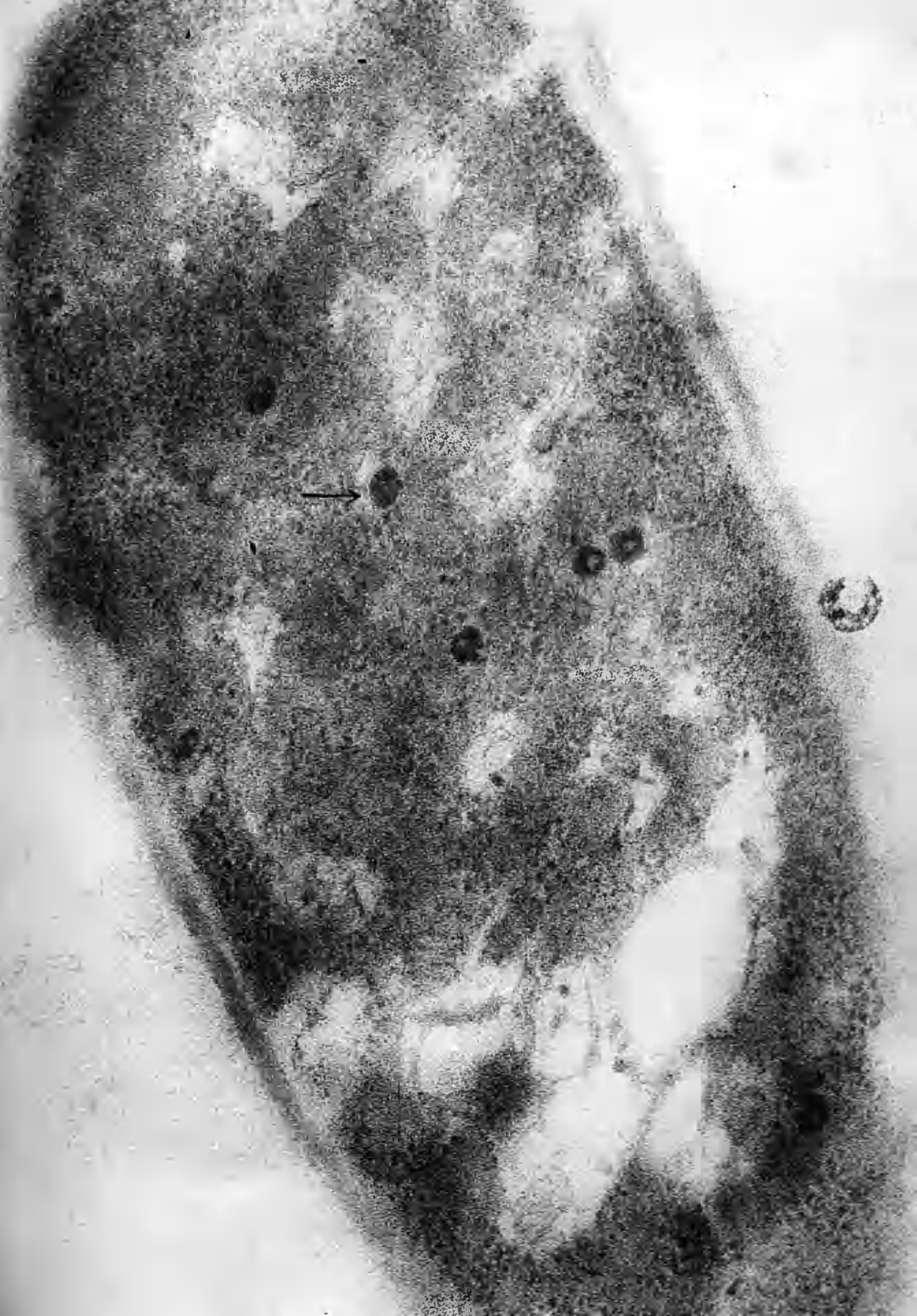


PLATE 9

Angular contours of some intracellular particles (Instrumental magnification x 60,000).

(S/62/1895) x 318,200

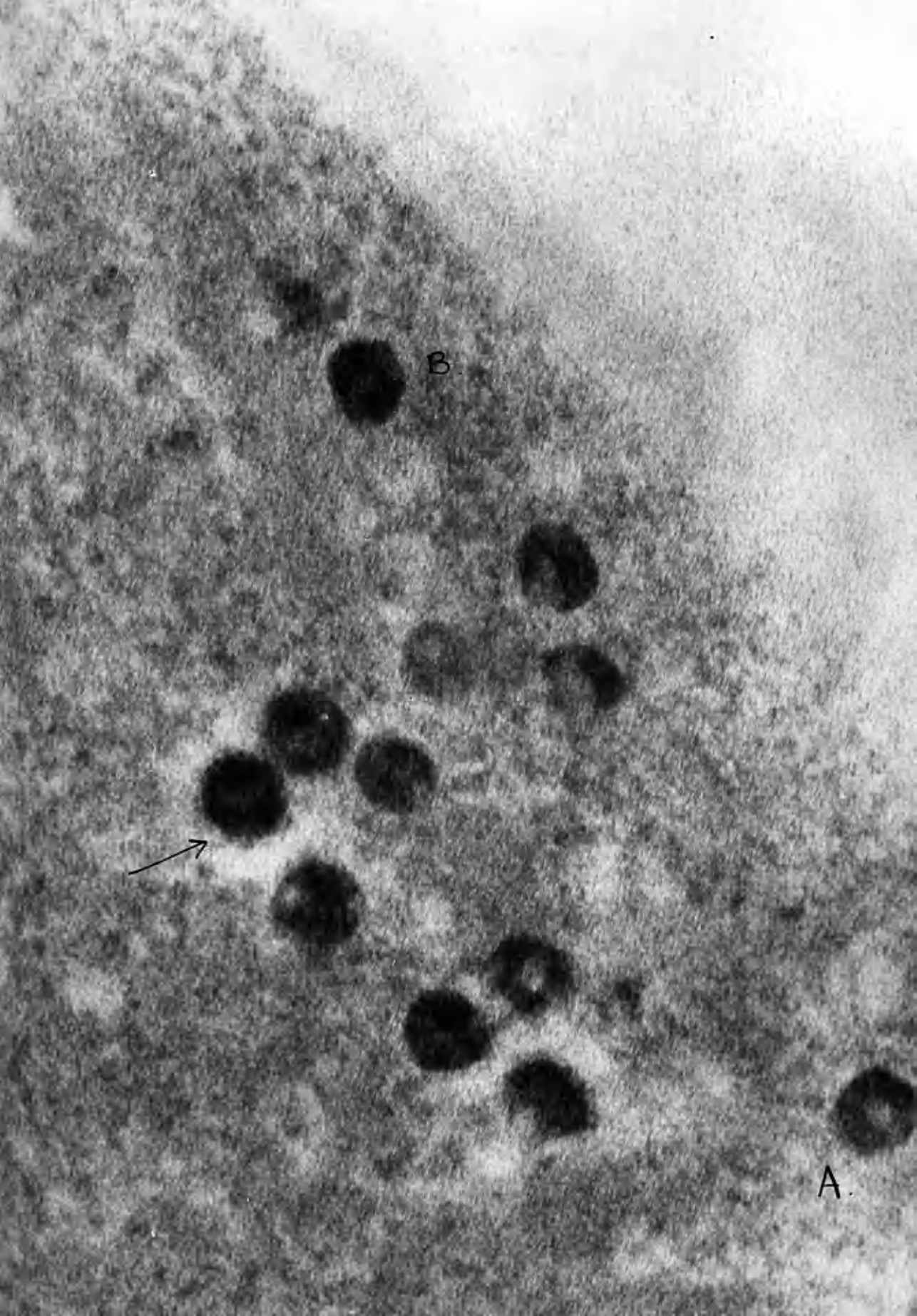

B
A

PLATE 10

Fully formed phages, several of which show clear external membranes.

(S/62/1878) x 142,250

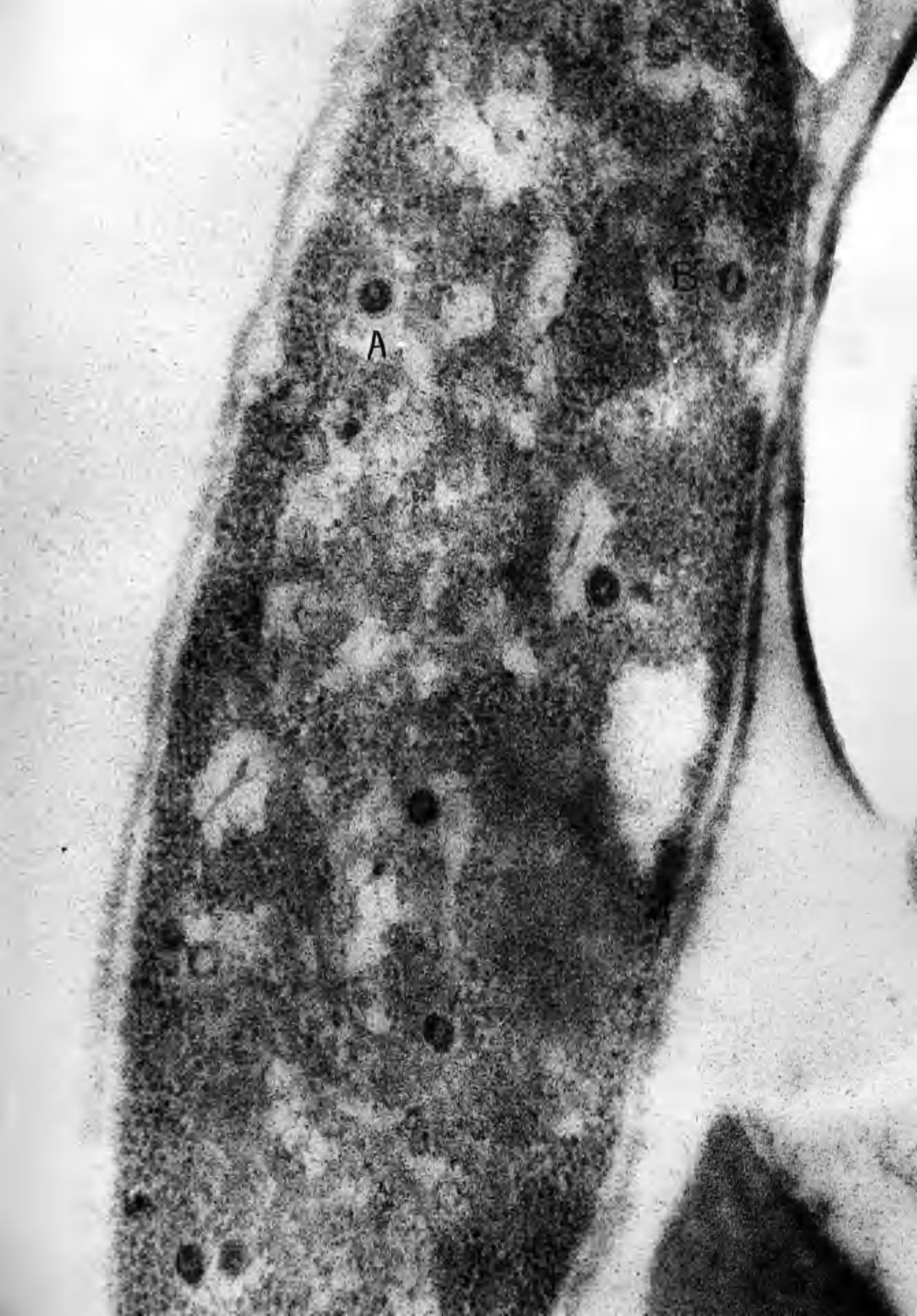

A
B

PLATE 11

Bacterium containing numerous phages, some of which may possess tails.

