THE FINE STRUCTURE OF *CHONDROCOCCUS COLUMNARIS*

II. Structure and Formation of Rhapidosomes

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ABSTRACT

When cells of *C. columnaris* were broken open, treated with PTA, and examined in the electron microscope, tubular structures (rhapidosomes) were present in the preparations. The rhapidosomes are approximately 300 A in diameter. Their length varies from about 500 to about 15,000 A. An axial hole which runs the length of the rhapidosomes appears to widen and narrow with a regular periodicity. End-on views of short segments of rhapidosomes revealed the presence of subunits around their outside peripheries. The results of studies of lysed cells and of sectioned cells indicate that the rhapidosomes are produced during the disintegration of cells. It seems likely that the compound membranes of the mesosomes break down to give rise to the tubular structures. The mesosomal origin of rhapidosomes is postulated only for the rhapidosomes of *C. columnaris*, since the origin of rhapidosomes from other organisms was not investigated during this study. The rhapidosomes of *C. columnaris* may be unrelated to those of *S. grandis*, *S. myxococcoides*, *A. violaceum*, and *Sorangium* 495, since there was a difference in the details of fine structure between rhapidosomes from *C. columnaris* and those found in the other four organisms.

INTRODUCTION

There have recently been reports of tubular structures occurring within the cells of certain organisms exhibiting a gliding type of motility. Such structures are released from cells of *Saprospira grandis* upon lysis (Lewin, 1963). Lewin called the tubular structures rhapidosomes and described them as being hollow rods 2,000 × 300 A. Some of these rods had "wick-like" appendages inserted into the axial holes. The "wick-like" appendages extended out from the hollow rods a distance of about 3,000 A. Lewin stated that similar particles had been observed in a lysate of *Chondrococcus columnaris*. Correll and Lewin (1964) reported that the rhapidosomes of *S. grandis* contained RNA. They were unable to determine whether these curious structures were a part of the bacterial anatomy or were bacteriophages.

Pate and Ordal (1965) have described tubular structures from *C. columnaris*. These appeared to differ from the rhapidosomes of *S. grandis* in having no "wick," and in having a central cavity that widened and narrowed with a regular periodicity. Reichenbach (1965) described structures similar to the rhapidosomes of *S. grandis* occurring in the myxobacterium *Archangium violaceum*. Gräf (1965) has reported the presence of rhapidosomes in cells of *Sporocytophaga myxococcoides*. He interpreted these structures as being breakdown products of a fibrillar sheath which envelops each cell and which is responsible for motility. Lewin and Kiethe

(1965) studied the formation of rhapidosomes in *S. grandis* by the thin section technique and concluded that these structures are formed inside the cell in connection with the nuclear material. Their electron micrographs demonstrated the presence of rhapidosomes only in cells in which the cytoplasm was undergoing degeneration. The nature of rhapidosomes—whether they develop as a normal cell constituent or are the result of a viral infection—is still undetermined. Bradley (1965) has expressed the opinion that rhapidosomes are related to the killer phages and bacteriocins.

Previous work (Pate and Ordal, 1967 *a*) has shown that cells of *C. columnaris*, when harvested from an actively growing culture, fixed with glutaraldehyde and OsO_4, sectioned, and examined in the electron microscope, contain no structures that resemble the tubular rhapidosomes. Intracytoplasmic membranes (mesosomes) were common. The mesosomes were shown to be made up of a series of compound membranes enclosed by unit membranes. When cells were fixed with OsO_4 alone (Ryter-Kellenberger method) the intracytoplasmic membranes appeared as convoluted tubules separated from the cytoplasm by a unit membrane. The tubules seen in sections of these cells appeared to be midway between mesosomes and rhapidosomes; they were enclosed within the outer limiting membrane and did not have the rigid appearance of rhapidosomes.

The present study was undertaken to determine the origin of the rhapidosomes within the cells of *C. columnaris* and to compare the ultrastructure of rhapidosomes from *C. columnaris* with that of rhapidosomes from other bacteria. The origin of the rhapidosomes was studied by breaking cells open by means of various techniques and examining the broken cells in the electron microscope. Also, rhapidosomes were looked for in lysates of cells broken open at various times during growth in a liquid culture and in cells sectioned at various times during growth.

MATERIALS AND METHODS

The principal organism used in this study was *C. columnaris* strain 1-R43. Other organisms examined were *Saprospira grandis*, *Sporocytophaga myxococcoides*. *Archangium violaceum*, and *Sorangium* 495.

Phosphotungstic Acid (PTA) Preparations (Brenner and Horne, 1959)

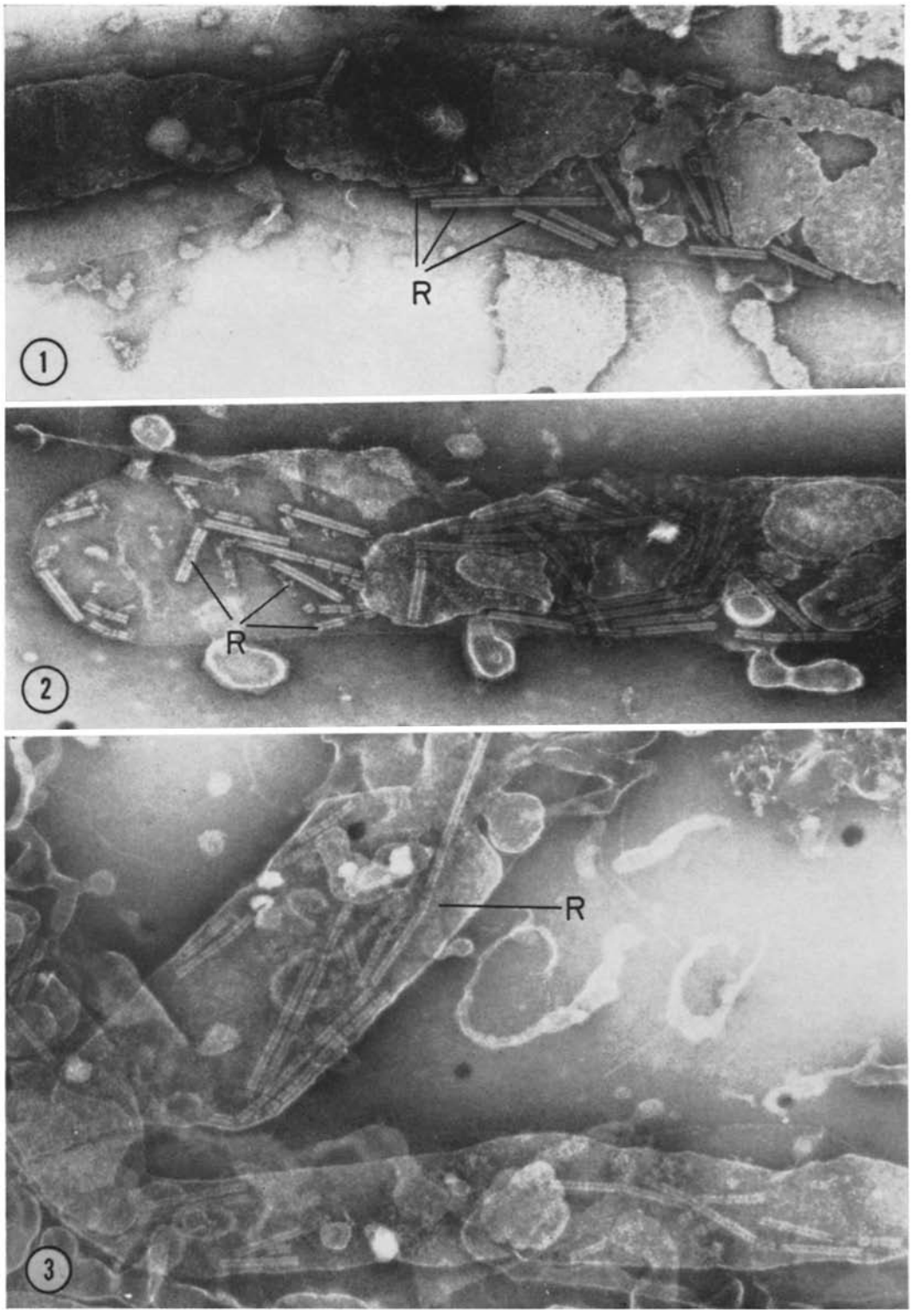
All of the specimens to be examined by the negative-contrast technique, whether whole cells or lysed cells, were prepared for electron microscopy in the following manner. The specimens were diluted until just barely turbid, placed on an electron microscope grid with a carbon supporting film, and the excess fluid was removed with filter paper. A solution of 2% phosphotungstic acid in distilled water, adjusted to a pH of 7.1 with NaOH, was then placed on the grid. The excess phosphotungstic acid was then drawn off with filter paper, and the grid was ready for examination. For the examination of lysed cells, one of the following methods was employed for breaking the cells open.

1. TREATMENT WITH SODIUM LAURYL SULFATE (SLS): The cells to be lysed were harvested from the culture medium by centrifugation, suspended in a small volume of 0.067 M phosphate buffer or distilled water, and a few drops of a solution of 25% SLS was added to the suspension. Lysis of the cells occurred within 3 min.

Abbreviations

CM, compound membrane of mesosome
DL, dense layer
F, fibrillar structures
ILM, inner limiting membrane
IUM, inner unit membrane of mesosome
M, mesosomes
N, nuclear material
OM, outer membrane
OLM, outer limiting membrane
PM, plasma membrane
R, ribosomes
Rh, rhapidosome
S, subunits
W, wick

FIGURES 1–3 PTA preparations of cells of *C. columnaris* broken open by grinding with dry ice. Lysis of the cells is rather poor, and many rhapidosomes (*Rh*) are seen associated with large fragments of cells. Notice that the rhapidosomes are not of uniform length and that they possess a periodic pattern along the central axis. This periodicity is especially well seen in Fig. 1. × 82,000.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1
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2. GRINDING WITH DRY ICE: Cells were harvested by centrifugation, and the pellets were frozen. The frozen pellets were placed in a mortar with crushed dry ice and ground with a pestle. As the dry ice evaporated, more was added. Usually 30–60 min of grinding was required to lyse even a small percentage of the cells.

