PARTICIPATION OF THE CYTOPLASMIC MEMBRANE IN THE GROWTH AND SPORE FORMATION OF BACILLI

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ABSTRACT

A polyester embedding technique was used to study the early stages of spore formation in members of the genus *Bacillus* in order to investigate further the origin and nature of the initial spore septum and the resulting forespore envelope. Whereas previously, with a methacrylate procedure, this layer had appeared to be continuous with the cell wall, this study reveals it as a double layer of cytoplasmic membrane. Perisporal, membranous organelles connected both to the developing forspore envelope and to the cytoplasmic membrane were encountered in the four species studied. Similar organelles were prominent during growth at the sites of transverse septa formation. These were connected to, or continuous with, the cytoplasmic membrane and often adherent to the chromatin bodies of the dividing bacilli.

INTRODUCTION

Recent studies of spore formation in members of the genus *Bacillus* have shown that, early in the process, a remarkable form of internal division occurs (28–29). The initial wall of the forespore was first detected as a septum, apparently continuous with the cell wall and invaginating centrally from the inner surface, thus forming between it and the end of the cell, a pocket, into which the future spore chromatin is enclosed. Although it was presumed that both surfaces of this spore septum might be covered by reflections of the cytoplasmic membrane, these could not be resolved in methacrylate embeddings. The process of sporulation has now been reinvestigated, using osmium fixation and polyester embedding procedure developed by Kellenberger and associates (9) since this procedure has proved useful in resolving cell layers (8). The result so obtained presented a more complex picture of the mechanism of forespore formation than that indicated earlier and thus prompted a comparative study of this division with that typical of vegetative growth.

MATERIALS AND METHODS

The organisms used in this study were *Bacillus cereus, Bacillus cereus* var. *alesti, Bacillus megaterium* strain KM, and an unclassified filamentous organism which forms both a spore and parasporal body. This latter organism, named *Bacillus medusa,* was originally isolated by Dr. C. F. Robinow from the dung of a Cambridge, England cow.

The medium described in reference 28 was used for obtaining fluid cultures of synchronously sporulating cells of *B. cereus* and *B. megaterium* and that described in reference 29 for *B. cereus* var. *alesti* and *B. medusa.*

The osmium tetroxide fixation and polyester (vestopal) embedding method described by Kellenberger *et al.* (9) was used without modification on

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most samples. In addition, potassium permanganate was applied with modifications for vestopal embedding to check the observations made on osmium-fixed material. Rapidly centrifuged cells were suspended in 0.6 per cent $KMnO_4$ in acetate-veronal buffer, pH 6.1 at 0°C. (10). After 3 hours on ice the samples were centrifuged in the cold, washed once in cold buffer, then worked into agar and treated as were the osmium-fixed specimens. Permanganate-fixed samples were also embedded directly without agar by dehydration first in cold acetone. However, preservation of membranous structures was as good, if not better, when agar blocks were used. Before dehydration all agar blocks were washed with either buffer or uranyl acetate (0.5 per cent in buffer). This latter treatment not only improved over-all contrast but resulted in better preservation of membrane and membranous inclusions.

Most sections were cut on a Porter-Blum microtone by glass knives with a cutting angle of about 40°; a few were cut with a diamond knife (Servall) fitted to a Reichert automatic microtome. Distilled water was used in the trough and sections were picked up on carbon-filmed grids. A few were subsequently stained with lead hydroxide, after the method of Watson (25). Preparations were examined in a Philips 100A electron microscope with an objective aperture of 40 μ and an accelerating voltage of 60 kv. The beam current varied from 18 to 22 μA. Photographs were made at an initial magnification of from 5,000 to 14,000 and enlarged 7 or 12 times during printing. A through-focus series was taken of each field and, where possible, prints made of the in and slightly under focused images. Because of the better contrast, the latter were usually selected for publication.

Sudan black B (0.3 per cent in ethylene glycol: ethanol:water, 40:40:20) staining was performed by observing wet mounts of living or osmium-fixed cells as they came in contact with the dye.

OBSERVATIONS

Development of the Forespore in Bacilli

The most striking advantage of the polyester embedding procedure has become particularly apparent in cells undergoing spore formation. Previously, in sections cut from methacrylate embeddings, the initial spore septum appeared as a thin, single layer continuous with the inner surface of the cell wall (28, 29). However, in sections cut from polyester embeddings the spore septum appears multi-layered. Furthermore, the origin of these layers becomes apparent when the formation of the spore septum is followed.

The general configurations and stages of development of the spore septum into the forespore wall or membrane were found to be very similar in the different bacilli included in this study. As before (28, 29), synchronously sporulating cultures greatly facilitated the interpretation of the development of this structure and its related appendages.

The earliest definite form of a developing spore septum is shown in Fig. 1. The thin dense line of the cytoplasmic or plasma membrane can be seen at this site (arrow, Fig. 1) reflected inwards to form the septum. That part of the axial filament of chromatin which is destined for the spore (see reference 28) projects into the developing pocket. In a high proportion of sections at these earliest stages, the perpendicular edge of the septum appeared to extend and to be adherent to the chromatin. In Fig. 2, it is seen as a simple loop. In slightly later stages, the connection between spore septum and chromatin is more complex and appears as a dense mass of membrane-like material still adherent to the chromatin (Figs. 3 to 5). This organelle or dense body[1] is invariably attached to the spore septum at, or near, its central closing rim and, although it may extend back into the cell (Fig. 4) it eventually appears to swing into the spore pocket to become enclosed with the spore chromatin (Figs. 3, 5, 7, and 14).

After the spore septum has completely traversed the cell it begins to bulge. Now, two or possibly three organelles or mesosomes make their appearance. In contrast to the occluded mesosome, these have no connection to the chromatin and are always found on the outside of the forespore region. Loops in the bulging septum, one of which is shown in Fig. 6, suggest a mode of formation. In an adjacent section of the cell shown in Fig. 6, this loop appears as a ring, separate from the complete septum. Henceforth, as the spore septum proliferates around the cell end to form the initial spore envelope and so complete the forespore, these membranous inclusions are found at, or near, the junction with the inner cell surface (Figs. 5 to 7). In many sections, they could be seen to have connections both with the plasma membrane lining the cell surface and the spore envelope.

The flexibility of the forespore membrane is ap-

[1] This is probably an unsuitable name. Certainly they are not always electron dense, being invisible in sections of resting spores (30). Since they appear to be attached to the surface membranes of the cell, one should adopt the suggestion of Robertson (15) for such membrane-attached cytoplasmic structures and use the term "mesosomes." Henceforth in this paper this term will be used.

parent from the indentations caused by those mesosomes which lie between the cell surface and developing forespore (Figs. 5 and 7). A section through two of these bodies on opposite sides of a well developed forespore produced an hourglass profile. Such indented spore outlines are also seen in methacrylate sections (reference 29, their Fig. 15) and presumably account for the peculiar profiles found in sporulating *B. subtilis* by Tokuyasu and Yamada (24, their Figs. 2 and 3). In neither of the micrographs accompanying these reports are such bodies clearly resolved. However, it has since been noted that by staining such methacrylate sections with phosphotungstic acid, ill defined dense areas can be seen at perisporal sites.