(S/62/1347) x 112,800

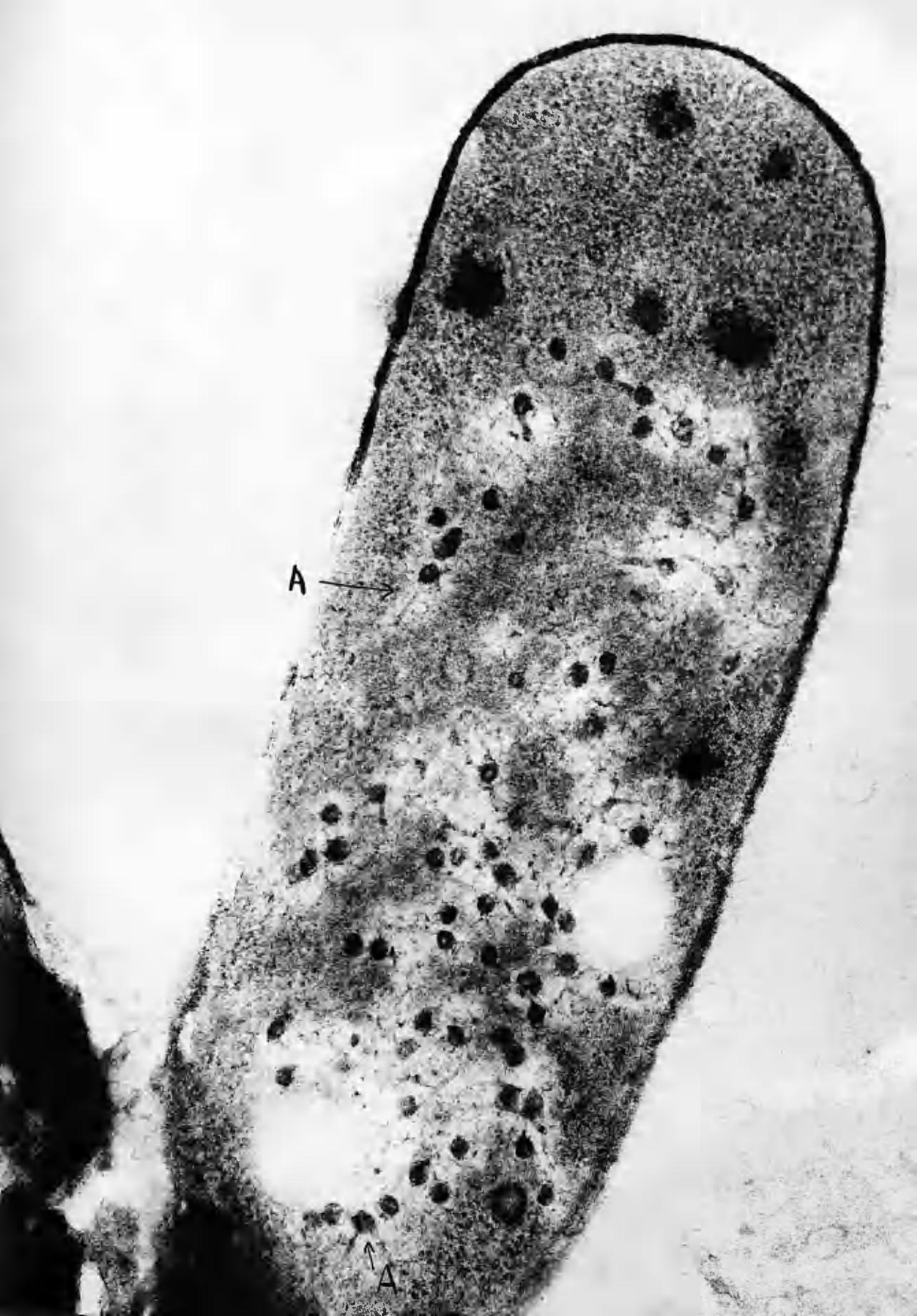

A
A

PLATE 12

Infected bacterium which contains fully formed phages and also two lattice structures.

(NS/63/194) x 145,000

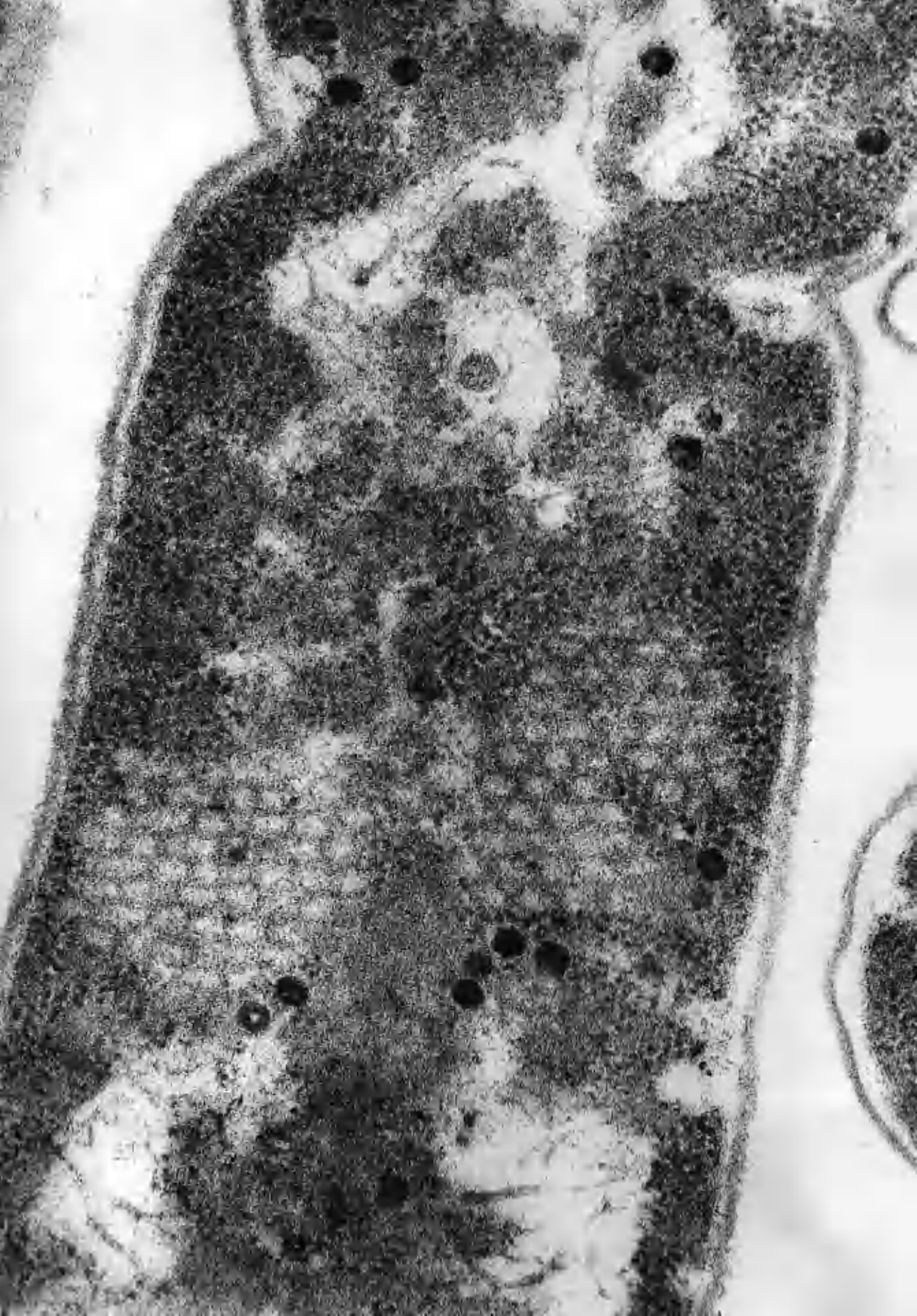


PLATE 13

The Bacterial Cell in this plate shows mature phage particles, and a lattice structure.

(S/62/1907) x 112,500

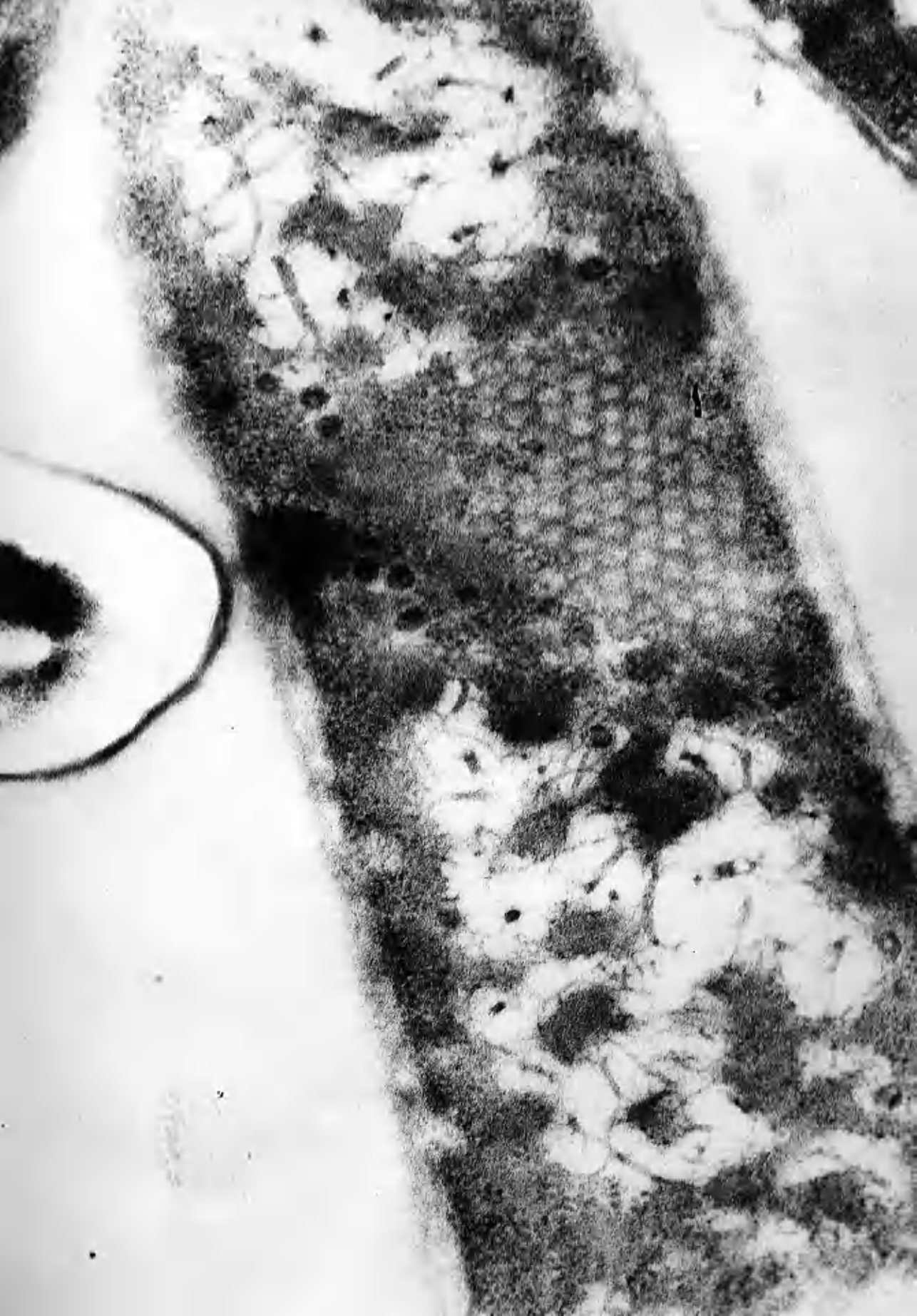


PLATE 14

Cell containing two lattices, the sub-units of which show varying intensities.