3. FRENCH PRESSURE CELL[1]: Cells were harvested by centrifugation, resuspended in a small amount of 0.067 M phosphate buffer or distilled water, placed in the pressure cell, and forced through a small aperture under a pressure of 5,000–10,000 psi.

4. In some cases, the cells were treated with a fixing agent before or immediately after lysis. The details of fixation vary with the experiment and are described in the text.

Assay for Rhapidosomes

The examination of cells of various bacteria for the presence of rhapidosomes was carried out in the following manner. Cells were grown in a liquid medium, harvested by centrifugation, resuspended in 0.067 M phosphate buffer or distilled water, and lysed with sodium lauryl sulfate or in the French pressure cell. The lysate was examined in the electron microscope by the negative-contrast technique. The examination of cells for the presence of rhapidosomes at various stages during the growth of the cells was carried out as follows. 1-ml suspensions of actively growing cells were inoculated into nine flasks containing 100 ml each of cytophaga broth. The flasks were placed on a rotary shaker at 25°C. At various time periods after inoculation, the optical density of one of the cultures was determined, the cells were harvested, lysed with SLS, and examined in the electron microscope for the presence of rhapidosomes. In addition, cells harvested after various periods of growth were fixed and sectioned, and the sections were examined for rhapidosomes.

Thin Sections

Cells were fixed by the double fixation method employing the fixatives glutaraldehyde and osmium tetroxide. The method of use has been described (Pate and Ordal, 1967). Fixed cells were dehydrated in a graded series of alcohols and embedded in Epon 812 (Luft, 1961). Sections were cut on an LKB Ultratome with diamond knives, picked up on copper grids with carbon support films, and stained for 15 min with 2% uranyl acetate followed by a 10-min treatment with lead citrate (Reynolds, 1963). All of the electron micrographs were made with an RCA EMU 3G electron microscope.

[1] Manufactured by the Medical Instrument Shop, University of Washington.

RESULTS

Grinding with Dry Ice

Grinding with dry ice, even for a long time, did not break more than a small fraction of the cells, and many of the ruptured cells were only partially lysed. Examples of cells lysed by this method are shown in Figs. 1–3. Large pieces of broken cells are common in these preparations. Many of the cells appear to be packed with rhapidosomes. The rhapidosomes are not arranged in any organized structure, but are distributed throughout the cells in a chaotic fashion. This is probably not an indication of their organization in the intact cells, but is a result of lysis. Bits of membranes are visible clinging to the broken cells. A very thin layer, which retains the shape of the cells, is often detected lying beneath the rhapidosomes and broken membranes. The rhapidosomes are seldom seen beyond the edge of this thin layer.

French Pressure Cell

An example of cells broken open in the French pressure cell under 5,000 psi is shown in Fig. 4. Examination of the lysate in the phase microscope has shown this method of lysis to be nearly 100% efficient for cells of *C. columnaris*. Large fragments of cells lysed in this manner still retain the shape of intact cells. These fragments are very thin, have scalloped edges, and appear to lose their structural integrity by a stripping-away process. Rhapidosomes are rarely seen associated with the cell debris, but are free in the lysate.

Lysis with Sodium Lauryl Sulfate

Lysis of the cells by treatment with sodium lauryl sulfate (SLS) resulted in complete lysis of the cells. No large fragments which retained the shape of the cells remained, the membranes were broken down into small pieces, and rhapidosomes were present in large numbers.

It was noticed that cells treated with 5% SLS for 60 sec at room temperature and then placed in an ice bath before placing them on grids for examination in the electron microscope were not so completely broken apart as those left at room temperature (Figs. 5–7). The damaged cells shown in Figs. 5 and 6 still retain the shape of the intact cell although the peripheral membranes are obviously not intact. These cell remnants appear to be covered by a thin layer of material, patches of which are missing from various areas, exposing

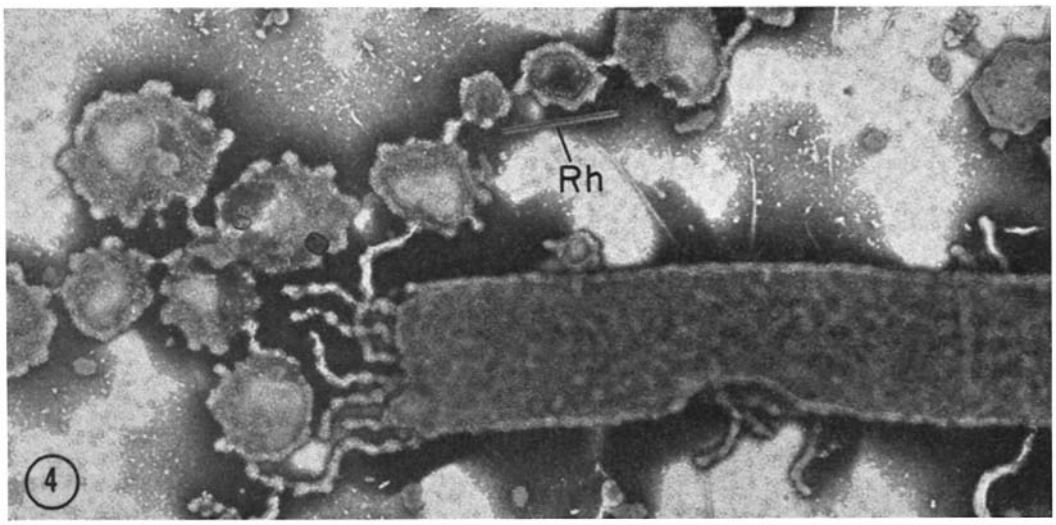


FIGURE 4 PTA preparations of cells of *C. columnaris* broken open in the French pressure cell. The cells appear to disintegrate by a stripping-away process. Rhapidosomes (*Rh*) can be seen free in the lysate. × 47,000.

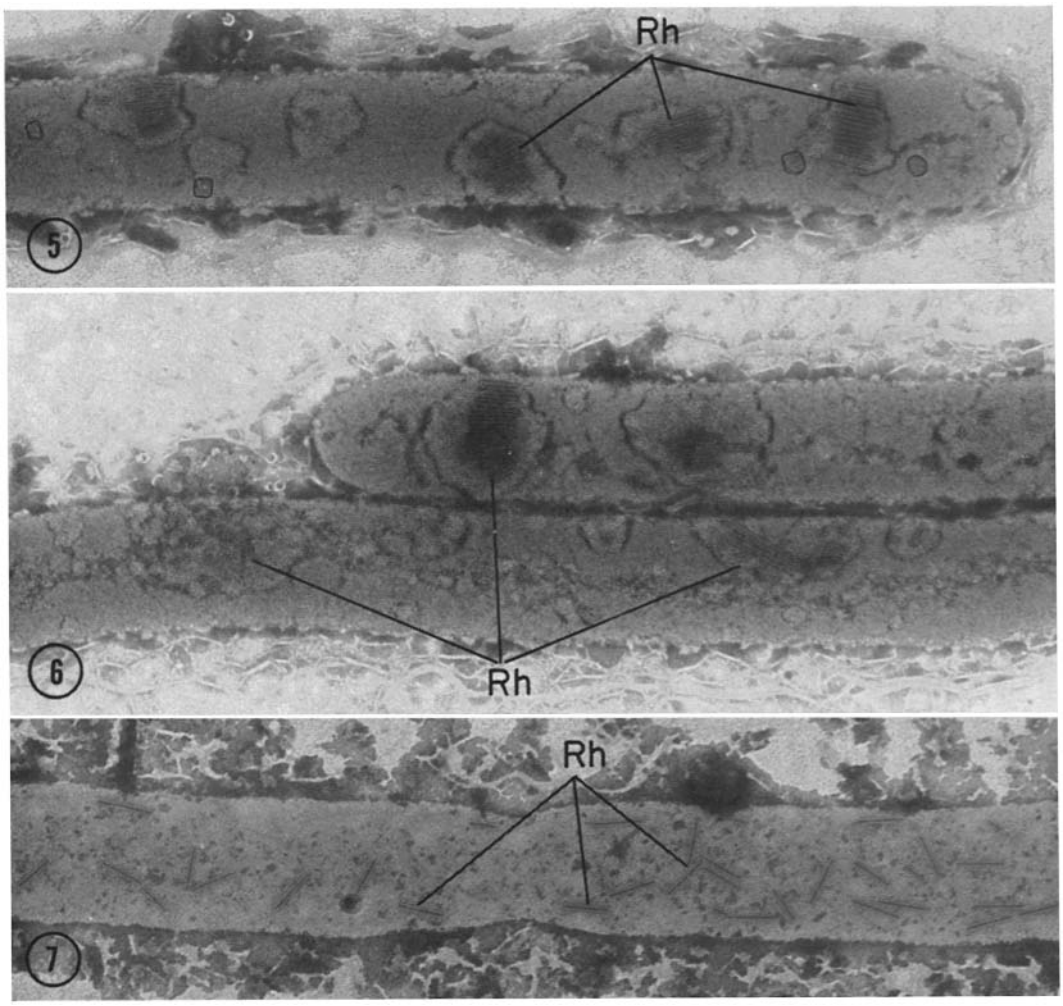
another similar layer beneath it. Often, the missing patches of the upper layer reveal the presence of rhapidosomes. Fig. 7 shows a structure which has retained the shape of the cell and which is extremely thin; its electron opacity is almost as low as that of the supporting film upon which it rests. Rhapidosomes are seen scattered along this structure.

Sodium Lauryl Sulfate Followed by Lysozyme

The structures remaining after 60-sec treatments with SLS were thought to represent the mucopeptide layer of the cells' surface. To test this idea, we placed intact cells on grids with parlodion and carbon support films, floated them on a solution of 5% SLS in distilled water for varying periods of time, washed them in distilled water to remove the SLS, and finally treated them with 2% PTA. Four of the grids were floated on the SLS solution for 15, 30, 60, and 120 sec, respectively. A fifth grid was floated on the SLS solution for 120 sec, washed in distilled water, and then floated on a solution of 0.16% lysozyme for 20 min. The cells treated with SLS for 15 sec are shown in Figs. 8 and 9. The peripheral membranes of these cells have been ruptured and can be seen folded back from the cells. The cells have become slightly electron-translucent, and the areas containing mesosomes can be seen. Rhapidosomes are not visible in any of the cells. Fig. 10 shows a cell after 30 sec in SLS. The pieces of broken membranes are no longer present, and some of the cells have become more electron-translucent. Outlines of rhapidosomes are just visible in the translucent cells. Figs. 11 and 12 show cells after treatment with SLS for 60 and 120 sec, respectively. The thin structures suspected of being the mucopeptide layers of intact cells are common in these preparations. Rhapidosomes are seen on their surfaces just as they were on the structures prepared by treating the cells with SLS followed by chilling. The thin structures were not found on the grids which had been floated on the lysozyme solution and are, therefore, considered to be the lysozyme-sensitive mucopeptide component of the cell.