A diagrammatic interpretation of this sequence of events is given in Fig. 9, *A* to *H*. The changes in chromatin arrangement have already been described (28, 29); hence only the sporulating ends of the cell are shown. The steps *A* to *D* are somewhat tentative but do account for most of the arrangements seen in sections of the cells from the four cultures studied. The sequence *E* to *H* is drawn without chromatin or lipid inclusions. After the formation of the initial envelope of the forespore, as depicted in Fig. 9, *H*, the mesosomes persist. They maintain their attachment to the cell surface and are found about the circumference of the developing spore during the formation of the spore coat. In contrast to the more delicate forespore membrane, the heavier coverings formed in later stages are not indented by these structures (Fig. 8).

The Fine Structure of the Spore Septum: The spore septum, and thus the forespore envelope or membrane, when well resolved, could be seen in osmium-fixed cells to possess five definite zones (Fig. 13). On each side of a moderately dense central layer ~100 A wide were two dense lines ~30 A in width each of which was found to be continuous at the outer rim of the septum with a similar dense line in the plasma membrane (Fig. 1). These dense lines were separated from the cytoplasm of the forespore and cell respectively by another zone of low density, ~50 A wide. Thus, the over-all width of the forespore membrane, from spore cytoplasm to cell cytoplasm, was found to be from 280 to 300 A.

Two interpretations are possible regarding the origin of the layers of the spore septum as seen in cross-sections. Each depends partly on what one regards as the limits of the plasma membrane and partly on the degree of resolution in decisive micrographs. One interpretation is that the central, moderately dense zone represents a thin inward extension of the cell wall. Hence, on either side, both the single, dense, presumably osmophilic line (~30 A wide) and the less dense zone (~50 A wide) would represent covering plasma membrane. Another view is that the cell wall is not involved and that the spore septum is a simple doubling of the plasma membrane. To decide the correct interpretation, the structure of the unit plasma membrane was studied in well resolved micrographs of these various bacilli in a variety of states.

The cell, of which Fig. 13 is detail, was at the stage of development shown in Fig. 9, *G*. Here, well resolved parts of both the plasma membrane and the forespore membrane can be compared. If the assumption is made that the entire space between cell wall substance and cytoplasm is occupied by the membrane, then from this and similar micrographs of both sporulating and vegetative cells (Figs. 11, 24, 25) of *B. medusa*, *B. megaterium* and varieties of *B. cereus*, the unit plasma membrane appears to occupy a space of some 140A and to be composed of a wavy dense backbone covered on both sides by less dense regions. A doubling of such a three-zone plasma membrane could thus account for the five-zone profile of the spore septum; the two apposed light wings forming the wide central zone of this spore envelope. Such an interpretation has been diagrammatically summarized in Fig. 16. (Fig. 9 was also drawn with this in mind). After uranyl acetate treatment (9), the borders of the unit plasma membrane (those marked ? in Fig. 16) are more prominent, as is also the central zone of the forespore membrane.

Permanganate Fixation of Sporulating Bacilli: Further evidence of the nature of the spore septum was obtained when permanganate-fixed cells were examined. Here, as with osmium, the preservation of the membrane is better when the specimens are treated and uranyl acetate following fixation. In permanganate-fixed cells not treated with uranyl acetate the spore septum was often partly split open in the central zone and its component zones poorly contrasted. After uranyl treatment, however, it was well preserved (aligned arrows, Fig. 11) and its junctions with the plasma membrane often clearly resolved (Fig. 10). However, the poor preservation of the cell wall after permanganate fixation prevented the accurate determination of the limits of the cytoplasmic membrane (Figs. 10

to 12). Although contrast was sufficient in some areas (aligned arrows, Fig. 11) to indicate that the basic structure of the plasma membrane was similar to that found after osmium fixation, the backbone line seen at the membrane site in osmium-uranyl–fixed sections was somewhat broader in the permanganate-uranyl–fixed cells and appared to extend more in the cell wall direction. This difference may in part account for the unexpected opacity of the central zone of the double form of this membrane, the spore septum, found after such fixation (Figs. 10 to 12).

The Development of the Transverse Cell Septum in Dividing Bacilli

The locations of the mesosomes during the development of the forespore (Figs. 6 and 7) (Fig. 9, *E* to *H*) are similar to those of the peripheral bodies found as poorly defined pockets by Chapman (2) in sporulating *B. cereus* and *B. megaterium*. Similar structures had been previously described by Chapman and Hillier (3) accompanying the ingrowing cross-wall of *B. cereus*. The finding here that membranous organelles occupied sites similar

Explanation of Figures

Legend

cm, cytoplasmic or plasma membrane
chr, chromatin, or nuclear material
trs, transverse cell septum
cw, cell wall
sps and *spm*, spore septum and forespore membrane
v, vacuole (site of lipid-containing inclusions)

FIGURES 1 to 5

Electron micrographs of early stages in the formation of forespores in members of the genus *Bacillus*. Only spore-forming ends of the rods are shown.

FIGURE 1

Bacillus cereus var. alesti. The earliest recognizable step in the development of the spore septum. The lower part is sufficiently well resolved to show the involvement of the cytoplasmic membrane (arrow). The part of the chromatin (*chr*) destined for the spore is still continuous with that in the rest of the cell. × 50,000.

FIGURE 2

A similar stage in another cell of *B. cereus var. alesti*. The beginnings of the spore septum in this off-centre cut are marked by arrows. At the top edge of the septum a loop of membrane is partly resolved and appears to be in contact with the partly enclosed but poorly contrasting spore chromatin. × 55,000.