(NS/62/204) x 115,300

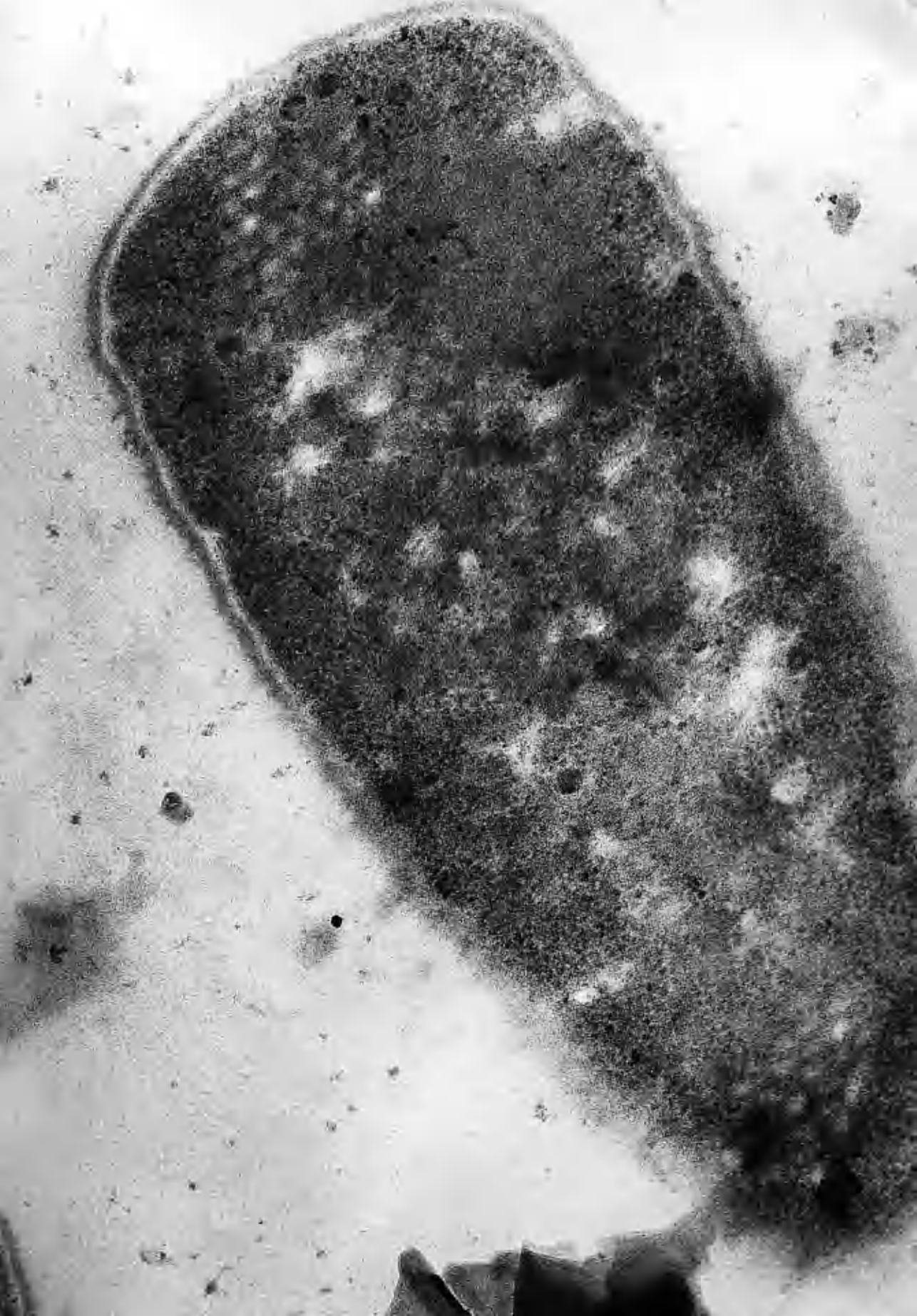


PLATE 15

Series I Section I

(NS/63/251) x 62,250

PLATE 16

Series I Section II

(NS/63/252) x 62,250

PLATE 17

Series I Section III

(NS/63/257) x 62,250

PLATE 18

Series I Section IV

(NS/63/258) x 62,250

PLATE 19

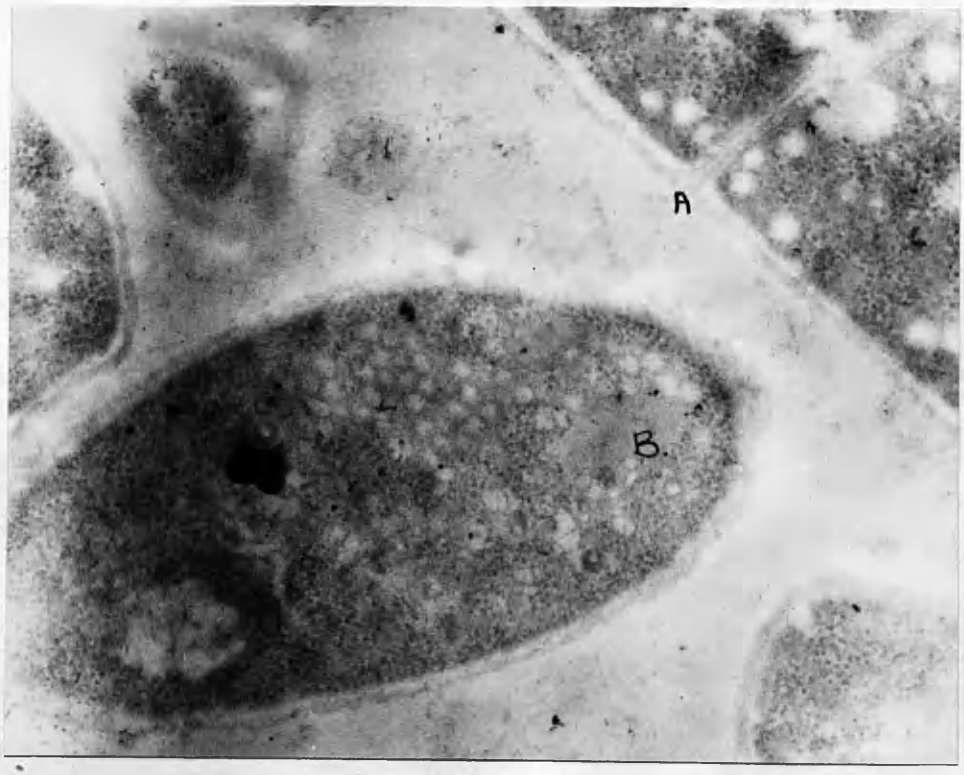
Series I Section V

(NS/63/262) x 62,250

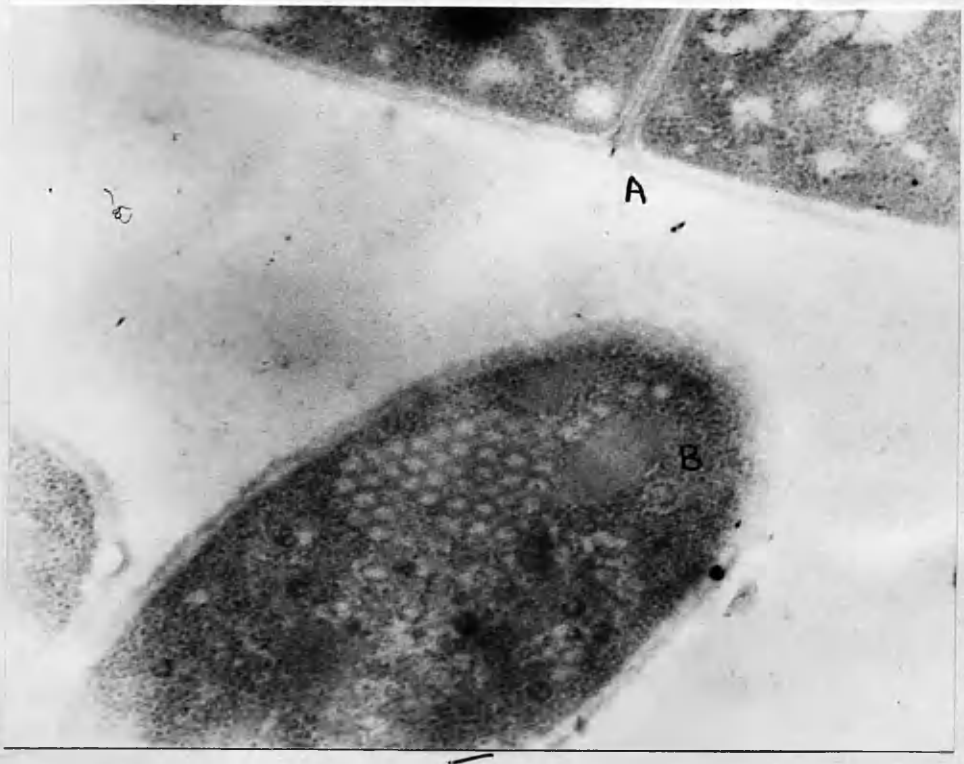
PLATE 20

Series I Section VI

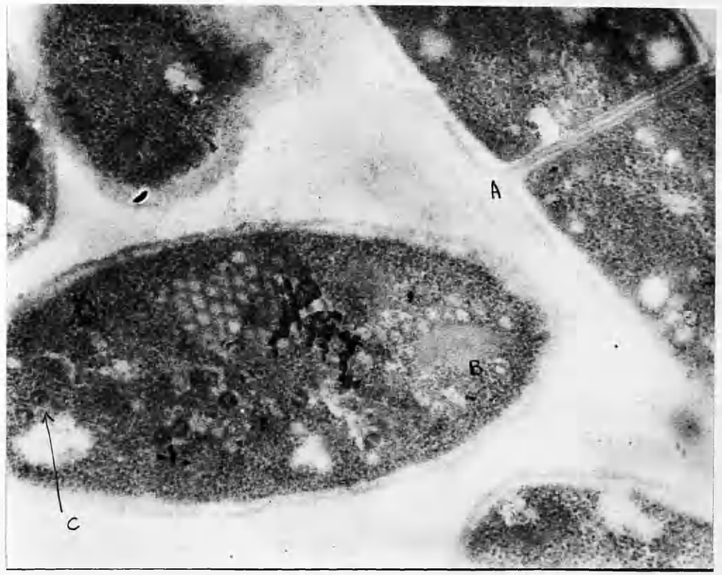
(NS/63/268) x 62,250



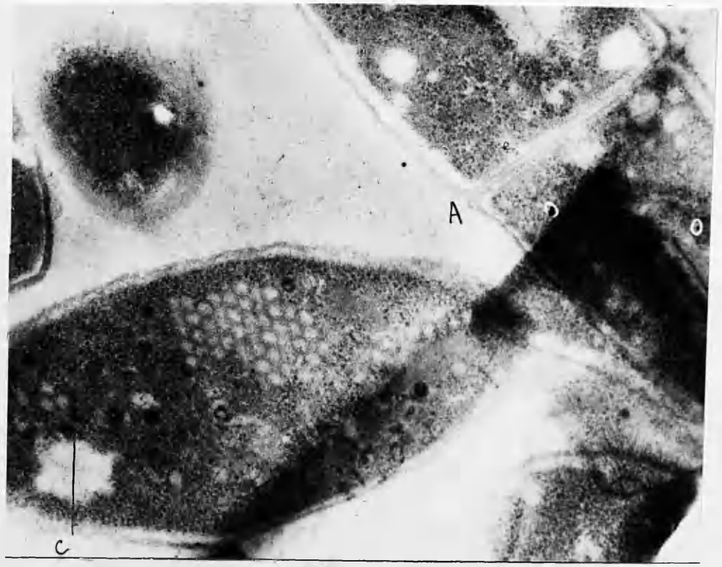
III.