Sodium Lauryl Sulfate Followed by Formaldehyde

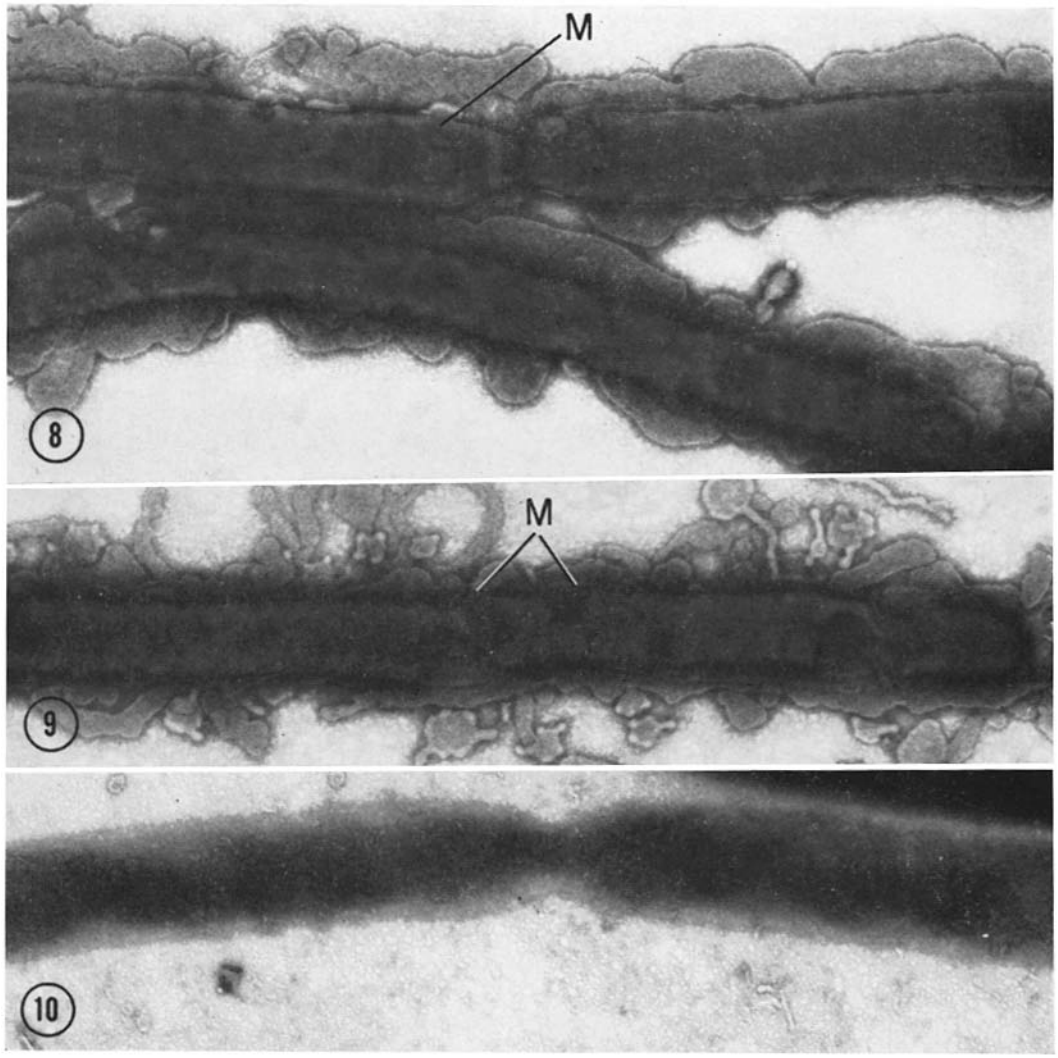
An attempt to determine the location of rhapidosomes before their release from the cells was made by treating the cells briefly with 5% SLS followed by fixation in 3.7% formaldehyde. The cells were grown and harvested as before, suspended in 0.067 M phosphate buffer with a pH of 7.4, and separated into three aliquots. SLS was added to each aliquot to a concentration



FIGURES 5–7 PTA preparations of cells of *C. columnaris* treated with SLS for 60 sec and then placed in an ice bath. Lysis of the cells is not complete. Rhapidosomes (*Rh*) are clustered in definite areas of the cells (Figs. 5 and 6). Fig. 7 shows a very thin layer which still retains the shape of the cell. × 47,000.

of 5%. Formaldehyde was added 15 sec after the addition of SLS to the first aliquot, after 30 sec to the second aliquot, and after 60 sec to the third aliquot. Negatively stained samples from each aliquot were examined in the electron microscope. After 15 sec of treatment with SLS (Fig. 13), the cells still retained their density to the electron beam, indicating no loss of cytoplasm had occurred. Damage to the outer membranes of the cells was apparent, and there were a few fibrils projecting out from the cells. Only a few rhapidosomes were seen; these were not associated with the relatively intact cells and had probably been released from cells which had lysed before fixation.

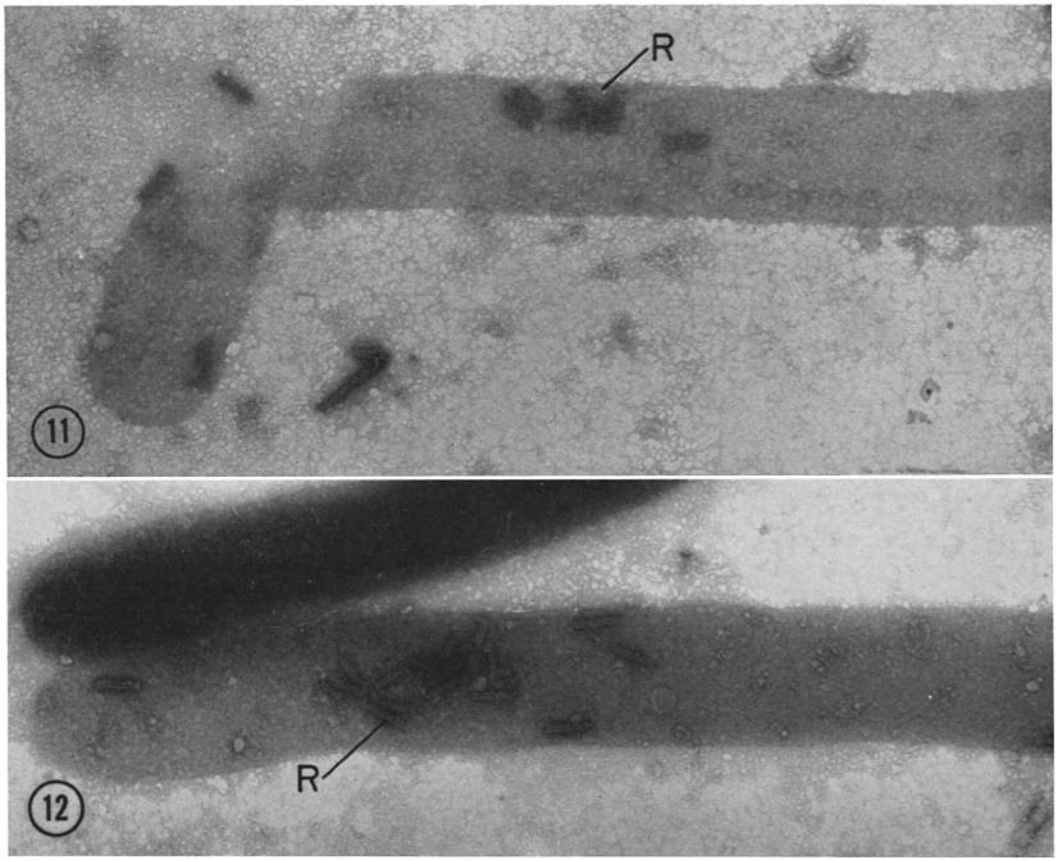
Most of the cells still retained their electron opacity after 30 sec in SLS. The number of rhapidosomes present at this stage of lysis was still small. At this time, fibrillar structures made their appearance (Figs. 14–16). Fig. 14 shows two of the fibrillar structures with fibrils lined up parallel to each other and apparently held together by some matrix. Strands of individual fibrils extend out from the edges of these struc-



FIGURES 8 and 9 PTA preparations of cells of *C. columnaris* treated with SLS for 15 sec. The peripheral membranes have been folded back from the cells, areas containing mesosomes (*M*) can be seen, and the damaged cells still retain their shape. Rhapidosomes are very rare in these preparations. × 47,000.

FIGURE 10 PTA preparation of cells of *C. columnaris* treated with SLS for 30 sec. The peripheral membranes are no longer visible, and the cells are losing their electron opacity. Rhapidosomes are still rare at this stage of lysis. × 47,000.

tures. A third structure is present which appears to be one of the fibrillar structures in the final stages of disintegration. The fibrillar structure shown in Fig. 16 is especially interesting. This structure has the appearance of a ball of yarn, the strands of one layer being wound in one direction with another layer wound in a different direction across the first layer. The diameter across this structure is about 0.7 μ, which is wider than the intact cells. The larger diameter of some of the fibrillar structures suggests that they are peeled off the surface of the cell in one piece, starting at one point and continuing completely around the cell back to the starting point, or that



FIGURES 11 and 12 PTA preparation of cells of *C. columnaris* treated with SLS for 60 sec (Fig. 11) and 120 sec (Fig. 12). At this stage of lysis, very thin structures which still retain the shape of the cells are present. Rhapidosomes (*Rh*) are present in large numbers and are often associated with the thin structures. × 47,000.

they come from the tips of the cells and include the end of the cell and part of the sides.

After 60 sec of treatment with SLS, no fibrillar structures were found, most of the cells had lost their density to the electron beam while still retaining the shape of the cell, and many rhapidosomes were associated with the damaged cells (Figs. 17 and 18).