FIGURE 3

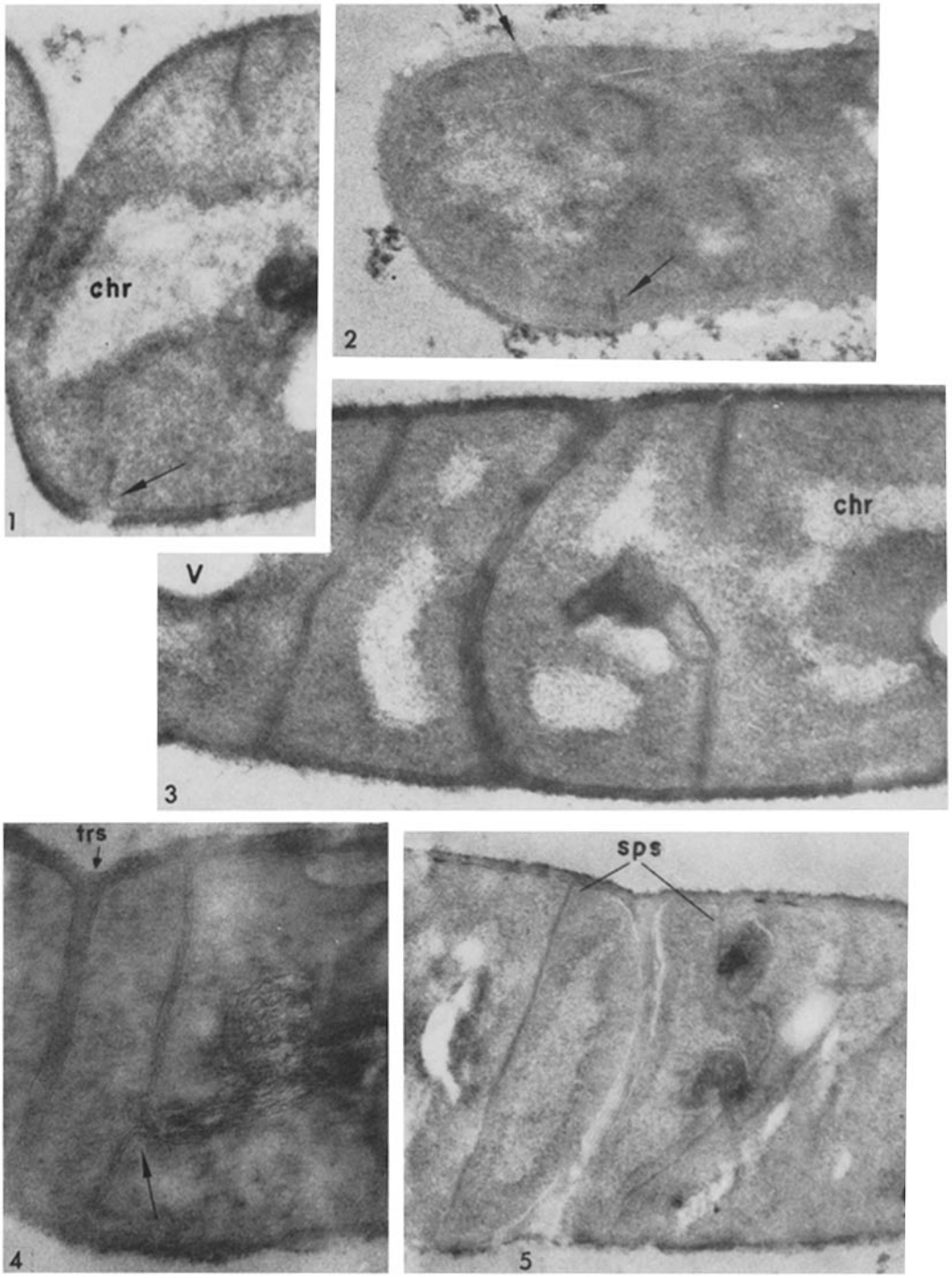
Partially formed spore septa on either side of a cell septum in the filamentous spore-former, *Bacillus medusa*. In the left cell, the future spore chromatin appears to be completely separated; in the right, the section has come closer to the point of separation of spore and cell chromatin (*chr*). The osmophilic body is in contact with the spore chromatin and continuous with the spore septum. Parts of lipid inclusions (*v*) are seen in each cell. × 60,000.

FIGURE 4

A spore septum and a transverse cell septum (*trs*) in *Bacillus megaterium*. A laminated structure continuous with the spore septum (arrow) and composed of similar material extends back into the cell. × 77,000.

FIGURE 5

Bacillus medusa. A slightly later stage than that shown in Fig. 3. In addition to the chromatin-attached osmophilic body, the right spore septum (*sps*) has another attached to it and is indented thereby. This section does not cut any such objects on the left spore septum. × 41,000.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to Chapman's perisporal bodies suggested that the peripheral bodies associated with vegetative division might also be membranous structures. Indeed, membranous organelles or mesosomes were found during late vegetative division in positions comparable to those of the vacuolated peripheral bodies described by Chapman and Hillier (3). In many sections these also showed a definite continuity with the plasma membrane covering the developing cross-wall (Fig. 15). It is interesting to note that, in the same way, the peripheral bodies described by Chapman and Hillier were continuous with a space between cell wall and cytoplasm.

In addition to their attachment to cytoplasmic membranes a high proportion of the vegetative cell mesosomes like that similar body occluded within the developing spore (Figs. 3, 5, 7, and 14) were also adjacent to, if not embedded in, the cell chromatin (Figs. 17, 23, 24, and 25).

As the transverse cell septum grows into the cell, the attachment of these organelles persists (Fig. 17). Occasionally, two and even three are traversed by the section (Figs. 18 and 25). On the other hand, the entire structure or its link with the plasma membrane may be missed.

The relation of the mesosomes of bacilli to the cell wall and to the formation of transverse cell septa was strikingly apparent in sections of cells rejuvenated from the stationary phase of growth. In such rapidly dividing cells of *B. megaterium* KM, very prominent mesosomes were found at the sites of septum formation. In addition, instead of the normal unit membrane between cell wall and cytoplasm, parts of the plasma membrane showed extensive proliferations around the base of these organelles (Fig. 23). Some of these bodies are found in close association with the vacuoles which mark the site of the lipid inclusions. Moreover, they seem particularly fragile, in that their inner components often appeared damaged by the knife (Fig. 24).

Fine Structure of the Mesosomes: In better resolved micrographs, the surface of each organelle has a limiting membrane which possesses a prominent dense line continuous with the similar line in the plasma membrane. Outside of this (*i.e.* towards the cytoplasm) a light zone and a finer cytoplasmic border are readily seen (Fig. 25, arrows at *b*). Within the organelle, the structure is seldom straightforward. However, in many places (arrows *c*, Fig. 25) the limiting heavier dense line appears to be directly involved in finger or lamellar like extensions into the lumen of the organelles. In some

FIGURES 6 and 7

Bacillus medusa showing the further development of the spore septum into the spore wall. Contrast in these figures has been accentuated by partial volatilization in the electron beam. Osmium-fixed, uranyl acetate–treated.