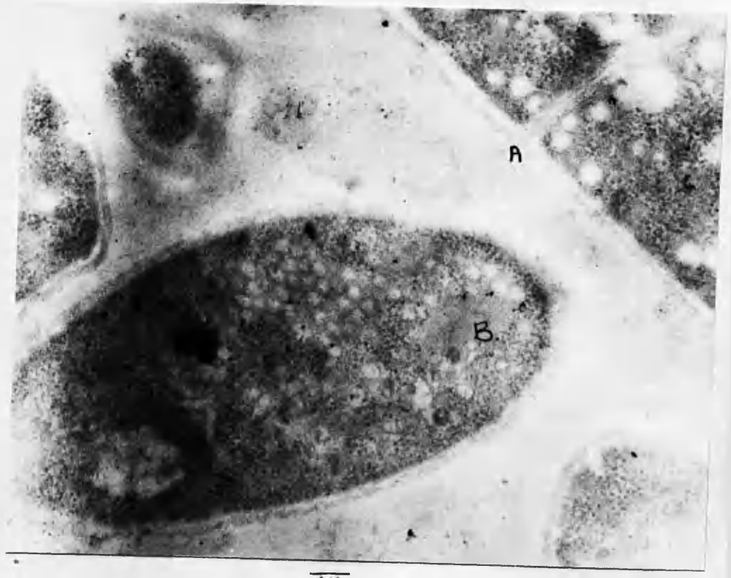
VI.



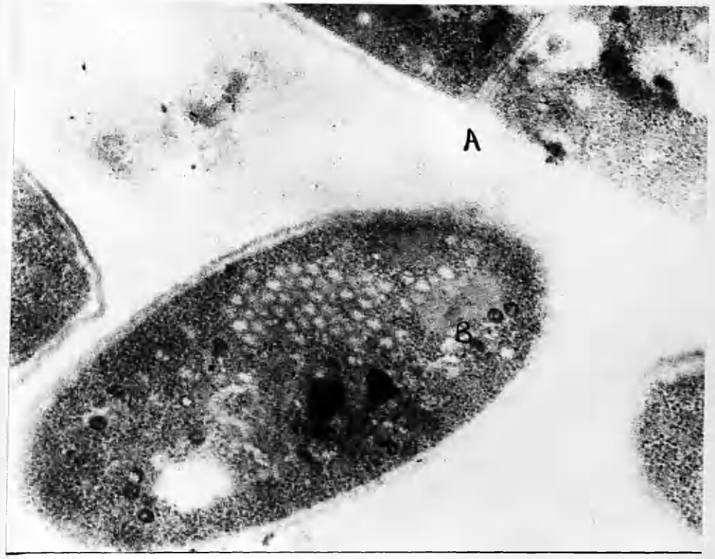
I.



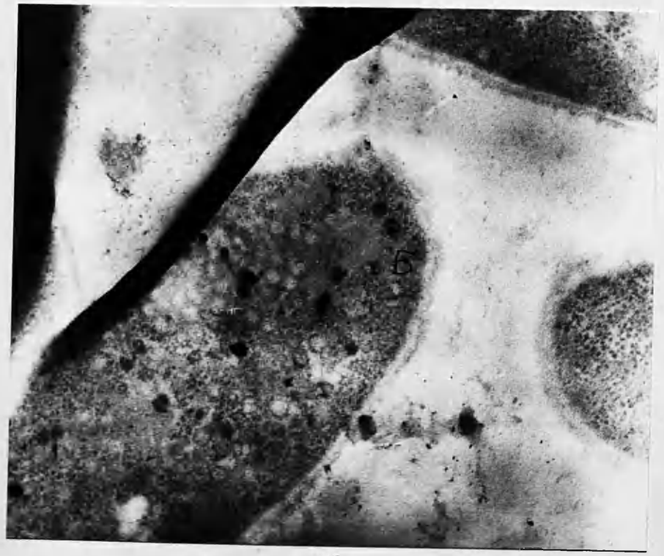
II.



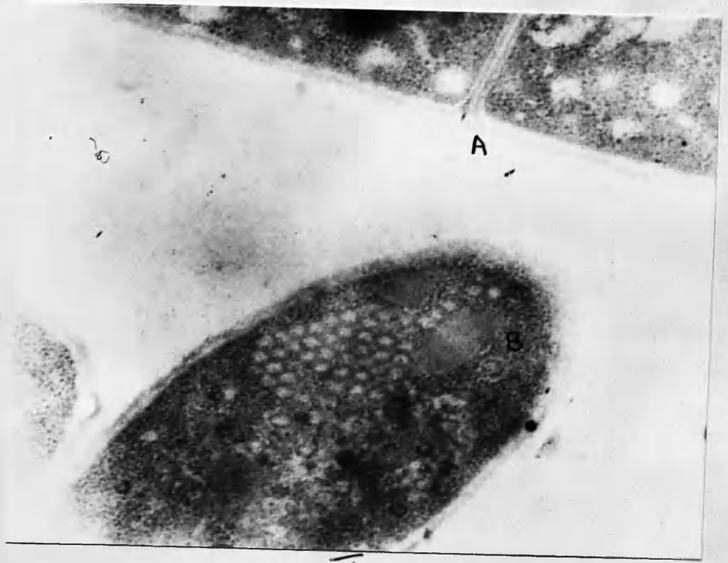
III.



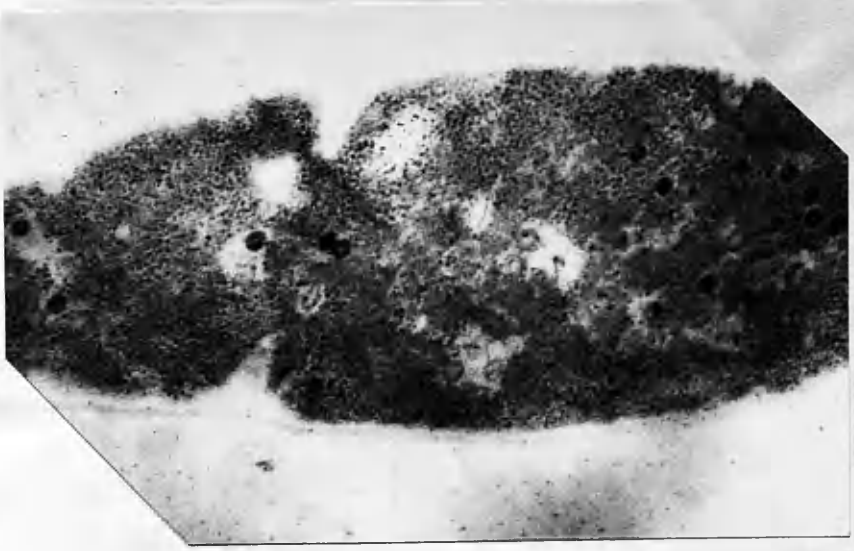
IV.



V.



VI.



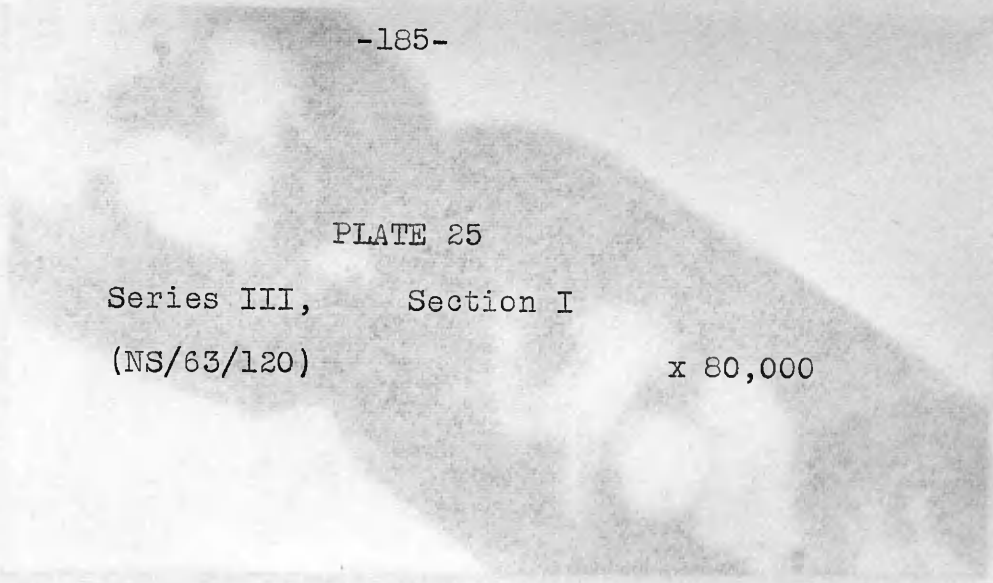


PLATE 25

Series III, Section I

(NS/63/120) x 80,000

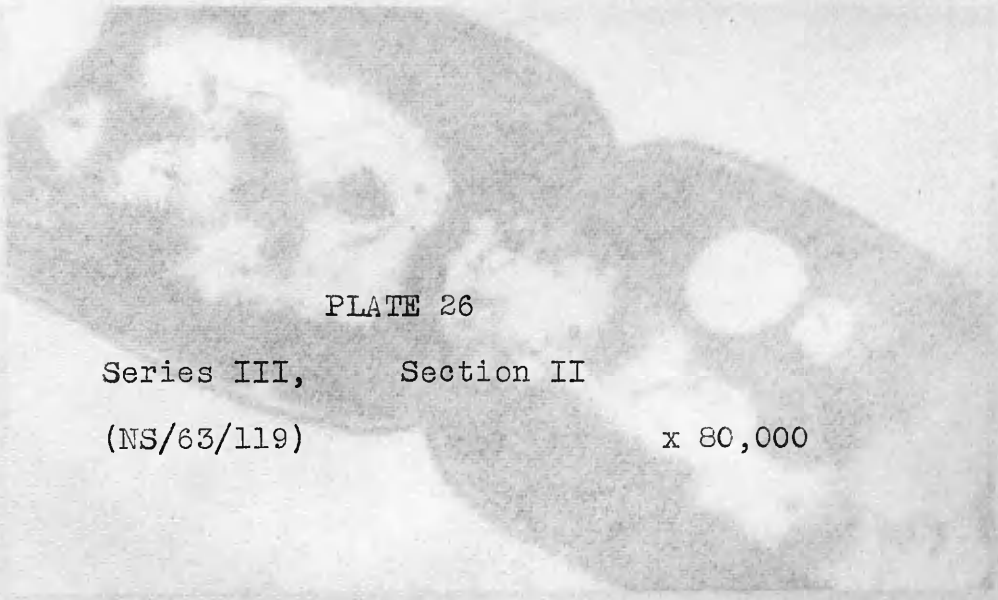


PLATE 26

Series III, Section II

(NS/63/119) x 80,000

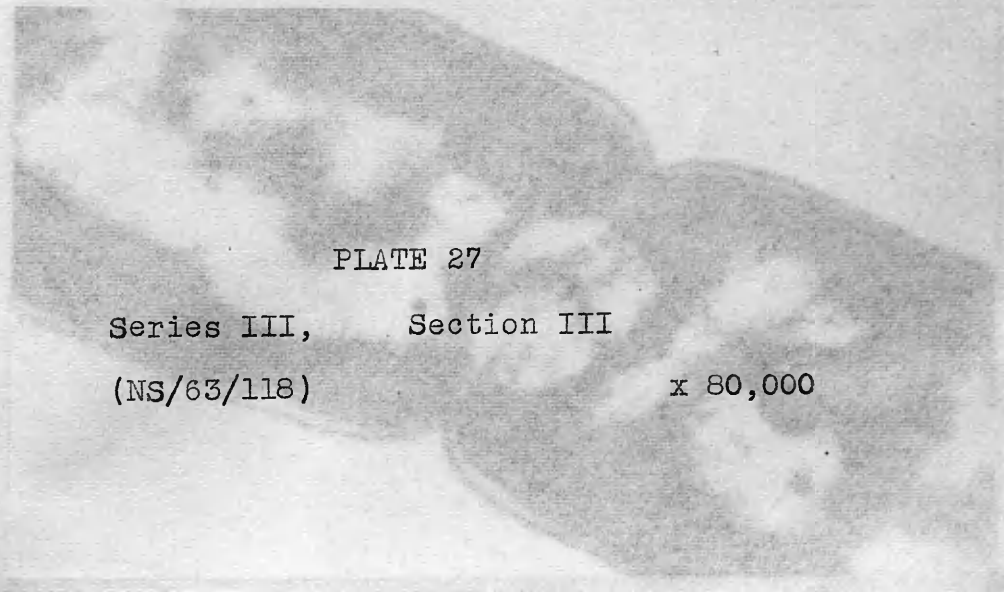


PLATE 27

Series III, Section III

(NS/63/118) x 80,000

PLATE 28

Osmium fixed sporulating organisms.