In this experiment, the disappearance of the fibrillar structures coincides with the appearance of rhapidosomes. However, it appears that the fibrillar structures are released from the surface of the cells, while the rhapidosomes are contained within the cells in one form or another until cell lysis is complete.

The Ultrastructure of Rhapidosomes

Figs. 19–21 show rhapidosomes which have been freed from their association with the cells. End-on views of short segments of rhapidosomes reveal the presence of subunits around their outside peripheries (Fig. 19).

These subunits can sometimes be seen along the length of the rapidosomes (Fig. 20). A cavity which runs the length of the rod-shaped structures appears to widen and narrow with a regular periodicity. The light areas in the centers of the rhapidosomes suggest the presence of some material within the cavity which is not permeable to PTA. However, these light areas may not actually be within the cavity. The outside diameter

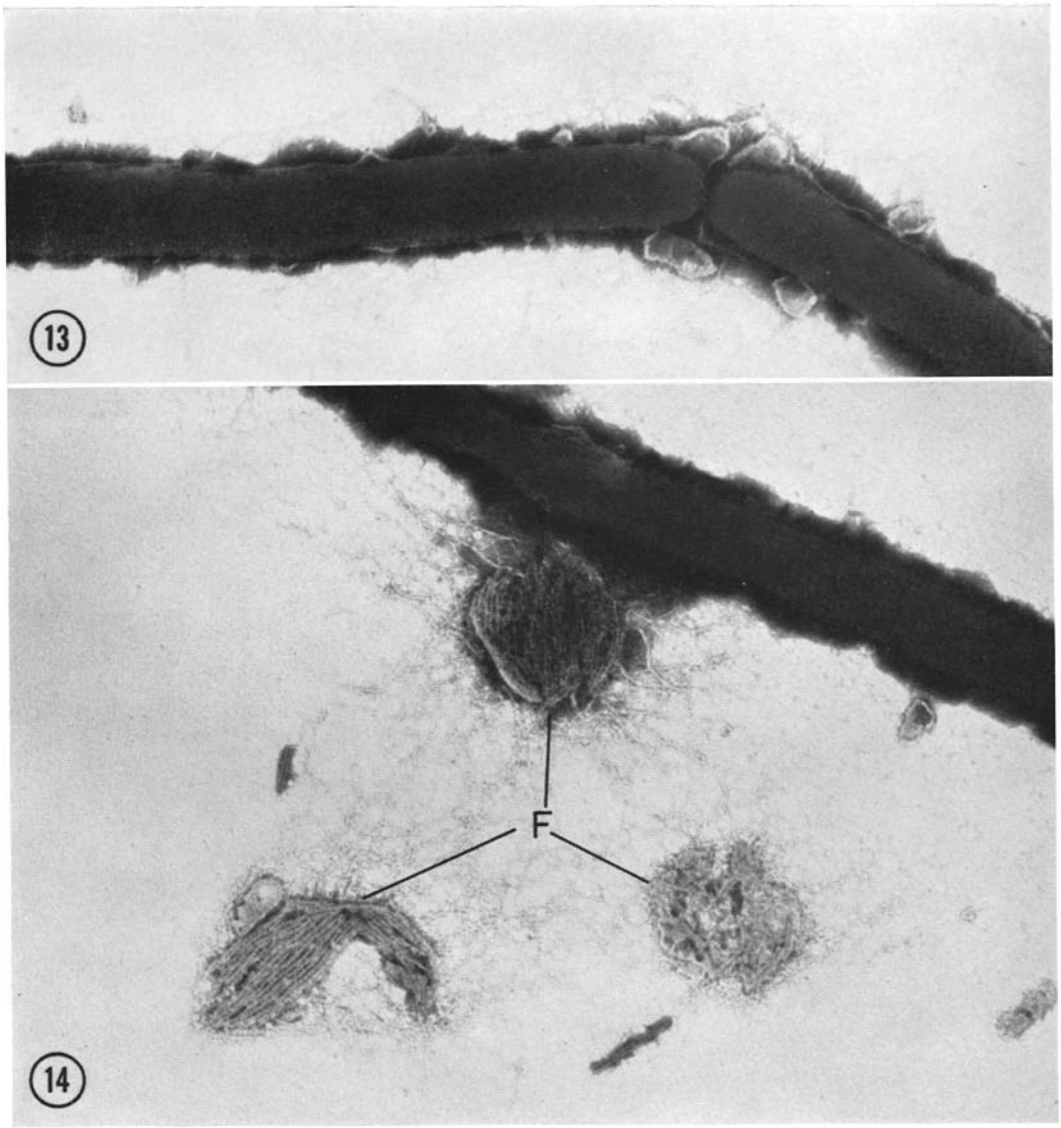
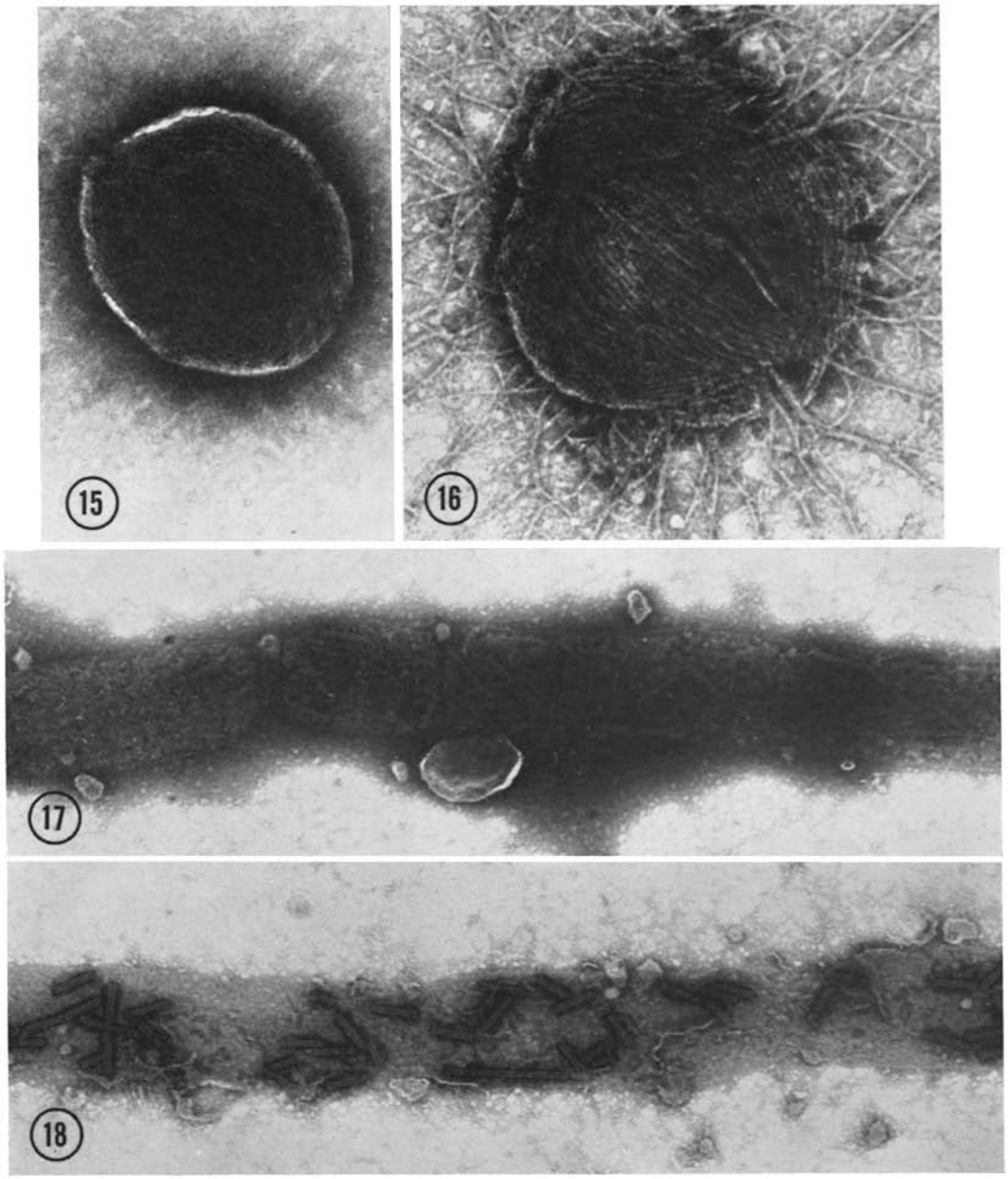


FIGURE 13 PTA preparation of cells of *C. columnaris* treated with SLS for 15 sec followed by fixation in formaldehyde. × 47,000.

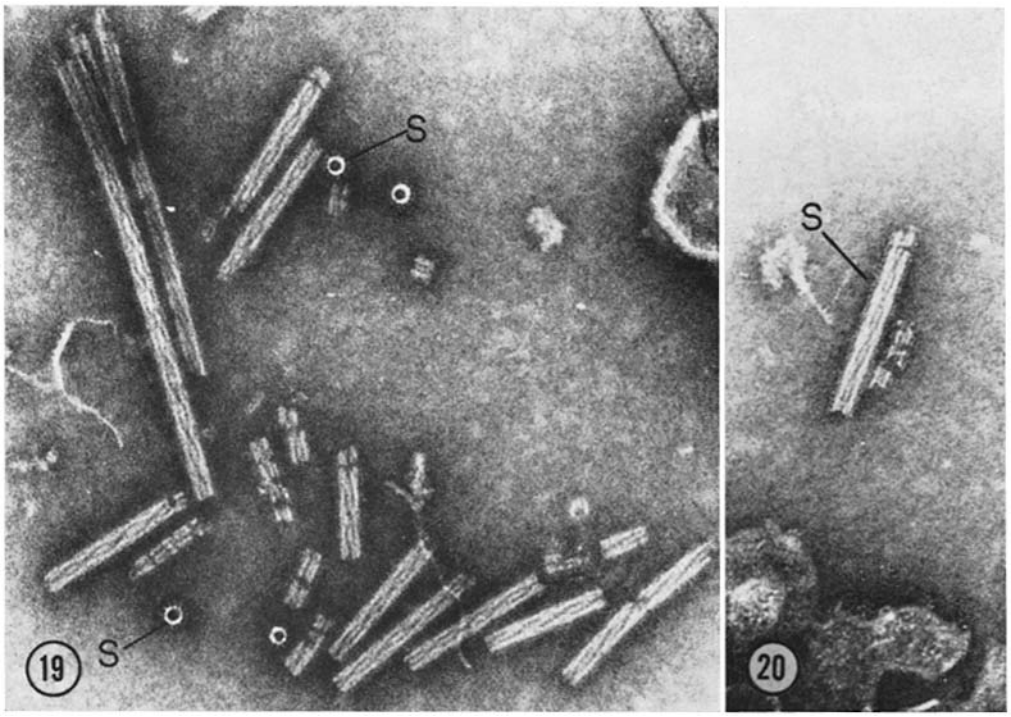
FIGURE 14 PTA preparations of cells of *C. columnaris* treated with SLS for 30 sec followed by fixation in formaldehyde. Fibrillar structures (*F*) were present in these preparations. Three of the fibrillar structures are shown in this micrograph. One of these structures appears to be in the final stages of disintegration. × 47 000.

of the rhapidosomes, including the subunits, is between 280 and 300 A. The length varies greatly from about 500 to about 15,000 A. The subunits which appear to cover the rhapidosomes are 45–50 A in length. The center-to-center distance of the subunits along the length of the rhapidosomes is about 60 A. The distance between subunits around the periphery of the rhapidosomes



FIGURES 15 and 16 PTA preparations of fibrillar structures released from cells of *C. columnaris* treated with SLS for 30 sec followed by fixation in formaldehyde. × 70,600.