FIGURE 6

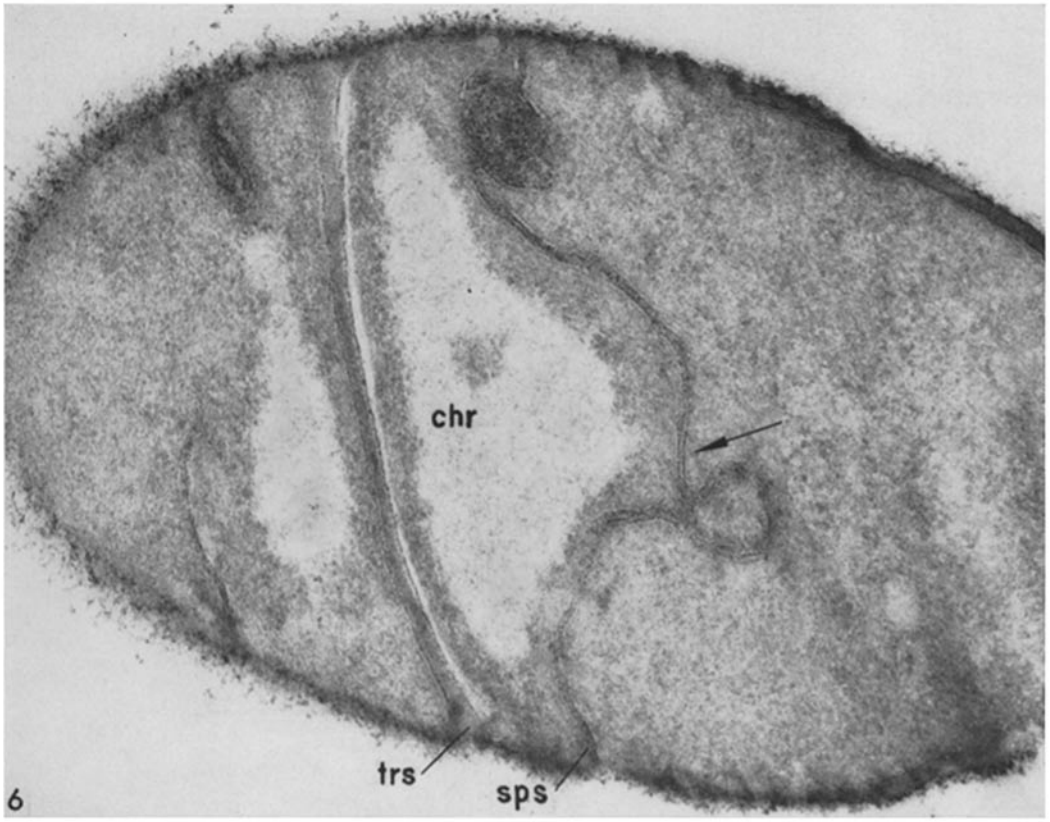
A dense, well stained granule is continuous with the membranous spore septum (*sps*) on the top right. The septum is best resolved near its centre (arrow). The loop of septum suggests a possible mode of formation of the perisporal mesosomes. × 83,000.

FIGURE 7

A completed septum developing into a spore wall and associated with two granules of laminated material. Although not well resolved, the surface of both bodies appeared in the original prints to be attached to the cytoplasmic surface and to the spore septum × 72,000.

FIGURE 8

Bacillus megaterium in a later stage of spore formation. The osmiophilic bodies of membranous material are still present and attached to the cytoplasmic membrane (large arrows). The spore coat is being formed and is partly resolved (small arrows). Osmium-fixed, uranyl acetate–treated. × 68,000.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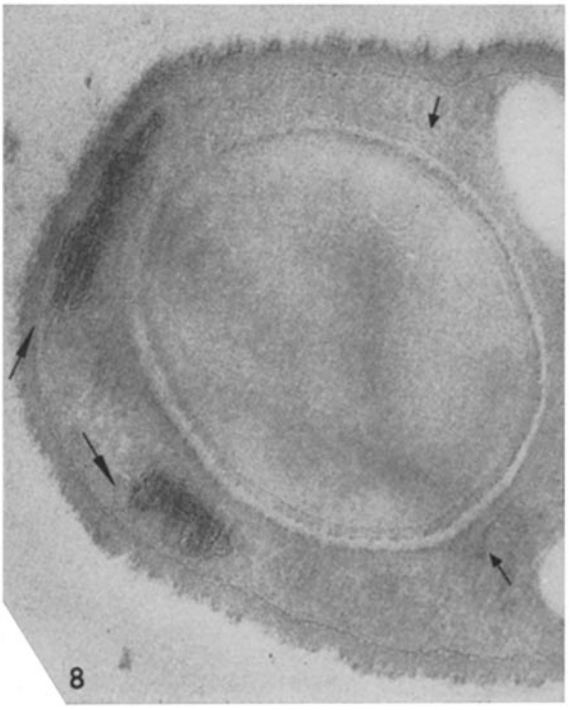
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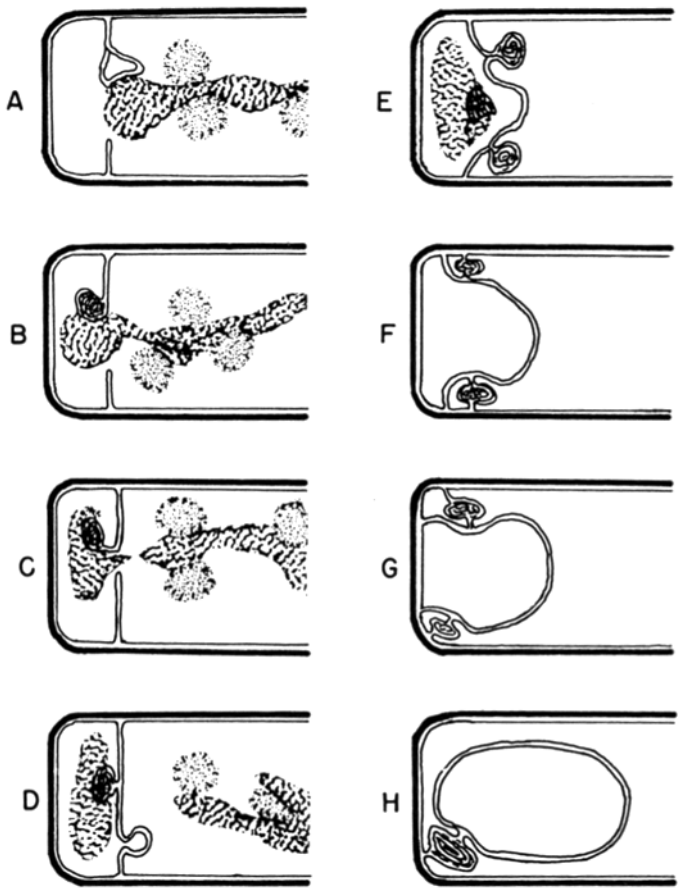


FIGURE 9

A diagrammatic interpretation of the electron micrographs of the developing forespore in bacilli. The steps *A* to *D*, the development of the spore septum, are less well established than steps *E* to *F*, the conversion of the spore septum to the forespore membrane. Chromatin and associated lipid granules have been omitted in steps *F* to *H* (see reference 25). The fine line represents the plasma membrane, the heavier envelope the cell wall.