(S/62/2212) x 103,800

N.	nuclear site.
S.C.	spore core.
I.M.	inner membrane.
C.W.	cortex wall.
C.	cortex.
O.M.	outer membrane.
S.W.	spore wall.
E.	exosporium.

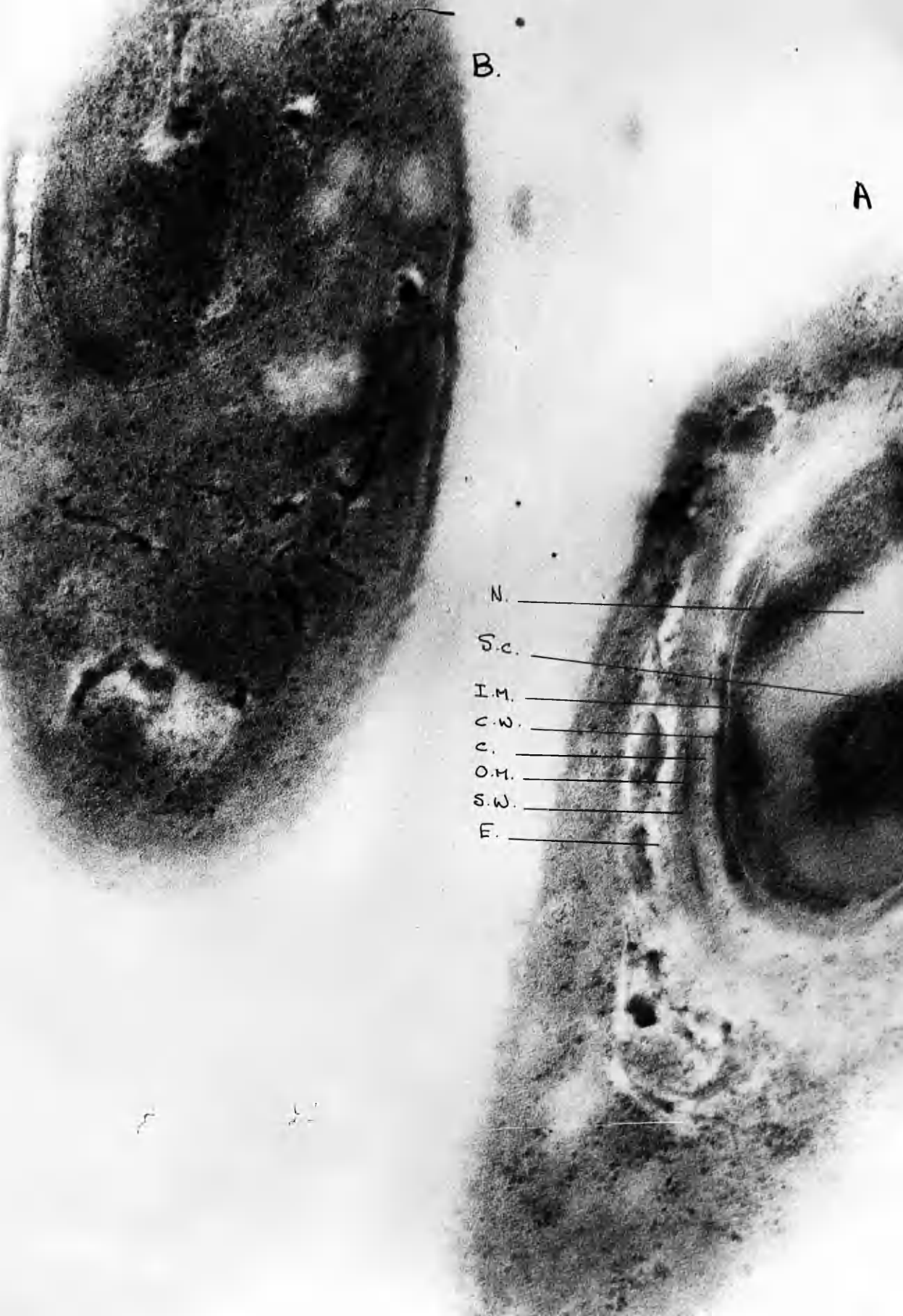

B.
A
N.
S.C.
I.M.
C.W.
C.
O.M.
S.W.
E.

PLATE 29

Osmium fixed sporulating organisms.

(NS/62/2203) x 120,000

N.	nuclear site.
S.C.	spore core.
I.M.	inner membrane.
C.W.	cortex wall.
C.	cortex.
O.M.	outer membrane.
S.W.	spore wall.
E.	exosporium.

PLATE 30

Spore forming cell, fixed with permanganate.

(S/62/2132) x 118,500

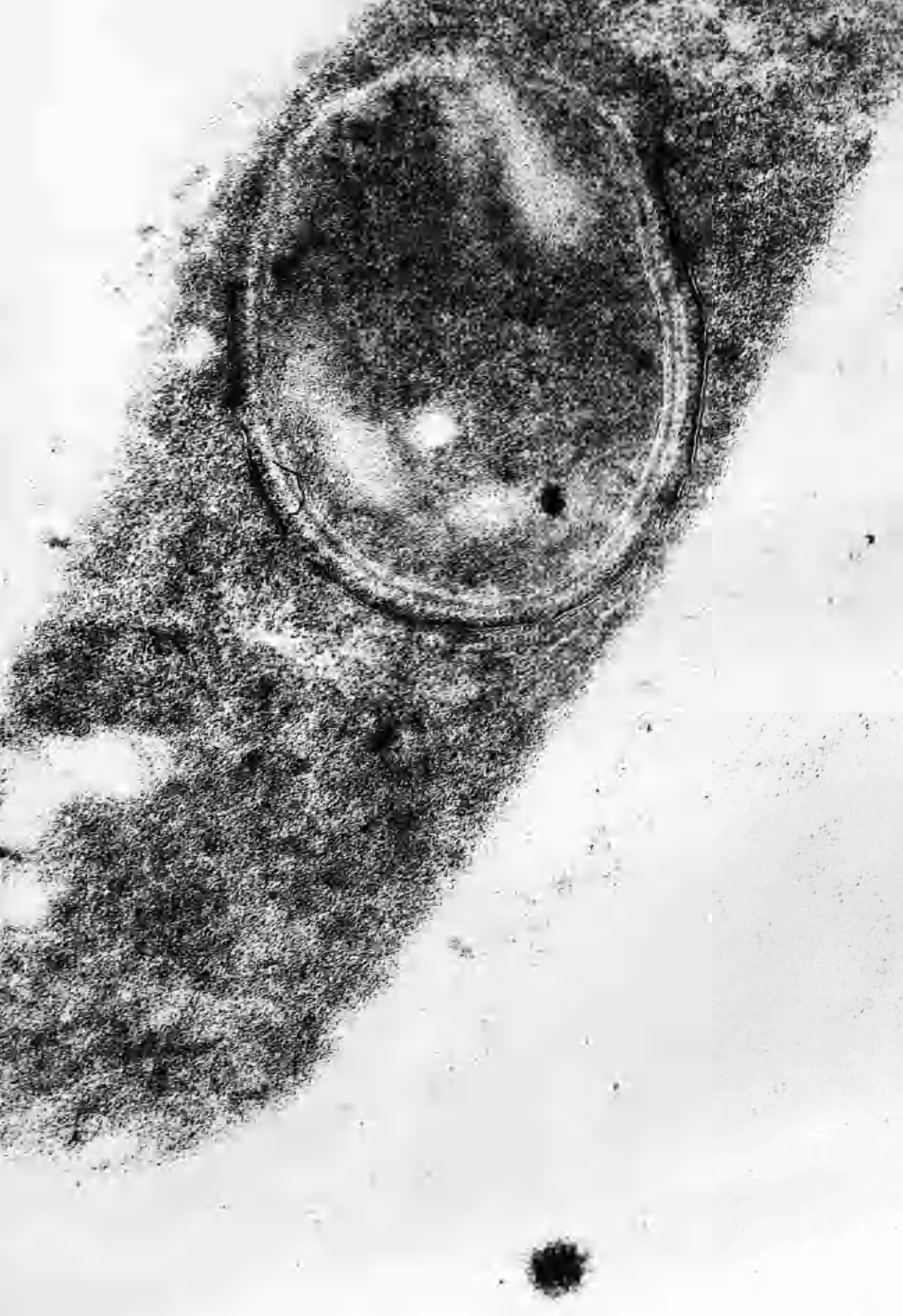


PLATE 31

Phosphotungstate stained bacterium showing "V" shaped ends of invaginating membranes.

(S/62/1085) x 233,000

PLATE 32

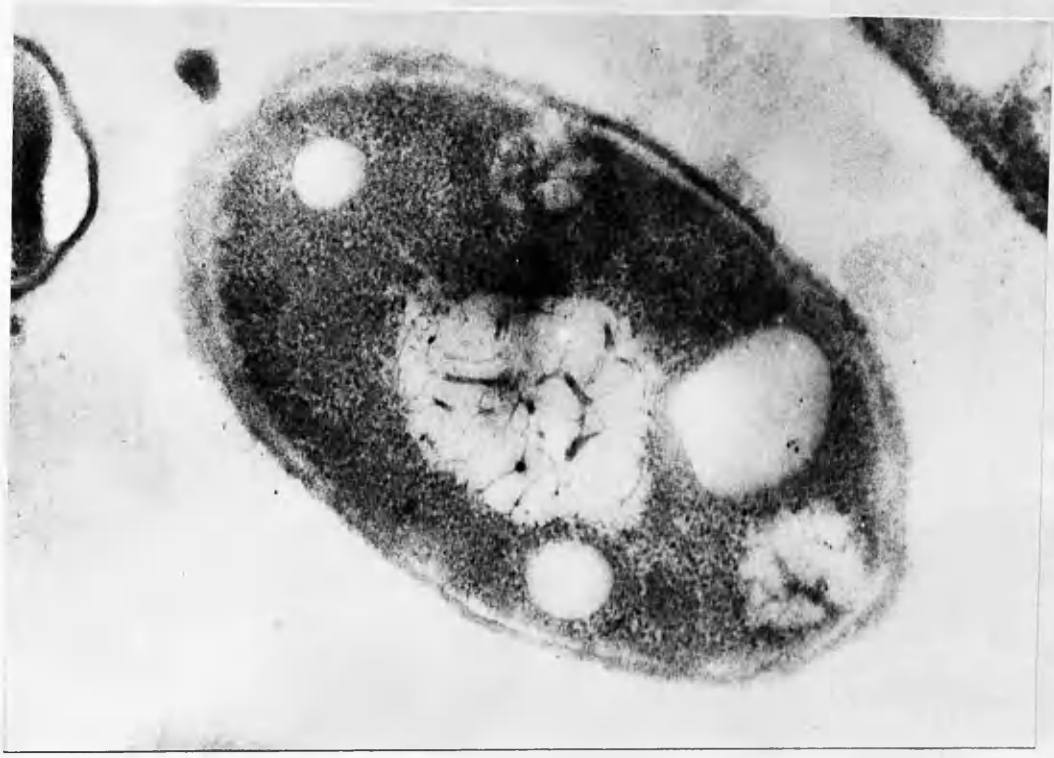
Sodium uranate stained bacterium.