FIGURES 17 and 18 PTA preparations of cells of *C. columnaris* treated with SLS for 60 sec followed by fixation in formaldehyde. At this stage of lysis no fibrillar structures can be seen, the cells are quite transparent, and many rhapidosomes are associated with the damaged cells. × 47,000.



FIGURES 19–21 PTA preparations of rhapidosomes of *C. columnaris*. Subunits (*S*) can be seen around the periphery of the rhapidosomes when viewed end-on. These subunits are shown along the length of a rhapidosome in Fig. 20. The axial hole of the rhapidosomes appears to widen and narrow with a regular periodicity. × 147 000.

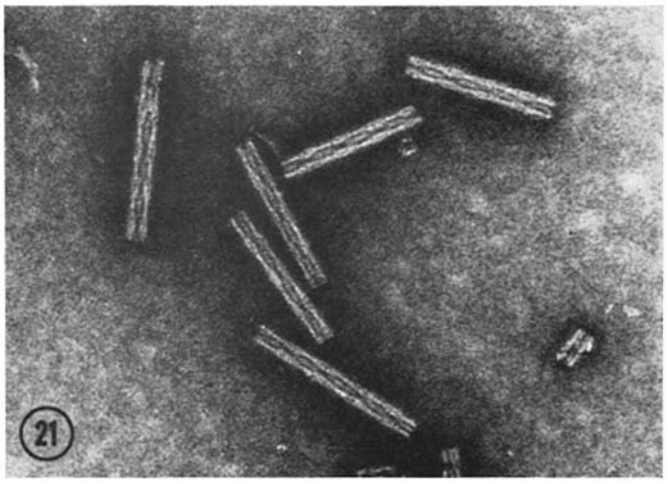


FIGURE 21 See legend under Figs. 19 and 20.

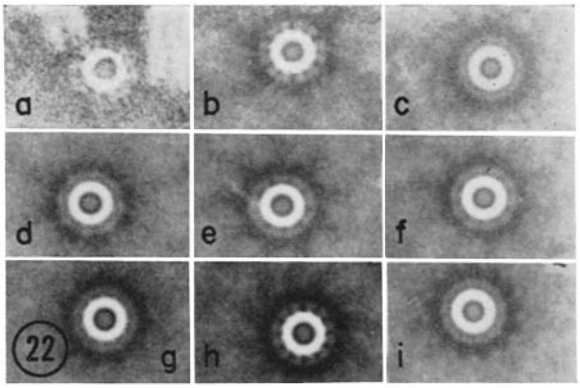


FIGURE 22 An end-on view of a rhapidosome embedded in PTA, printed by the Markham rotation technique. Where n equals the number of equal angular divisions through which each rhapidosome was rotated, in 22 *a*, $n = 0$; 22 *b*, $n = 6$; 22 *c*, $n = 7$; 22 *d*, $n = 8$; 22 *e*, $n = 9$; 22 *f*, $n = 10$; 22 *g*, $n = 11$; 22 *h*, $n = 12$; 22 *i*, $n = 13$. Reinforcement of the subunits occurs at $n = 6$ and $n = 12$, which would be expected if the number of subunits around the rhapidosome were 12. × 300,000.

is about 75 A. The diameter of the cavity varies between 30 and 100 A. The periodicity of the cavity as it widens and narrows is approximately 400 A. There is some evidence for a helical pattern in the rhapidosomes.

The rotation technique described by Markham, Frey, and Hills (1963) and Agrawal et al. (1965) was used so that we could determine the number of subunits around the outside of the rhapidosomes. The photographic paper to be exposed was attached to a circular card which had been marked off into equal angular divisions. The center of an end-on rhapidosome was projected onto the center of the card. If n equals the number of divisions on the card, an exposure time of $1/n$ times the required exposure time was given, the card rotated by one division, and another exposure made. This was continued until n exposures had been made. The paper was then removed from the card and developed. This procedure was repeated with a series of circular cards, each one marked off into different numbers of divisions ($n = 6$, 7, 8, 9, 10, 11, 12, and 13).

When n is equal to the number of subunits around the periphery, or an integral fraction of that number, the subunits should be reinforced by this technique. The results are shown in Fig. 22. It appears that the number of subunits around the rhapidosomes is 12. This number is what would be expected from the diameter of the rhapidosomes and the center-to-center distance between subunits around the periphery. A circle with a diameter of 280 A has a circumference of 879.2 A. A circle made up of 12 subunits with a center-to-center distance of 75 A between them would have a circumference of 900 A.

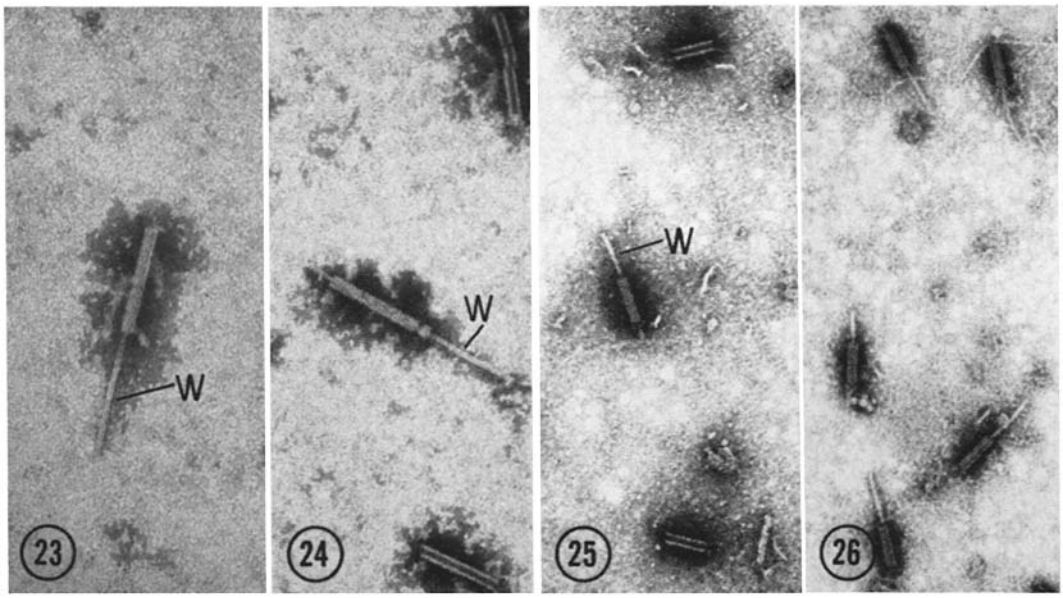
TABLE I

Presence of Rhapidosomes during the Growth of Cells of C. columnaris

Hours of growth	Klett units	Rhapidosomes present
8	25	+
9.5	59	+
11	116	+
12.5	155	+
14	182	+
15.5	178	+
17	177	+
19.5	166	+
36.5	148	+

Assay for Rhapidosomes

THE OCCURRENCE OF RHAPIDOSOMES DURING THE GROWTH OF C. COLUMNARIS: In order to determine whether the rhapidosomes (or precursors to rhapidosomes) are produced at a particular time during growth of the cells or are present throughout the growth of a culture, we inoculated actively growing cells into nine culture flasks containing 100 ml of cytophaga broth per flask, grew them for various periods of time, harvested them by centrifugation, lysed them, and then examined them in the electron



FIGURES 23 and 24 PTA preparations of rhapidosomes from *S. grandis*. These rhapidosomes differ from those of *C. columnaris* in having a "wick" (*W*) inserted in the axial hole. The wicks appear hollow as shown by the dark lines down their centers. Empty rhapidosomes without wicks were present in these preparations, as were wicks isolated from the rhapidosomes. × 70,600.

FIGURES 25 and 26 PTA preparation of rhapidosomes from *Sporocytophaga myxococcoides*. These rhapidosomes are also of the *S. grandis* type. × 70,600.

microscope for rhapidosomes. Before we harvested each flask, the optical density of the culture fluid was determined with a Klett-Summerson colorimeter with a green filter (540 mμ). The results are shown in Table I.

The ratio of cell debris to rhapidosomes appeared constant in all the samples; there was no noticeable increase in number of rhapidosomes at any time during the growth curve. After 14 hr of growth, the optical density of the culture decreased, indicating that autolysis of the cells was occurring at this time.

OCCURRENCE OF RHAPIDOSOMES IN VARIOUS GLIDING BACTERIA: 40 different strains of *C. columnaris* were examined, and every strain possessed rhapidosomes of the type found in strain 1-R43. These 40 strains were stock cultures that had been isolated from infected fish over a period of years. Other bacteria with this same type of rhapidosome were *M. xanthus* (FB), and *Chondrococcus coralloides* (M18). No rhapidosomes of any kind were found in cells of nine different *Cytophaga* species. Rhapidosomes of the type described by Correll and Lewin for *S. grandis* were found in lysates of *S. grandis* (Figs. 23 and 24), *Sporocytophaga myxococcoides*, and *Sorangium* #495 (Figs. 25 and 26). No rhapidosomes of either kind were seen in lysates of *Beggiatoa* and *Leucothrix*.