FIGURES 10 and 11

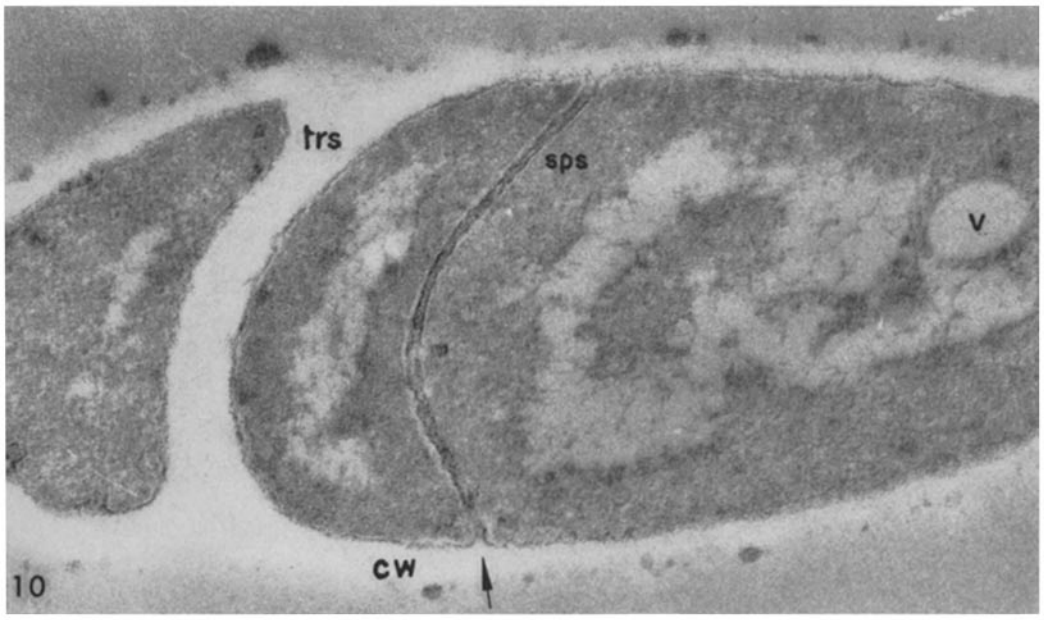
Sporulating cells of *B. medusa* fixed with $KMnO_4$ and stained with uranyl acetate.

FIGURE 10

The well developed spore septum shows a layering not greatly different from that obtained with osmium-uranyl treatment. In contrast to the cytoplasmic membrane, the cell wall (*cw* and *trs*) is poorly preserved and of low contrast. The arrow marks the junction of the spore septum with the cytoplasmic membrane. × 80,000.

FIGURE 11

The spore septum is partly developed into the initial envelope of the forespore. Part of an occluded membranous organelle is seen in the spore chromatin. × 100,000.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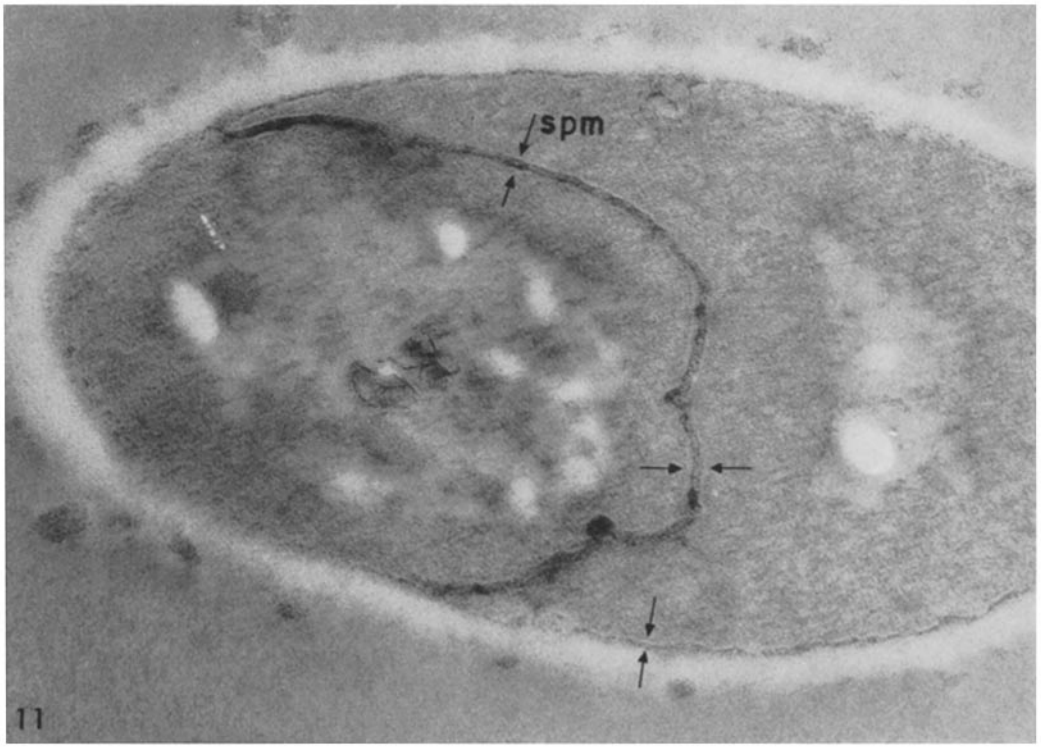
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regions bulbous enlargements are seen on these extensions and, when properly cut, appear double-walled (Fig. 26). A simplified diagram, suggesting a possible relationship of the membrane layers to these inner extensions is shown in Fig. 28.

The two types of internal structure found in the mesosomes—the mass of closely packed and often concentrically arranged lamellae and the more loose, open arrangement of tubules with bulbous enlargements can conceivably develope by variations of the same mechanism of membrane proliferation suggested in Fig. 28. Although these structural differences may in part be due to minor variations in preparation as well as in thickness and plane of the sections, the lamellar type was more prevalent in perisporal bodies, the tubular in those associated with the nuclear bodies and transverse septa of rapidly dividing bacteria. This tubular pattern is, in some respects, similar although on a smaller scale to that described by Pappas and Brandt (14), in the mitochondria of an ameba.

The Effect of Lysis on the Membrane Structure of Bacilli: During the course of separate studies of the effects of inhibitors on spore formation, it was observed that after prolonged blocking of the protein synthesis with chloramphenicol the cytoplasmic density of the cells was partly reduced and the number of the mesosomes seemed more plentiful. When such cells were stained with lead, the over-all improvement in contrast permitted a more detailed analysis of these membranous appendages (Fig. 25). In most such cells, the space between cell wall and cytoplasm was occupied as usual by a dense line and two lighter zones (arrows at *a*, Fig. 25). However, parts of the membrane in these inhibited cells showed considerable increase in density along the line of the inner or cytoplasmic border and appeared now as a double dense line (~70 A wide) close to the cytoplasm, but still separated by a less opaque zone from the cell wall (Fig. 26).