(NS/63/641) x 77,200

PLATE 33

Ammonium molybdate staining.

(S/62/1068) x 85,350.



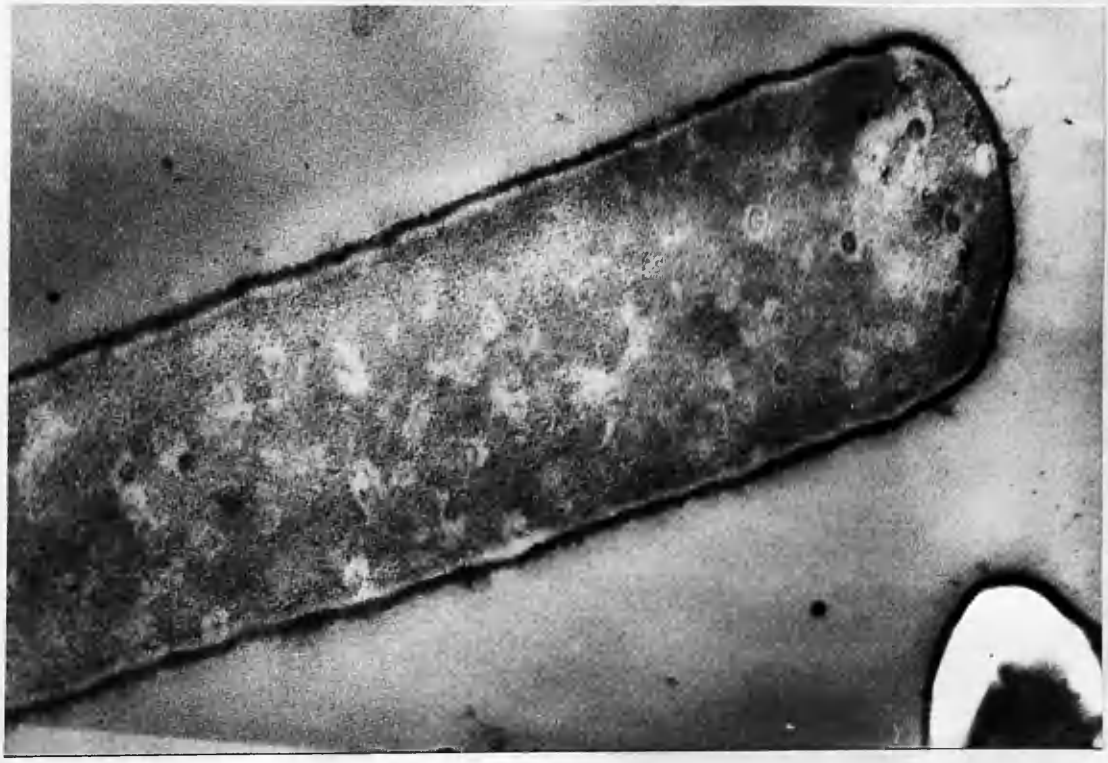


PLATE 34

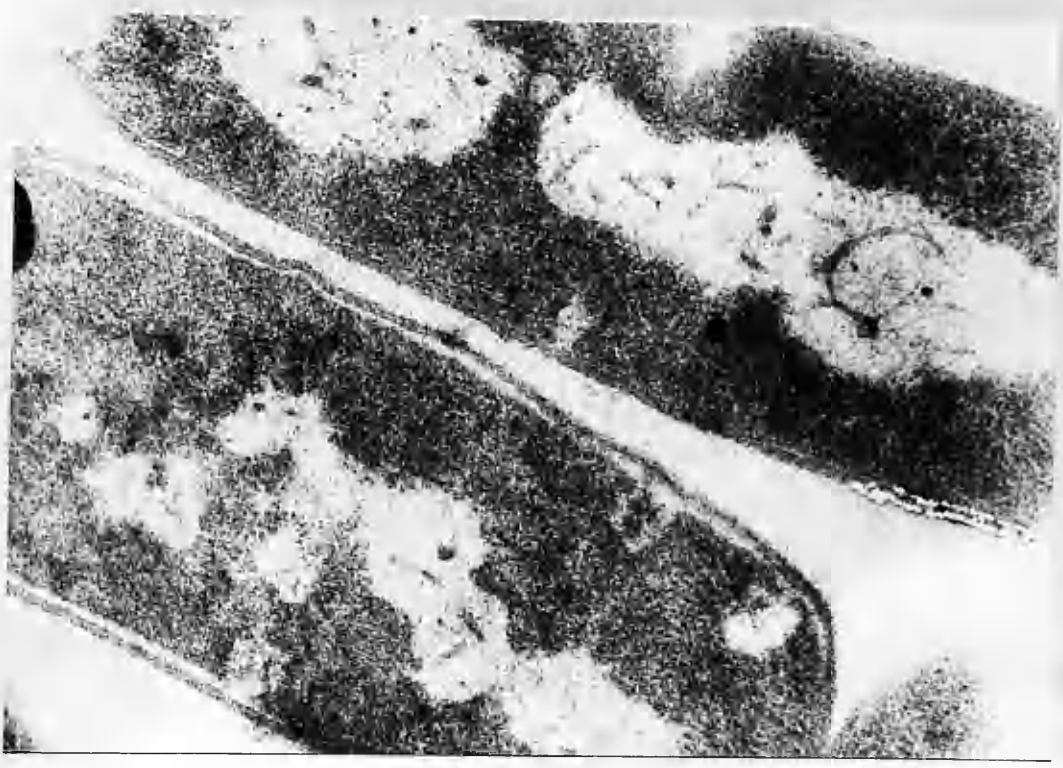
Lanthanum nitrate staining.

(S/62/1020) x 76,800

PLATE 35

Permanganate staining.

(NS/63/591) x 72,000



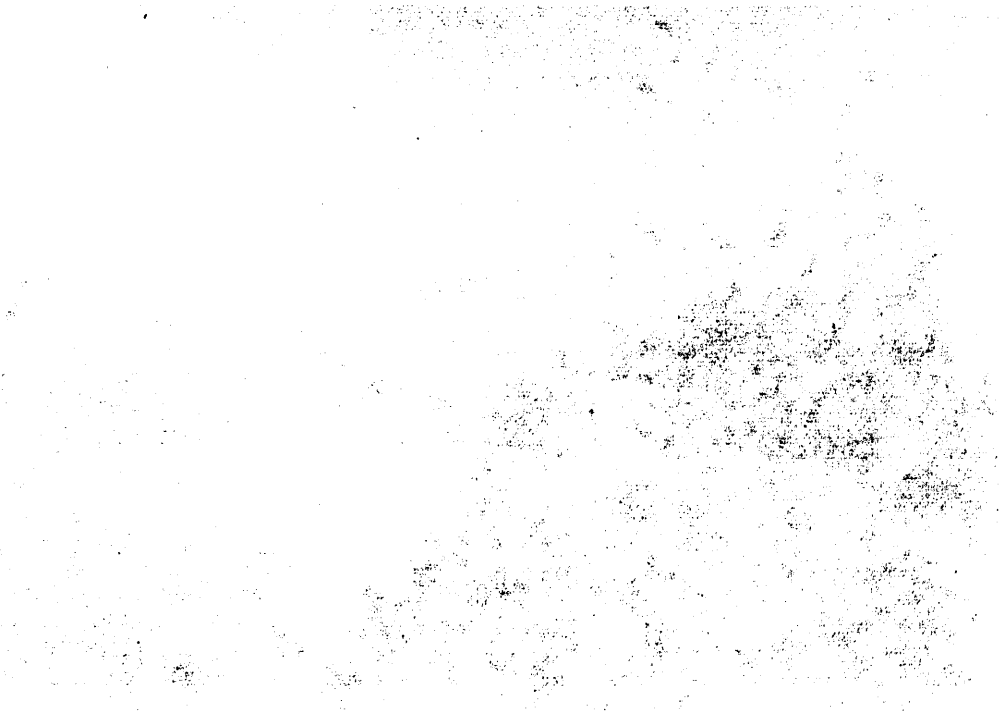


PLATE 36

Transverse section of cell containing several mature phages. Lysis is about to occur.