SECTIONED CELLS: Sections made from cells harvested after 16 hr of growth are shown in Figs. 27–30. These cells differ from those fixed after 12 hr of growth (Pate and Ordal, 1967*a*) in one important respect. Many of the mesosomes contain structures which appear to be tubular. The mesosomes in these micrographs are beginning to resemble those in cells fixed by the Ryter-Kellenberger method, since they consist of convoluted tubules enclosed within a unit membrane (Pate and Ordal, 1967*a*).

When sections of cells harvested after 20 hr of growth were examined, it was seen that some cells were intact, some were in an advanced state of lysis, and some were completely lysed. Tubular elements were abundant in the cells undergoing lysis. Cross-sections of the tubular

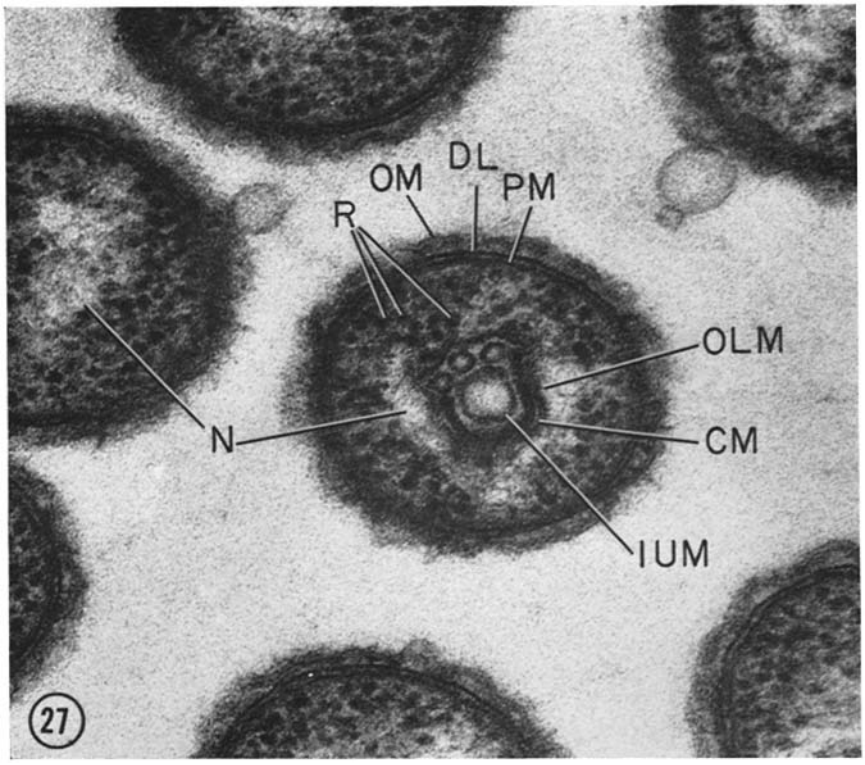


FIGURE 27 Section through cell of *C. columnaris*. Glutaraldehyde and OsO_4 fixation, 16-hr culture. The compound membrane of the mesosome appears to be breaking down to form tubular structures. × 126,000.

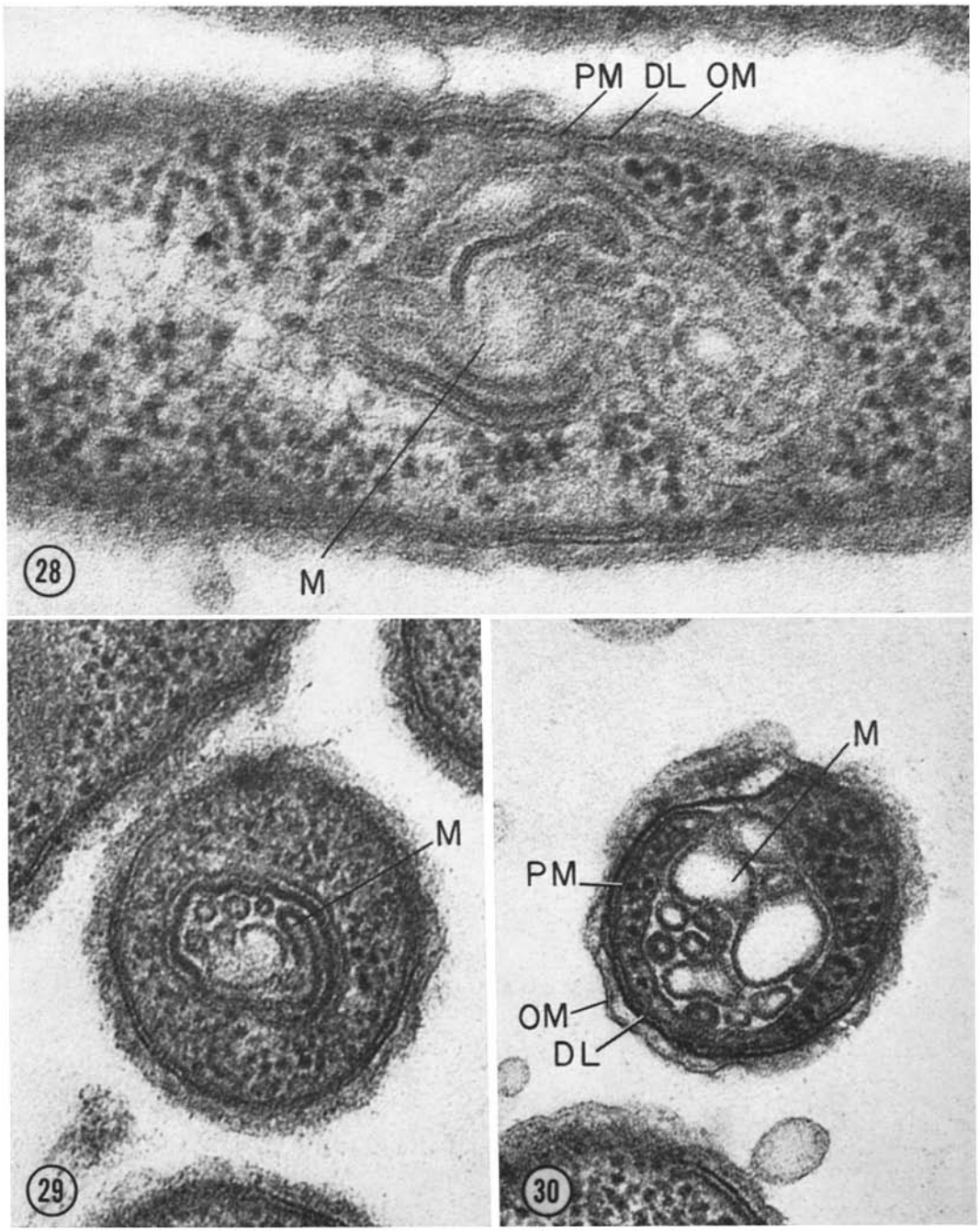
structures are shown in Figs. 31–34. In cross-section, these structures are doughnut-shaped and have a diameter of 280–300 A; the diameter of the hole varies from being just visible to slightly more than 100 A. The walls of the tubules shown in Figs. 32 and 33 have the appearance of unit membranes. In Fig. 31, a cell is shown in which 25 of the tubular structures are clustered. The tubules are located within the plasma membrane of the cell. This is shown especially well in Fig. 34, in which the plasma membrane is clearly intact, and six of the tubular structures are inside the cell.

Longitudinal sections of the tubules are shown in Figs. 35–39. Unlike the tubules seen after Ryter-Kellenberger fixation, these are straight and have a rigid appearance. They look, in fact, like the rhapidosomes seen in PTA preparations of cell lysates, and must surely be the same structures. Notice that the tubules do not show a helical pattern; the walls continue straight through from one end to the other (Figs. 37–39). Figs. 38 and 39 show longitudinal sections of tubules inside cells which still retain some cellular organization. From these micrographs, it appears that the tubules are not dispersed in the cytoplasm, but are organized in clusters in definite areas within the cells. The above observations indicate a developmental relationship between the membranes of the mesosomes and the rhapidosomes. This possibility will be considered further in the Discussion.