A similar double line structure of the cytoplasmic membrane has also been observed during

FIGURE 12

The sporulating end of a cell of *B. medusa* fixed in permanganate and stained with uranyl acetate. The central zone of the forespore membrane (*spm*) is heavily stained and the bordering light zones well contrasted. Part of the membrane has been cut tangentially. The cell wall (*cw* and *trs*) is practically invisible. × 100,000.

FIGURE 13

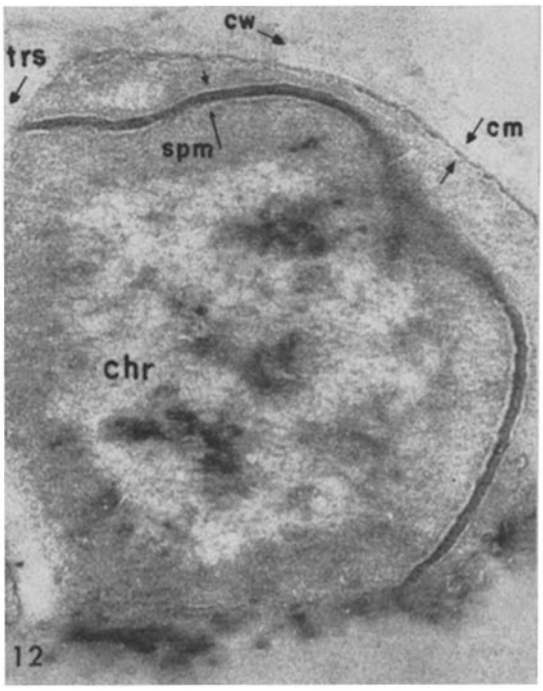
A comparison of the cell membrane and spore membrane in *B. megaterium*. This cell is at the same stage of sporulation shown in Fig. 12 or Fig. 9, *G*. The space between the cell wall (*cw*) and cytoplasm (the plasma or cytoplasmic membrane, *cm*, small arrows) is some 145 A; that between the cell cytoplasm and forespore (*spm*, large arrows) is 280 to 300 A and can be accounted for in both structure and width by a doubling of the unit cytoplasmic membrane. Osmium-fixed, uranyl acetate–stained. × 197,000.

FIGURE 14

A thin section through a sporulating filament of *B. medusa* showing to the right of the transverse cell septum (*trs*) the enclosure of a membranous, laminated body with the spore chromatin and to the left the extension of similar material from the cell surface into a chromatin body not apparently involved in spore formation. The disposition of the remaining part of the chromatin about lipid vacuoles is also shown. Osmium-fixed, uranyl acetate–treated. × 38,000.

FIGURE 15

The last cell division in *B. medusa* prior to spore formation. The arrows indicate the developing transverse cell septum (*trs*). The lower rim has an attached mesosome, whose surface is continuous with the cytoplasmic membrane and whose interior is filled with apparent proliferations thereof. Osmium-fixed, uranyl acetate–treated. × 45,000.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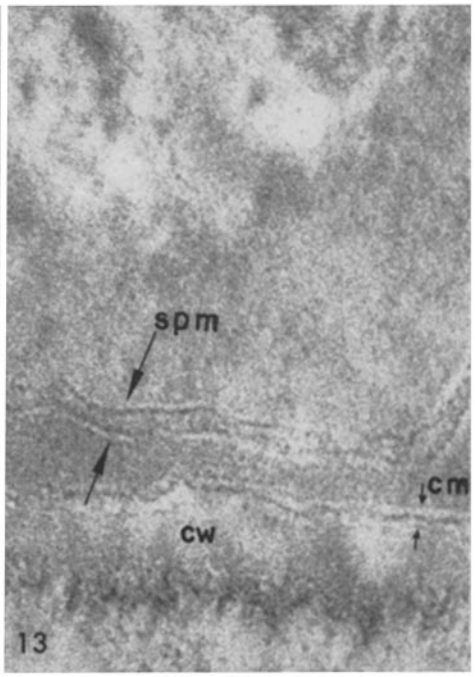
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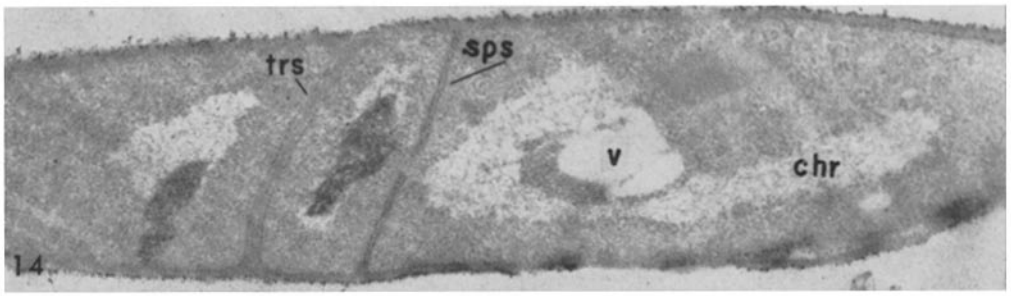
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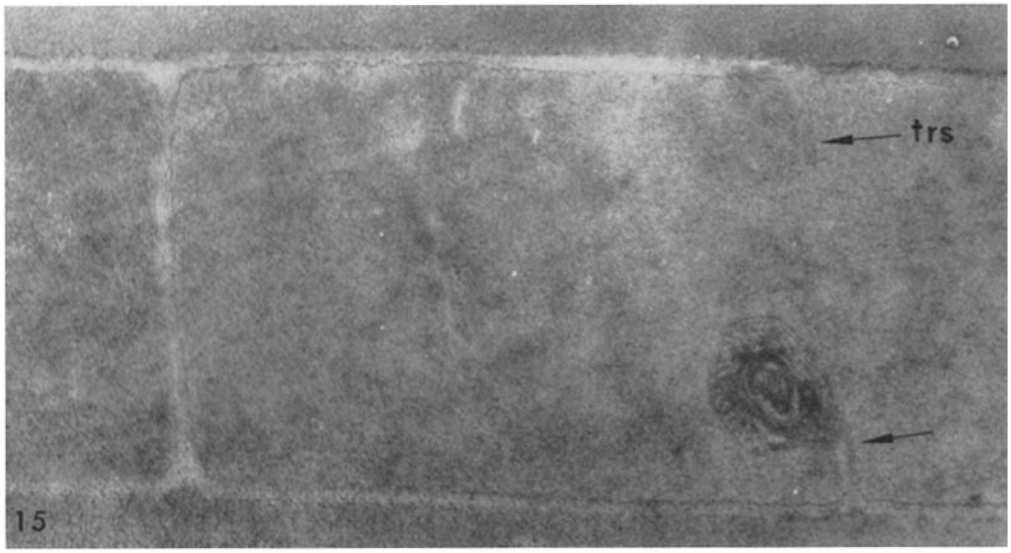
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the extensive lysis which follows the completion of spore formation (30). Fig. 27 is a cross-section of such a lysed cell, the remnant ring of the cell wall being beyond the frame of the picture. A similar double line membrane could be seen in lytic cells fixed with permanganate. In such lytic systems, the original backbone line of the membrane formed the outer dense line, while the inner cytoplasmic border formed the second line when the cytoplasm was lost (extreme left of Fig. 28). The mechanism of this change in membrane appearance is at present obscure but does not seem to be simply the deposition of protein remnants onto the inner surface of a membrane. However, even in membranes of well lysed cells, adherent protein and folds make a clear interpretation hazardous.