(S/62/1909) x 148,200

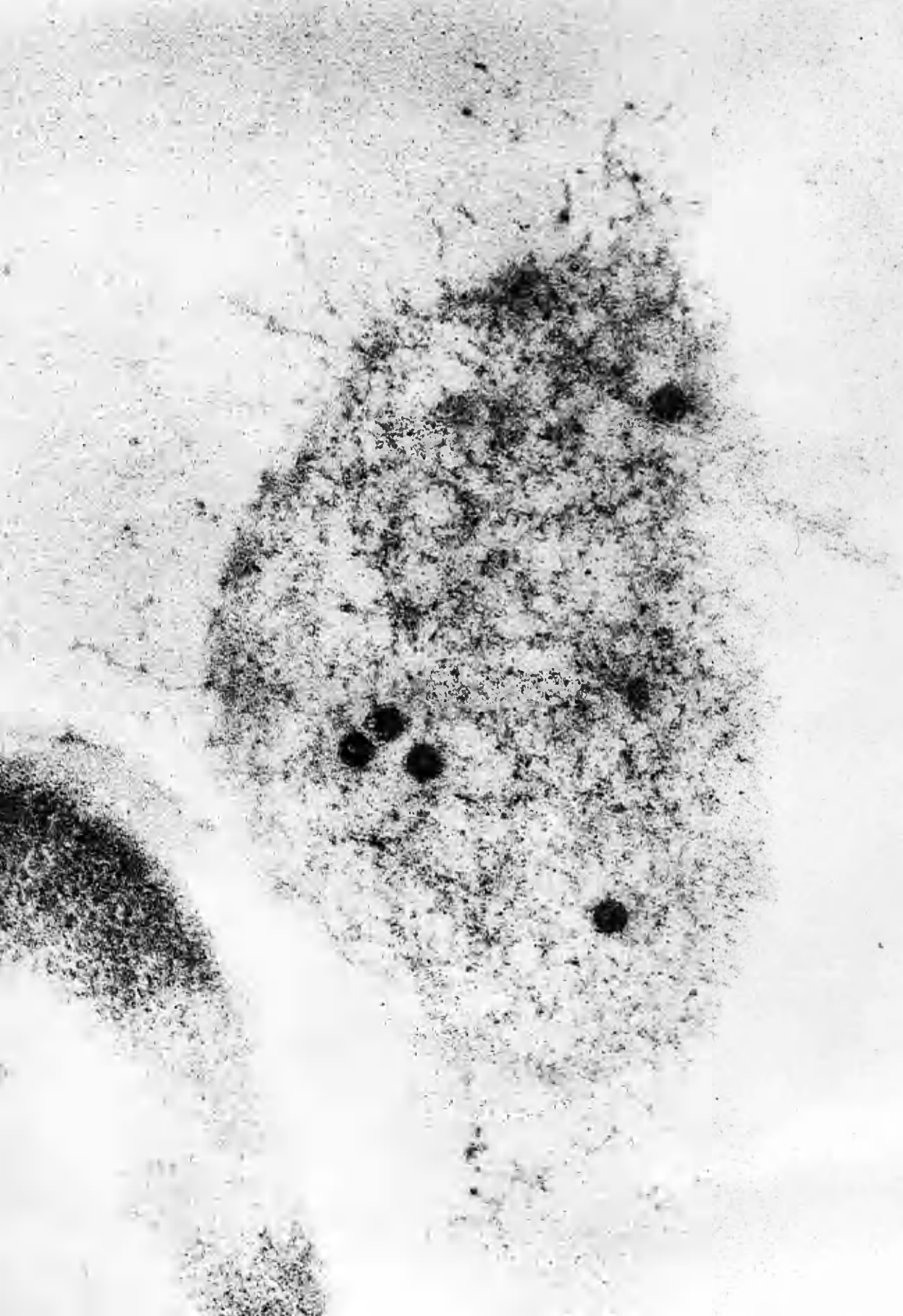
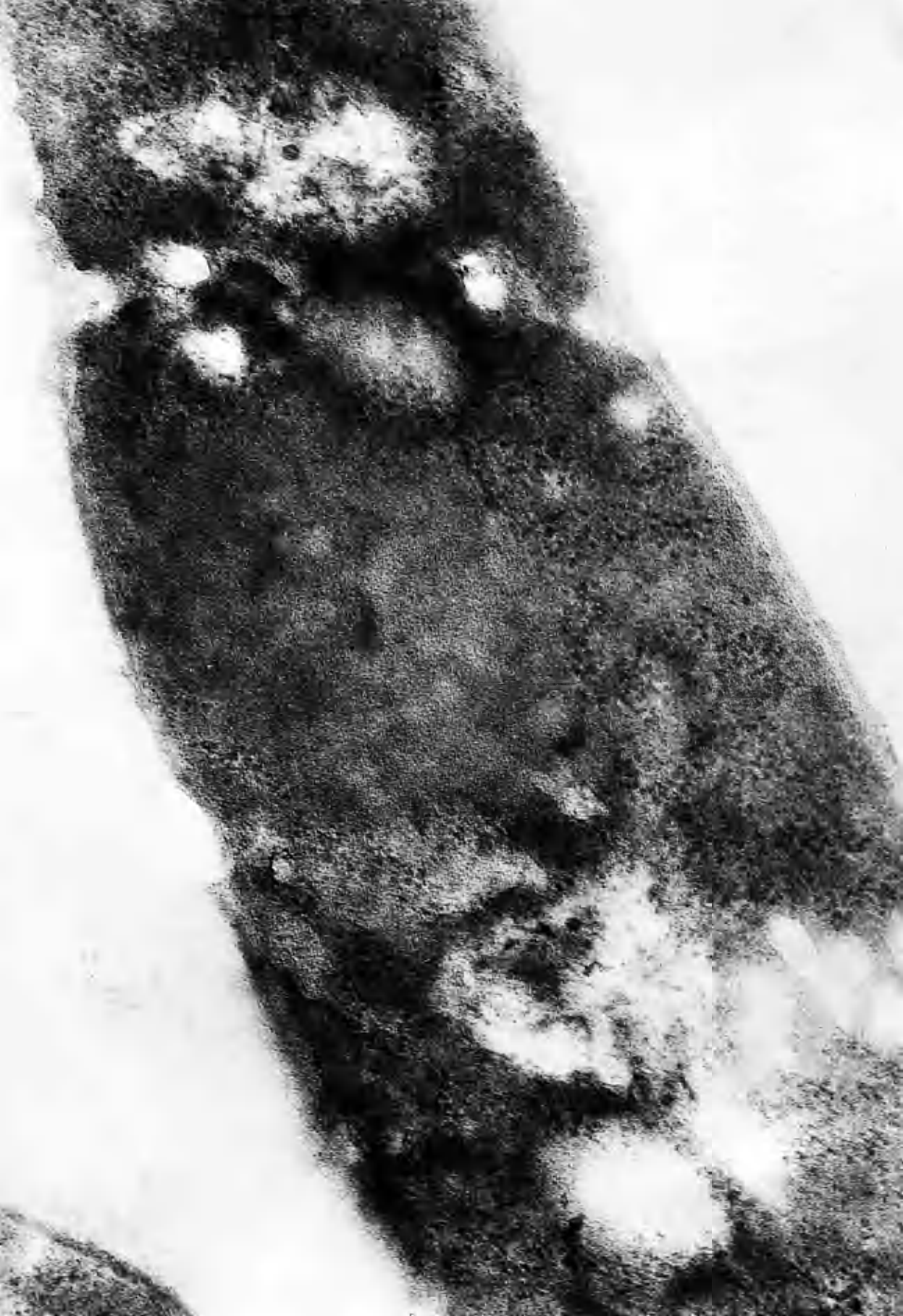


PLATE 37

Infected cell showing area of cytoplasm, which is completely clear of ribosomes.

(S/62/1787) x 128,500



GENERAL CONCLUSIONS

Part I

In morphological studies, the four B. cereus bacteriophages appeared to be very similar. Evidence derived from shadowed and negatively stained preparations suggests that the heads of all four are icosahedral, although as yet the bipyramidal hexagonal prism cannot be ruled out completely. Also, from a study of fine structure, it seems that the tails of these phages are pentagonal in cross-section, being composed either of a series of concentric rings, or of a helix with almost five capsomeres per turn of the helix. The base-plate therefore may also be pentagonal in cross-section, and some evidence in favour of this was obtained.

Additional fine structure was detected in the negatively stained preparations containing empty and partially empty heads. The tail appears to penetrate some distance into the head, terminating in two spherical structures, which may well be associated with injection of the phage DNA into the bacterial cell.

Part II

Judged on the basis of the degree of shrinkage and general appearance of the bacterial cell, the fixation

and embedding techniques used in this study seem suitable for a study of intracellular phage growth. However, it would be of interest to compare these results with those obtained by the Kellenberger fixation procedure (Kellenberger et al., 1958).

From a study of the thin sections of uninfected and infected cells, it emerged that the structures of the organisms of the B. cereus group are very similar to those observed by Glauert et al., (1961) for a closely related organism, B. subtilis. In interpreting such findings however, it must be remembered that the appearance of the bacterial cell in electron microscopy to a large extent depends on the methods used for its study, and so exact comparisons are difficult.

The sequence of events during the intracellular growth of the B. cereus bacteriophages is strikingly similar to that observed recently by Kellenberger (1961) for T_2 phage of E. coli. An important point of difference, however, was noted; in the present study, no marginal vacuoles were observed, suggesting that the distribution of DNA following infection may vary for different phage/host-cell relationships.

A comparison of the appearance of the intracellular and extracellular phage particles was also made. This revealed similarities in size and shape, and the

hexagonal outlines often seen in section is further evidence in favour of an icosahedral or bipyramidal head form. Some evidence obtained suggests that the tail is formed at a late stage of phage development, but as yet this cannot be conclusively stated. From a study of the head in the course of intracellular growth, it is suggested that it grows from the head rather than being added on as a completed unit.

In the course of work, it was noted that inclusions of a "lattice" nature commonly appeared in infected cells, in which the nuclear delocalisation was extensive. The nature and function of these inclusions remains doubtful, although several possibilities were considered. From structural considerations, it is likely that the inclusions are crystalline bodies which are in a transient phase of phage growth, or represent a by-product of the disrupted metabolism of the cell.

Observations were also made on the structure of the spores produced by these organisms. These closely resembled the spores of B. cereus described by Young and Fitz-James (1962), although the actual form of the spore seems to vary greatly in the course of its development.

Finally a comparison of various materials as post-stains was included, as future studies may be aided by the use of a stain specific for the components of the phages and the host cell. A fairly dilute alcoholic solution of uranyl acetate applied for 60 minutes at room temperature was the most effective general stain. Further work on this aspect is necessary, however, taking into account such conditions as concentration of stain, and the time and temperature of its application.

BIBLIOGRAPHY

Adams, M.H. (1959) "Bacteriophages" New York Interscience Publishers Inc.

Afrikian, E.G. (1960) J. Insect Path. 2, 299.

Ajl, S.J. (1950) J. Bact. 60, 393.

Anderson, T.F. (1943) J. Bact. 45, 303.

Anderson, T.F. (1950) J. Appl. Phys. 21, 70.

Anderson, T.F. (1951) Trans. N.Y.Acad. Sci., 13, 130.

Anderson, T.F. (1960) Eur. Eg. Conf. Electron Microscopy, Delft. 2, 1008

Anderson, T. F., Doermann, A.H. (1952) J. Gen. Physiol., 35, 657.

Anderson, T.F., Rappaport, C., Muscatine, N.A. (1953) Ann.Inst.Past. 84, 5

Backus, R.C., Williams, R.C. (1950) J. Appl. Phys., 21, 11.

Beer, M. (1962) 5th Int. Conf. E. Mic. Philadelphia, 1, A6.

Bendettini, E.L., Bertolini, B. (1963) J.Roy.Mic.Soc., 81, Ser.3, 219.

Bernal, J.D., Carlisle, C.H. (1948) Nature, 162, 139.

Bernal, J.D., Fankuchen, I. (1941) J. Gen. Physiol. 25, 111.

Bernal, J.D., Fankuchen, I., Riley, D.P. (1938) Nature, 142, 1075.

Birbeck, E.H., Mercer, M.S.C. (1961) "A handbook for Biologists", Blackwell Scientific Publications, Oxford.

Borysko, E. (1956) J. Biochim. Biophys. Cytol.2, (supple) 3.

Bradbury, S., Meek, G.A. (1960) Quart. J. Microscop. Sci., 101, 241.

Bradley, D.E. (1954) Brit. J. Appl. Phys., 5, 65.

Bradley, D.E. (1958) 4th Int. Conf. E. Mic. Berlin, 1, 428.

Bradley, D.E. (1959) Brit. J. Appl. Phys., 10, 198.

Bradley, D.E. (1962) 5th Int. Conf. E. Mic. Philadelphia, S.7.

Bradley, D.E. (1963) J. Gen. Microbiol., 31, 435.

Bradley, D.E., Kay, D. (1960) J. Gen. Microbiol., 23, 553.

Bradley, D.E., Kay, D. (1962) J. Gen. Microbiol., 27, 195.

Brenner, S., Horne, R.W., (1959) Biochim. Biophys. Acta 34, 103.

Brenner, S., Streisinger, G., Horne, R.W., Champe, S.P., Barnett, L., Benzer, S., Rees, M.W. (1959) J. Mol. Biol., 1, 281.

Brody, I (1959) J. Ultrastruct. Res., 2, 482.

Busch, H. (1926) Ann. Physik., 81 (ser.4) 974.

Caulfield, J.B. (1957) J. Biophys. Biochem. Cytol., 3, 827.

Caspar, D.L.D. (1956) Nature, 177, 475.

Chapman, G.B. (1959) J. Biophys. Biochim. Cytol., 6, 221.

Chapman, G.B., Hillier, J. (1953) J. Bact., 66, 362.

Chiozotto, A., Coppo, A., Donini, P., Graziosi, F. (1960) Eur.Reg.Con. E. Mic. Delft, 1002.

Claude, A., Fullam, E.F. (1946) J. Exptl. Med., 83, 499.

Claude, A. (1962) 5th Int. Con. E. Mic. Philadelphia, 2, L.14.

Coupland, J. H., (1954) Proc. Into. Con. E. Mic. London, 159.

Daems, H.T., Van de Pol, J.H., Cohen, J.A. (1961) J. Mol. Biol.,3, 225.

Davisson, C., Germer, L.H. (1927) Phys. Rev., 30, 705.

Dawson, I.M., Smillie, E.R., Norris, J.R. (1962) J. Gen. Microbiol.,28,517

De Broglie, L. (1924) Phil. Mag., 47, 446.

Delbrück, M. (1946) Biol. Revs. Camb. Phil. Soc., 21, 30.

De Mars, R.I., Luria, S.E., Fisher, H. Levinthal, C. (1953) Ann. Inst. Past., 84, 113.

Doermann, D.H. (1952) J. Gen. Physiol., 35, 645.

Dupouy, G., Perrier, F. (1962) 5th Int. Con. E. Mic. Philadelphia, 1, A.2

Elford, W.J. (1931) J. Bact., 34, 505.

Elford, W.J. (1938) "Handbuch der Virusforchug" p.126.