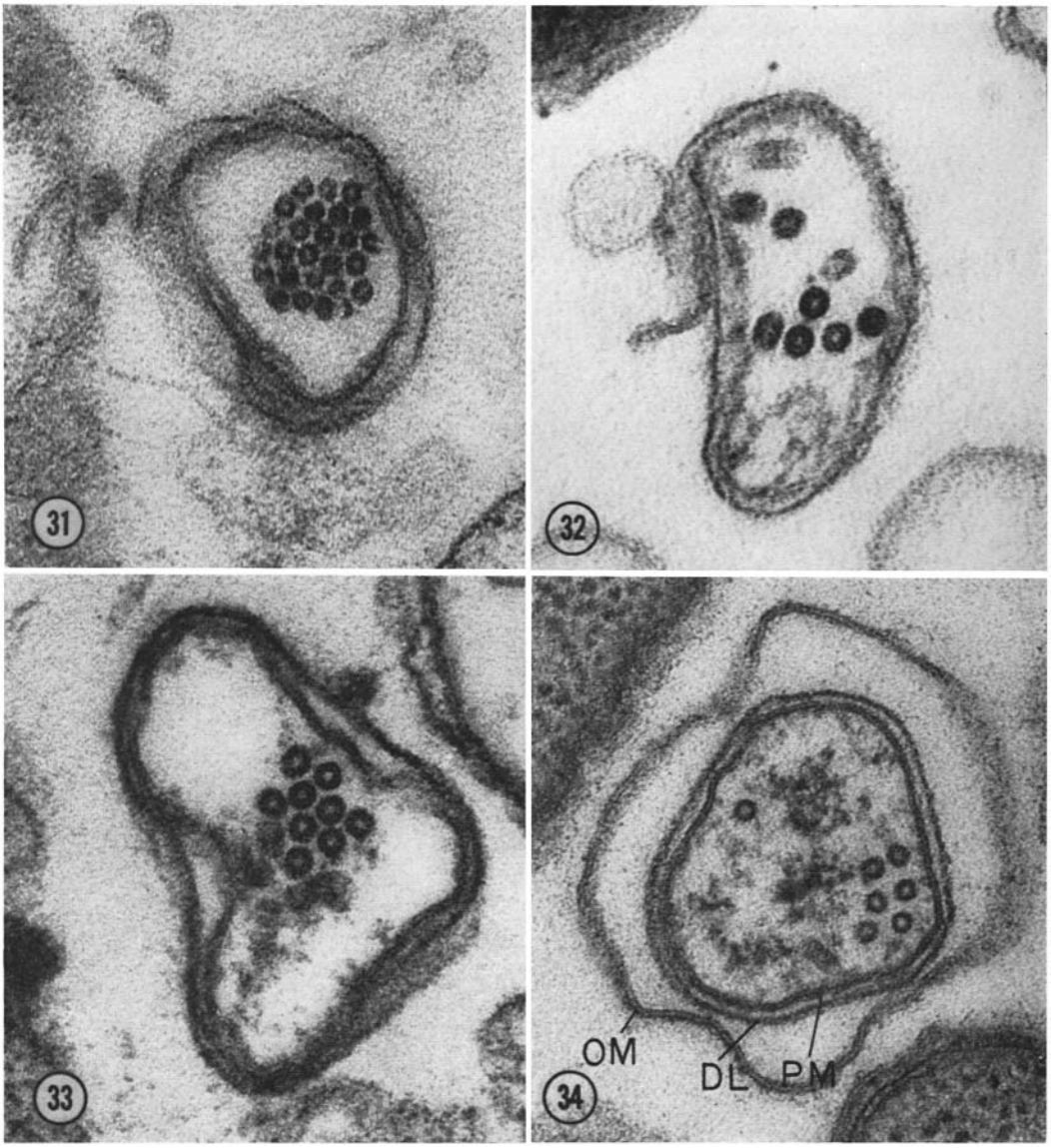
DISCUSSION

Structural Organization of the Cells

The observations on lysed and partially lysed cells of *C. columnaris* permit some statements to be made concerning the architecture of the intact cell. The cells contain a lysozyme-sensitive component which lies interior to a surface membrane or membrane-like structure which is destroyed by treatment with sodium lauryl

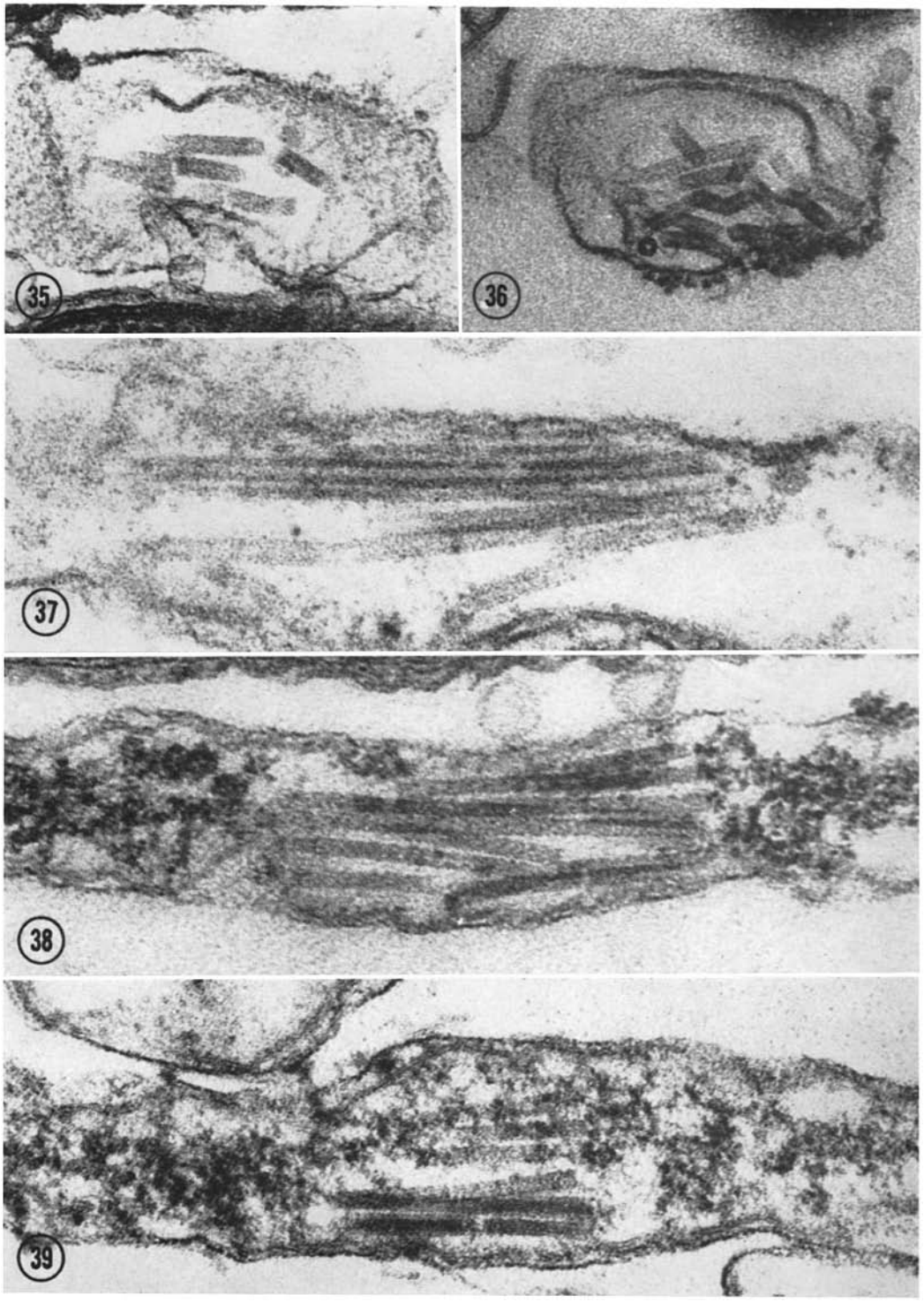


FIGURES 28–30 Sections through cells of *C. columnaris*. Glutaraldehyde and O_3O_4 fixation, 16-hr culture. In these sections, the compound membranes of the mesosome appear to be breaking down to form tubular structures. In Fig. 28, the outer limiting membrane of the mesosome can be seen to be continuous with the plasma membrane. Fig. 28, × 172,000; Figs. 29 and 30, × 126,000.



FIGURES 31–34 Sections through cells of *C. columnaris*. Glutaraldehyde and OsO_4 fixation, 20-hr culture. The cells are in an advanced state of degeneration. Tubular structures can be seen in cross-section in the center of the cells. The walls of the tubules in Fig. 33 appear as unit membranes. In Fig. 34, the peripheral membranes of the cell have separated and can be distinguished clearly. Figs. 31, 32, and 34, × 126,000; Fig. 33, × 172 000.

sulfate. The lysozyme-sensitive component is considered to be the mucopeptide layer, is extremely thin, and appears to be quite fragile when the membranes have been removed by SLS treatment. If the dissolution of membranes is halted by lowering the temperature, by washing the SLS away, or by treatment with a fixative, the mucopeptide layer retains the shape of the cell. But if the dissolution of membranes by SLS is allowed to proceed to completion, the muco-



FIGURES 35–39 Sections through cells of *C. columnaris*. Glutaraldehyde and OsO_4 fixation, 20-hr culture. These micrographs show longitudinal sections through tubules in disintegrating cells. The walls of the tubules appear to continue from one end to the other; there is no indication of a spiraling pattern. Figs. 35, 36, 38, and 39, × 126,000; Fig. 37, × 172,000.

peptide layer also falls apart. There is evidence that the mucopeptide layer is quite porous. After the membranes had been removed from the cells adhering to the grids by treatment with SLS, the cells gradually lost their density to the electron beam, suggesting that the cytoplasm was leaking out, even though the mucopeptide layer was still intact. Eventually, only rhapidosomes remained with the thin mucopeptide layer. It may be that the rhapidosomes were too large to pass through the pores of the mucopeptide sheath. Almost all of the rhapidosomes were dissociated from larger remnants of cells when a more violent method of lysis was employed, such as the French pressure cell. More gentle methods of lysis, e.g. limited treatment with SLS or grinding with dry ice, left many rhapidosomes still associated with fragments of cells. The reason for the difference in release of rhapidosomes may be that the gentle methods of lysis left the rhapidosomes trapped within the mucopeptide sheath, while the more violent methods opened up the sheath and allowed their escape. One of the outer layers of the cell contains a network of fibrils. This fibrillar structure quickly disintegrates upon lysis of the cell unless it has been stabilized by fixation. The organization of these fibrils in intact cells is considered in another paper (Pate and Ordal, 1967 *b*).

Structure and Formation of Rhapidosomes

It has been demonstrated that some structural characteristic of rhapidosomes produces a 400-A periodicity along the length of these particles when embedded in PTA and examined in the electron microscope. In some of the micrographs, there was evidence for a helical pattern in the walls of rhapidosomes treated with PTA (Figs. 20 and 21). Longitudinal sections indicated that the walls of rhapidosomes continue straight from one end to the other, rather than spiraling around. The subunits around the peripheries of the rhapidosomes embedded in PTA do not appear to be the ends of fibrils which run the length of the rhapidosomes, since the same subunits were seen along the length of some rhapidosomes. Therefore, the possibility that the surfaces of rhapidosomes are made up of fibrils coiled around from one end to the other and somehow giving rise to the 400-A periodicity seems unlikely. Cross-sections of the rhapidosomes indicated that the diameters of the cavities were not constant, so that there may actually be a regular variation in the size of the cavity with no corresponding variation of the outside surface. At the moment, there is no information as to how the variation in diameter is brought about.

It has been shown that rhapidosomes are only released from the cells of *C. columnaris* when the cells have been completely lysed. From the studies on controlled lysis of the cells and from sections of cells containing rhapidosomes, it appears that rhapidosomes of *C. columnaris* are produced inside the cells, rather than from a surface structure as suggested by Gräf for rhapidosomes of *Saprospira* and *Sporocytophaga* (Gräf, 1965). That rhapidosomes were present in every strain of *C. columnaris* examined (40 strains) suggests that they are present as a normal component or are produced from a normal component of the cells, rather than being infectious viruses or particles related to the bacteriocins as Bradley suggested for the rhapidosomes of *S. grandis* (Bradley, 1965).