Light Microscopy of Mesosomes in Growing and Sporulating Bacilli: The size of the mesosomes (0.15 to 0.20 μ diameter) in most growing cultures is just at the limit of resolution of the light microscope and hence they are difficult to demonstrate in living phase contrast preparations. However, after osmium fixation, some of them can be seen in phase contrast micrographs of fixed cells (Fig. 19). Similarly, it is difficult to detect the mesosomes associated with spore formation in living preparations, although the spore septum may be seen readily (Fig. 20). Neither the mesosomes of rapidly dividing cells nor the spore septum and the associated perisporal mesosomes showed any immediate staining on exposure to Sudan black, with or without prior fixation. It should be noted moreover that rapidly growing cells such as those shown in Fig. 19 did not show any staining with Sudan black. In fact, with the aerated fluid medium used, sudanophilic granules did not usually appear until late growth or early spore formation (Fig. 21). Then those small refractile granules which become sharply visible in living phase contrast smears and are presumably inclusions of β-hydroxybutyrate polymer common to these bacilli showed rapid staining with Sudan black. In contrast to the mesosomes, these sudanophilic sites appear to have little affinity for osmium and are found as vacuoles in thin sections (29).

Janus Green Staining: The sites of the peripheral bodies could also be demonstrated by the application of Janus green B to the living cells. The most convenient method of following the effects of such staining was to observe continually a wet mount of cells from an actively aerated culture as the stain was run under the coverslip. Otherwise undetected granules were the first site of dye binding and with continued exposure these stained more prominently (Fig. 22). Eventually, however, as the cytoplasm itself became stained, the contrast of the inclusions lessened. Similarly, Janus green B was added to a culture at the stage of forespore forma-

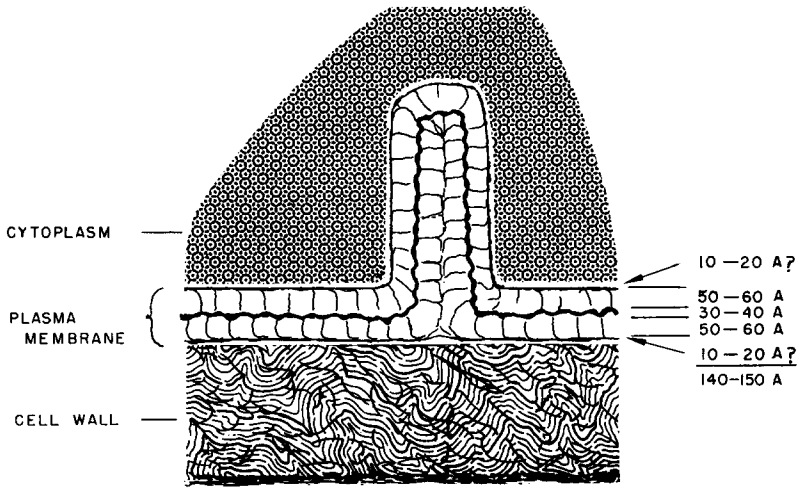


FIGURE 16

A diagrammatic interpretation of the cross-section of the plasma membrane and its connection with the developing spore septum from micrographs of osmium-fixed, uranyl-treated material. The 10 to 20 A lines marked ? are at the limit of resolution of the instrument used and represent either a border to the membrane or a dense line which is accentuated by uranyl acetate treatment.

tion shown in Fig. 20; the spore septum and associated mesosomes became clearly outlined. The resulting resolution of the spore septum was momentarily as good as that possible by phase microscopy.

DISCUSSION

During the past few years improved preservation and resolution of structures in thin sections have led to the demonstration of electron-dense, membranous, and laminated organelles in the cells of a number of bacterial species. Both the ability of bacterial cells to show localized reduction of redox dyes (12) and the similarity of structure to mitochondria have led many workers to assume these organelles are analogous structures. However, Weibull (26) found that the large deposits of formazan which develop in bacteria by the reduction of triphenyl tetrazoleum are not centers of reduction themselves and cautions against concluding that such staining indicates the presence of mitochondria in bacteria. Nevertheless, Niklowitz (13) found that in thin sections of tetrazoleum-treated *Escherichia coli* formazan vacuoles were often adjacent to ill defined, dense central or polar bodies which he termed "mitochondrial equivalents" and which could also be seen in untreated cells. A similar structure, somewhat better resolved, appears in a thin-section of *E. coli* published by Kellenberger *et al.* (9) (their Fig. 2*a*) while concentric laminated bodies in the cytoplasm of *B. subtilis* cells were shown by Ryter *et al.* (17). In cells of this bacillus grown from germinated spores, Dr. Woutera van Iterson, University of Amsterdam, has obtained excellent micrographs of intracellular organelles associated both with the chromatin bodies and with the cross-wall membrane, as described here (personal communication).

Recently Hickman and Frenkel (5) have published micrographs of laminated organelles in older cells of *Rhodospirillum rubrum* and a remarkable system of intracytoplasmic membranes has now been seen in *Streptomyces coelicolor* by Glauert and Hopwood (4) and in *Mycobacterium leprae* by Brieger, Glauert, and Allen (1). Laminated cytoplasmic structures have also been seen in avian tubercle bacilli (19) and in *Mycobacterium tuberculosis* by Shinohara *et al.* (20). These latter workers isolated a particulate fraction which partly resembled the inclusion in thin-sections and which possessed terminal oxidative enzyme systems.

The now established presence in bacterial membranes of enzyme systems normally found in mitochondria of plant and animal cells (11, 23) as well as intermediate reactions of protein synthesis (6) focuses fresh attention on the structure and function of this layer or its extensions in the life cycle of the bacilli. Indeed, it now appears that membranous inclusions are a consistent component of bacillus cells and play an active role in the proliferation processes occurring during rapid growth and spore formation. Thus the definition of the cytoplasmic membrane achieved here by the polyester-embedding procedure reveals that the spore septum is a proliferation of the cytoplasmic membrane and not, as was suggested by observations of methacrylate sections, continuous with the cell wall (28, 29).