Elford, W.J., Andrewes, C.H. (1932) Brit. J. Exptl. Path., 13, 446.

Epstein, H.T. (1953) Adv. Virus Res., 1, 1.

Fernandez-Moran, H. (1956) J. Biophys. Biochim. Cytol., 2 (supple) 29.

Fitz-James, P.C. (1960) J. Biophys. Biochim. Cytol., 8, 507.

Franklin, R.E., Klug, A., Holmes, K.C. (1957) C.I.B.A. Foundation Symposium "Nature of Viruses", p.39.

Fraser, D., Williams, R.C. (1953) J. Bact., 65, 167.

Garen, A.G., Kozloff, L.M. (1959) "The Viruses" p.203 (Burnet & Stanley)

Gettner, M.E., Hillier, J. (1950) J. Appl. Phys., 21, 68

Gibbons, I.R., Bradfield, J.R.G. (1956) Proc. 1st Eur.Reg.Con.ElMic. Stockholm, 121.

Glauert, A.M. (1961) "Techniques in Electron Microscopy" Ed. Kay Blackwell Scientific Publications, Oxford Press.

Glauert, A.M., Brieger, E.M., Allen, J.M. (1961) Exptl.Cell Res., 22, 73.

Glauert, A.M., Glauert, R.H. (1958) J. Biophys. Biochim. Cytol., 4, 191.

Glauert, A.M., Rogers, G.E., Glauert, R.H. (1956) Nature, 178, 803.

Hall, C.E. (1951) J. Appl. Phys., 22, 655.

Hall, C.E. (1955) J. Biophys. Biochim. Cytol. 1, 1.

Hall, C.E., Jackus, M.E., Schmidt, P.O. (1945) J. Appl. Phys., 16, 459.

Hall, C.E., MacLean, E.C., Tessman, I. (1959) J. Mol. Biol., 1, 192.

d'Herelle, F. (1917) C. R. Acad.Sci., 165, 373.

d'Herelle, F. (1926) "The Bacteriophage and its Behaviour" Williams & Wilkins, Baltimore.

Hercik, F. (1955) Biochim. et Biophys. Acta. 18, 1.

Herriott, R.M. (1951) J. Bact., 61, 752.

Hillier, J., Baker, R.F. (1946) J. Bact., 52, 411.

Hodgkin, D. (1949) Cold Spring Harbour Symposium of Quantitative Biology, 14, 65.

Horne, R.W. (1962) 5th Int. Con. E. Mic. Philadelphia, S.1.

Horne, R.W., Brenner, S. (1959) 4th Int. Con. E. Mic. Berlin, 2, 625.

Horne, R.W., Wildy, P. (1961) Virology, 15, 348.

Horne, R.W., Brenner, S., Waterson, A.P., Wildy, P. (1959) J.Mol.Biol. 1,

Hughes, D.E. (1962) J. Gen. Microbiol., 29, 39.

Huxley, H.E. (1956) Proc. E. Mic. Con. Stockholm, 260.

Huxley, H.E. (1957) J. Biophys. Biochem. Cytol., 3, 631.

Iwanowski, D. (1892) Bull. Acad. Imp. Sci., 3, 67.

Jackus, M.A. (1956) J. Biophys. Biochim. Cytol., 2 (Supple) 243.

Kaesberg, P. (1956) Science, 124, 626.

Kausche, G.A., Pfankuch, E., Ruska, H. (1939) Naturwiss., 27, 292.

Kay, D. (1961) "Techniques in Electron Microscopy" Blackwell Scientific Publications, Oxford Press.

Kay, D., Bradley, D.E. (1962) J. Gen. Microbiol., 27, 195.

Kellenberger, E. (1961) Adv. Virus Res., 8, 1.

Kellenberger, E. Arber, W. (1955) Z. Naturforsch., 10b, 698.

Kellenberger, E., Séchaud, J. (1957) Virology, 3, 256.

Kellenberger, E., Ryter, A., Séchaud, J. (1958) J. Biophys. Biochim.Cytol. 4, 671.

Kellenberger, E., Schwab, W., Ryter, A., (1956) Experimentia, 12, 421.

Kellenberger, E., Séchaud, J., Ryter, A. (1959) Virology, 8, 478.

Kendall, P.A., Barnard, E.A. (1963) J. Roy. Mic. Soc. 81, ser.3, 203.

Koch, G., Dreyer, W.J. (1958) Virology, 6, 291.

Kozloff, L.M., Henderson, K. (1955) Nature, 176, 1169.

Kozloff, L.M. Lute, M. (1959) J. Biol. Chem. 234, 539.

Latta, H., Hartmann, J.F. (1950) J. Exptl. Biol., 74, 436.

Levinthal, C., Fisher, H. (1952) Biochim. et Biophys. Acta, 9, 419.

Lominski, I., Cameron, J., Wyllie, G. (1958) Nature, 181, 1477.

Luft, J.H. (1956) J. Biophys. Biochim. Cytol., 2, 799.

Luria, S.E., Anderson, T.F. (1942) Proc.Nat.Acad.Sci. U.S., 28, 127.

Luria, S.E., Delbrück, M., Anderson, T.F. (1943) J. Bact., 46, 57.

Luria, S.E., Williams, R.C. Backus, R.C. (1951) J. Bact., 61, 179.

Lwoff, A., Anderson, T.F., Jacobs, F. (1959) Ann. Inst. Past., 97, 281.

Maaløe, O., Birch-Anderson, A. (1956) Bacterial Anatomy Symp. Soc. Gen. Microbiol., 6, 261.

Maaløe, O., Birch-Anderson, A., Sjöstrand, F.S. (1954) Biochim. et Biophys. Acta., 15, 12.

Mach, F. (1962) 5th Int. Con. E. Mic. Philadelphia, 2, S10.

Mahler, H.R., Fraser, D. (1961) Adv. Virus Res., 8, 63.

Marton, L. (1936) Physica, 3, 959.

Mercer, E.H. (1963) J. Roy. Soc. ser.3, 81, 179.

Müller, H.O. (1942) Kolloid Z. 99, 6.

Murray, J. (1957) Can. J. Microbiol., 3, 531.

Neurath, H., Saum, A.M. (1938) J. Biol. Chem., 126, 435.

Newman, S.B., Borysko, E., Swerdlow, M. (1949) Science, 110, 66.

Nixon, H.L., Gibbs, A.J. (1960) J. Mol. Biol., 2, 197.

Nixon, W.C. (1958) 4th Int. Con. E. Mic. Berlin, 1, 302.

Noda, H., Wyckoff, R.W.G. (1952) Biochim. Biophys. Acta., 8, 381.

Norris, J.R. (1961) J. Gen. Microbiol., 26, 167.

Norris, J.R., Watson, D.H.,(1960) J. Gen. Microbiol., 22, 744.

Northrop, J.H. (1938) J. Gen. Physiol., 21, 335.

O'Brien, H.C., McKinley, G.M. (1943) Science, 98, 455.

Palade, G.E. (1952) J. Exp. Med., 95, 285.

Pease, D.C., Baker, R.F. (1948) Proc. Soc. Exp. Biol., 67, 470.

Pfankuch, E., Kausche, G.A. (1940) Naturwiss., 28, 46.

Putnam, F.W. (1950) Science, 111, 481.

Robertson, J.D. (1957) J. Biophys. Biochim. Cytol., 3, 1043.

Robinow, C.F. (1953) J. Bact., 66, 300.

Rohdin, J. (1954) "Techniques in Electron Microscopy". Ed. Kay, Blackwell Scientific Publications, Oxford Press, 1961, p.193.

Ruska, H. (1940) Naturwiss. 28, 45.

Ruska, E. (1962) 5th Int. Cong. E. Mic. Philadelphia, A.1.

Ryter, A., Kellenberger, E. (1958) J. Ultrastructure. Res. 2, 200.

Sabatini, D.D., Bensch, K.G., Barnett, R.J., (1962) 5th Int. Con. E. Mic. Philadelphia L.3.

Salton, M.R. (1962) J. Gen. Microbiol., 29, 15.

Schiff, L.I. (1941) Phys. Rev., 60, 743.

Swift, H., Rasch, E. (1958) Sci. Instr. News. 3, 1.

Stanley, W.M. (1935) Science, 81, 644.

Stanley, W.M., Lauffler, M.A. (1948) "Viral and Rickettsial Infections of Man". Ed. Rivers.

Takeya, K., Koike, M., Nori, R., Toda, T. (1961) J. Biochim. et Biophys. Acta. 11, 441.

Thomson, J.J. (1897) Phil. Mag., 4, 293.

Thomson, G.P. (1928) Proc. Roy. Soc. A. 117, 600. 119, 615.

Tokuyuasu, K., Yamada, E. (1959) J. Biophys. Biochim. Cytol., 5, 129.

Tromans, W.J., Horne, R.W., (1961) Virology, 15, 1.

Twort, F.W. (1915) Lancet, 2, 1241.

Valentine, R.C. (1961) Adv. Virus Res., 8, 287.

Valentine, R.C., Hopper, P.K. (1957) Nature, 180, 928.

Van Dorsten, A.C., Oosterkamp, W.J., Le Poole, J.B. (1947) Philips Tech. Rev., 9, 105.

Van Iterson, W. (1961) J. Biophys. Biochim. Cytol., 9, 183.

Von Ardenne, M. (1938) Zeits. F. Physik., 111, 152.

Von Ardenne, M. (1939) Z. Wiss. Mikroskop., 56, 8.

Von Borries, B., Glaser, W., (1944) Kolloid Z., 106, 123.

Von Borries, B., Ruska, E., (1940) Z. Phys. 116, 249.

Watson, M. L. (1952) J. Bact., 63, 473.

Watson, M.L. (1958) J. Biophys. Biochim. Cytol., 4, 475.

Watson, M.L. (1958) J. Biophys. Biochim. Cytol., 4, 6, 727.

Watson, J.D., Crick, F.H.C. (1956) Nature, 177, 473.

Watson, J.D., Crick, F.H.C. (1957) C.I.B.A. Foundation on the Nature of Viruses, 5.

Wildy, P., Russell, W.C., Horne, R.W. (1960) Virology, 12, 204.

Williams, R.C. (1952a) Biochim. Biophys. Acta., 8, 227.

Williams, R.C. (1952b) Biochim. Biophys. Acta., 9, 237.

Williams, R.C. (1953) Cold Spring Harbour Symposium Quantitative Biology, 18, 185.

Williams, R.C. (1955) Adv. Virus Res., 2, 183.

Williams, R.C. (1957) C.I.B.A. Foundation Viruses, 19.

Williams, R.C., Backus, R.C. (1949) J.Am.Chem.Soc., 71, 4052.

Williams, R.C., Fraser, D. (1953) J. Bact., 66, 458.

Williams, R.C., Fraser, D., (1956) Virology, 2, 289.

Williams, R.C., Kass, S.J., Knight, C.A. (1960) Virology, 12, 48.

Williams, R.C., Smith, K.M. (1957) Nature, 179, 119.

Williams, R.C., Smith, K.M. (1958) Biochim. Biophys. Acta., 28, 464.

Williams, R.C., Wyckoff, R.W.G. (1945) J. Appl. Phys., 15, 712.

Wilska, A.P. (1960) Proc. Eur. Reg. Con. Delft. 1, 105.

Wyckoff, R.W.G. (1948) Biochim. et Biophys. Acta., 2, 246.

Wyckoff, R.W.G. (1949) "Electron Microscopy, Techniques & Applications". Interscience New York London.

Young, I.E., Fitz-James, P.C. (1962), J. Cell. Biol., 12, 115.

Zworykin, V.K., Hillier, J., Vance, A.W. (1941) J. Appl. Phys. 12, 738.

Zetterquist, H. (1956) "Techniques in Electron Microscopy". Ed. Kay. Blackwell Scientific Publications, Oxford Press. p. 193.