In cells sectioned after fixation by the Ryter-Kellenberger method, tubular structures with the same diameters as rhapidosomes were seen enclosed by a unit membrane (Pate and Ordal, 1967*a*). In cells sectioned after fixation by glutaraldehyde followed by osmium tetroxide, the structures seen varied with the age of the cultures from which the cells were harvested. Cells from young cultures contained no tubular structures, but mesosomes composed of systems of compound membranes surrounded by a unit membrane were seen. Sections of cells from older cultures showed cells in various stages of disintegration. Rhapidosomes were seen which were clearly inside the plasma membranes of the disintegrating cells. End-on views of sectioned rhapidosomes showed a unit membrane structure. In one micrograph, a cross-section of a cell was seen which contained 25 rhapidosomes, closely packed together. Longitudinal sections were seen which contained many rhapidosomes localized in one small area of the cell. From these sections, it appears that rhapidosomes are produced in fairly large numbers in defined areas of the cells.

The time of appearance of rhapidosomes in the cells was investigated by electron microscopic examination of lysates of cells harvested at different times during the growth curve. The results of this investigation indicated that rhapidosomes were present at all times during growth of the cells. But the results from electron microscopy of

cells sectioned during different periods of the growth curve clearly indicated that rhapidosomes were present only late in the growth curve, when cell lysis was in progress.

All of these observations concerning the location and time of appearance of rhapidosomes in the cells of *C. columnaris* lead to the conclusion that they are produced by the disintegration of some structural component of the cell and strongly suggest that that structural component is the mesosome.

The question now arises, how are rhapidosomes produced from the membranes of mesosomes? Is it possible to form the tubular rhapidosomes from the compound membranes of mesosomes in one rapid transition? If certain assumptions are made concerning the breakdown of membranes, such a transition could be explained. When the compound membranes of the mesosomes disintegrate, they may do so along certain lines, determined by the molecular construction of the membranes. The peripheral membranes of the cells, when ruptured in the French pressure cell, were seen to come apart by a stripping-away process. Boatman (1964) also reported that Versene and lysozyme appeared to remove the wall of *Rhodospirillum rubrum* by a stripping process. This kind of breakdown of membranes would seem to indicate that their structure had preferred lines of dissociation.

Let us assume that such preferred lines of dissociation do exist in the compound membranes of the mesosomes, and assume, furthermore, that these lines run parallel to the front of the ingrowing compound membrane. As seen in the diagram (Fig. 40), this arrangement would permit formation of tubules by a separation of the membranes along their preferred lines of dissociation followed by an immediate fusion of the resulting free edges of the membranes. As each loop is removed from the compound membrane, a new loop is produced by the fusion of the free edges. The formation of a new loop requires that the forces acting to separate the surfaces of the compound membranes are greater than the forces holding them together. It is likely that the small radius of curvature where fusion of the free edges had occurred would create a tension which would force the surfaces of the membranes apart. The immediate fusion of free edges of unit membranes after bursting of closed spheres has been postulated by Mercer (1962) to account for the absence of planar structures. This fusion of free edges is thought to be a characteristic of lipid membrane systems.

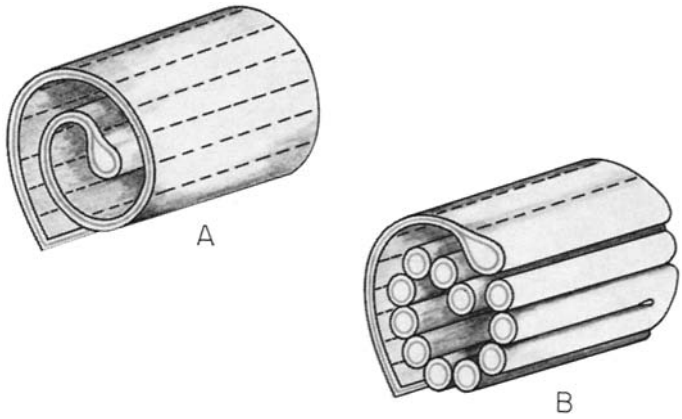


FIGURE 40 Formation of rhapidosomes from mesosomes of *C. columnaris*. *A*, diagram of a compound membrane of a mesosome. The broken lines indicate preferred lines of dissociation. *B*, the same membrane during disintegration. Tubular structures are being produced from the compound membrane.

Some of the electron micrographs of mesosomes sectioned after double fixation with glutaraldehyde and osmium tetroxide show structures which could be interpreted as the formation of rhapidosomes (Figs. 27, 29, and 30). The structure of the mesosome shown in Fig. 27 is particularly similar to the diagram showing formation of rhapidosomes. If rhapidosomes are produced from the membranes of the mesosomes, the occurrence of the subunits seen in end-on views of rhapidosomes

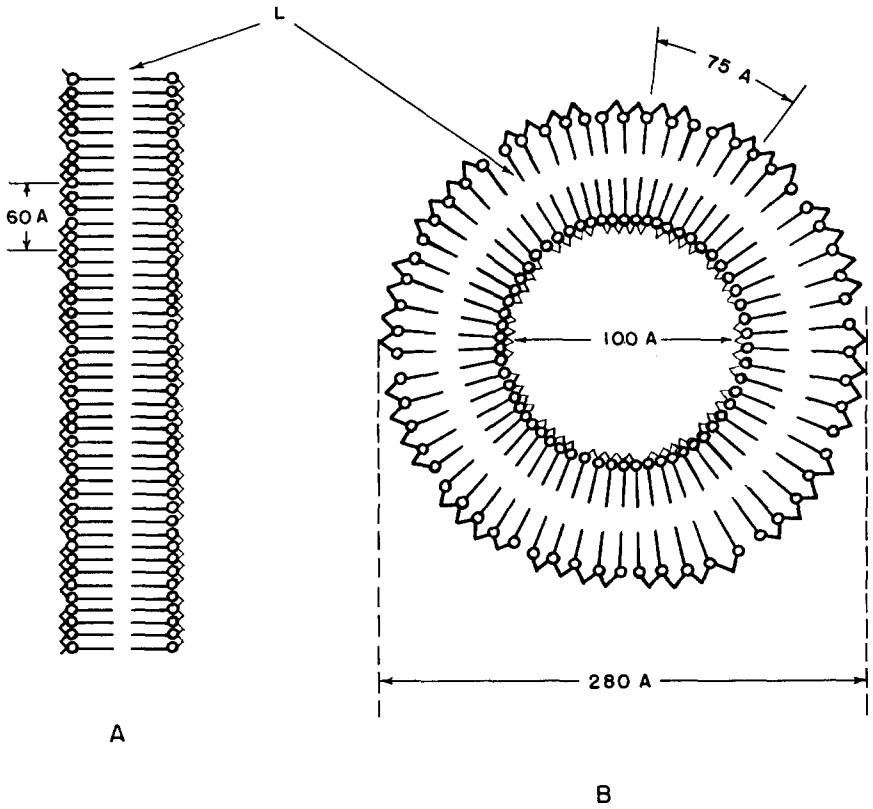


FIGURE 41 Diagram of a unit membrane; *A*, linear presentation showing a bimolecular layer of lipid molecules (*L*) lined up parallel with each other and bounded on both sides by nonlipid material; *B*, the same membrane arranged in a circle.

treated with PTA might be explained in the following way. One conception of the molecular construction of membranes is that of a bimolecular layer of phospholipids lined up parallel with each other with the hydrophobic nonpolar ends of the molecules facing inward and the polar ends of the molecules facing inward and the polar ends of the molecules facing the surfaces of the membrane. This lipid core is covered on both surfaces by a nonlipid material, probably not the same material on both sides. A diagram of a unit membrane is presented in Fig. 41 *A*. Figure 41 *B* is a representation of the same membrane arranged in a circle of 280 A diameter. In this diagram, the polar ends of the molecules on the inside of the circle are closely packed, while the polar ends of the molecules on the outside of the circle are forced apart by the curvature of the membrane. The figure is drawn so that each nonlipid molecule on the outer surface of the membrane covers five of the lipid molecules (*L*), holding them together as a unit. Separation of the lipid molecules by the bending of the membrane would occur principally in the areas between these units. If this sort of structure were treated with PTA and examined in the electron microscope, the spaces between the individual units would be filled with PTA and would be opaque to the electron beam, and the units composed of lipid molecules joined together by a molecule of nonlipid material would not be permeable to the PTA and would show up as light areas.

The thickness of the walls of the rhapidosomes, from the edge facing the cavity to the end of the subunits, as measured in micrographs of PTA preparations showing end-on views, is 80–90 A. This is in agreement with the measurements of the walls of rhapidosomes made from micrographs of sectioned cells. Thus, the subunits seen in PTA preparations must be contained within the wall of the rhapidosomes, rather than project-

ing from its surface. This observation supports the hypothesis that the rhapidosomes are made up of unit membranes and that the subunits are composed of several molecules of lipid joined together by a molecule of nonlipid material (probably protein) as shown in the diagram. There is some evidence that biological membranes are not constructed according to the Robertson unit membrane model (Robertson, 1964), but are made up of repeating units of lipoprotein (Green and Perdue, 1966). If this proves to be the case, the presence of subunits around the peripheries of the rhapidosomes could be explained by the same argument: the subunits are spread apart by the small radius of curvature and become easier to detect in the electron microscope.

This model for the formation of rhapidosomes of *C. columnaris* is presented as an attempt to explain the observations made on their fine structure, location within the cells, and time of appearance in the cells. It is not meant to represent final conclusions reached on the formation and structure of the rhapidosomes, but only a hypothesis based on the evidence presently available. It will be possible to examine this hypothesis more critically once the chemical composition of the rhapidosomes is known. Since we have recently been able to obtain highly purified preparations of rhapidosomes by density gradient centrifugation in CsCl (unpublished results), information regarding their chemical make-up should soon be available.

The rhapidosomes of *C. columnaris* differ in structure from those of *Saprospira*, *Sporocytophaga*, *Sorangium*, and *Archangium*. These two types of rhapidosomes may be completely unrelated. However, since the term "rhapidosome" has already been used to refer to the tubular structures from *C. columnaris* (Pate and Ordal, 1965), it is thought best to continue with this name, rather than suggest a new one, until more information is available for comparing the two types of rhapidosomes.

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