The Fine Structure of the Plasma Membrane: In presenting the observations above, it has been assumed that the cytoplasmic membrane occupies the entire space between the inner border of the cell wall and the cytoplasm and consists of a dense backbone line bordered by two lighter zones. Such an interpretation presents a unit membrane of rather wide dimensions, yet when doubled accounts for the dimensions of the spore septum (Fig. 9). Such a structure is rather different from the pair of parallel dense lines ($\sim$75 A) found as the unit membrane in a great variety of animal cells, mainly through the extensive studies of Robertson (15). However, with the loss of cytoplasmic density in a bacillus cell, either by lysis or by the blocking of protein synthesis, the inner, cytoplasmic border of the membrane attains a density equal to that of the original backbone and the whole structure now appears similar in profile to that of animal cells.

This suggests an alternate interpretation, that the bacillus "unit membrane" is not different from that described for animal cells but that *in situ* only one of its dense lines is visible. Then the outer light border of the membrane would represent some non-membrane substance perhaps analogous to the "gap" substance or "basement membrane" of certain animal membranes (15). By the same reasoning, the spore septum could then be compared to the Schwann cell mesaxon (15). However, both the remarkable uniformity of this outer light zone and its persistence as a layer twice as wide between the two backbone lines in the spore septum suggest that it is an integral part of the unit membrane, not a variable by-product of it. In any event, at present it seems more reasonable to interpret the limits of the bacillus membrane from its appear-

ance *in situ* in normal physiological states rather than in abnormal conditions and after lysis.

Analyses of isolated protoplast membranes or ghosts show a high content of phospholipid and protein, indicating that bacterial membranes are probably a phospholipoprotein structure (27). It is tempting to speculate on the molecular arrangement of the structure as it exists in the membrane of the bacillus cell. Two hypotheses are available for such speculation and are based on the bimolecular phospholipid model proposed by Finean (see reference 15). One assumes the dense lines in the ~75 A double image are the sites of the hydrophilic groups. This type of molecular model could be applied to the double dense-lined structure found after lysis of the cell and so presented as evidence for a single bimolecular leaflet in the same way that Robertson (15) has applied it to animal membrane systems.

The other hypothetical scheme of molecular configurations has been recently presented by Stoeckenius (22) from studies of sections of artificial myelin figures formed in water and protein solutions and presents evidence that the unsaturated carbon atoms in the fatty acid chains of the bimolecular leaflet are the sites of osmium binding,

FIGURE 17

A section of *B. medusa* showing a mesosome attached to the side of the closure of the transverse septum. The dense line of the cytoplasmic membrane is reflected over the surface of the body which contains parallel tubules made up of material similar in structure to the cytoplasmic membrane. The peripheral ends of the inner tubules terminate at the site of septum formation. The central surface of the body is embedded in poorly contrasted chromatin (*chr*). Part of another peripheral body is seen in the adjacent cell. × 73,000.

FIGURE 18

A cell from the same embedding as those shown in Figs. 17 and 18. Three osmophilic mesosomes are grouped near a recently completed transverse septum. The arrow marks the midline of the cell section. Any attachments of these bodies to the cytoplasmic membrane have not been revealed in this cut. × 82,000.

FIGURE 19

Dark phase contrast photomicrographs of *B. medusa* at the same stage of growth (11 hours) shown in Figs. 15, 17, and 18. Osmium staining (vapor 1 per cent) has revealed the mesosomes. The positions of the completed transverse septa are marked with arrows. × 3,880.

FIGURE 20

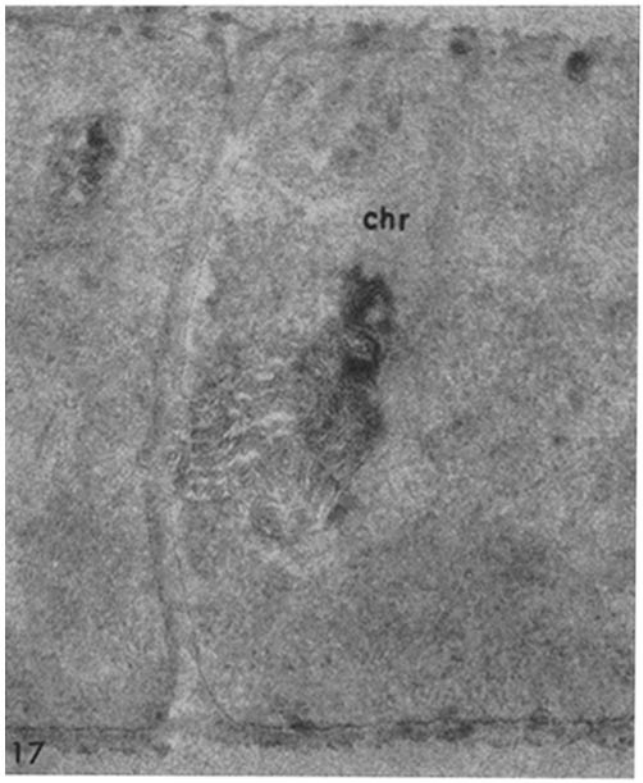
Dark phase contrast of living cells of *B. medusa* durnig the formation of the forespore. Arrows mark the position of two forespore membranes on either side of a transverse septum. The small dense granules are not osmophilic but stain with Sudan black (Fig. 21). The large oval, refractile profiles are parasporal protein inclusions characteristic of sporulation in this organism.

FIGURE 21

A Sudan black B stain of osmium-fixed cells from a slightly earlier stage than that shown in Fig. 20. (Brightfield. × 3600).

FIGURE 22

Brightfield photomicrograph of cells in a smear of *B. megaterium KM* exposed to Janus green B showing various degrees of staining.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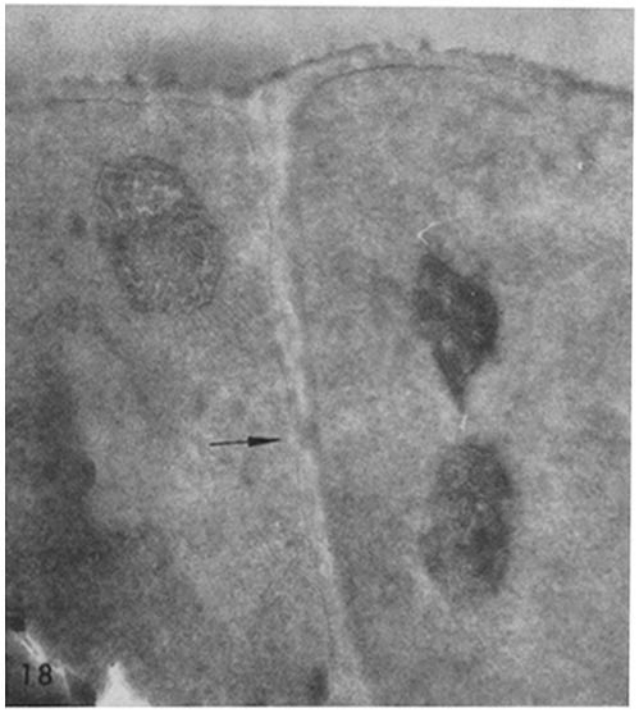
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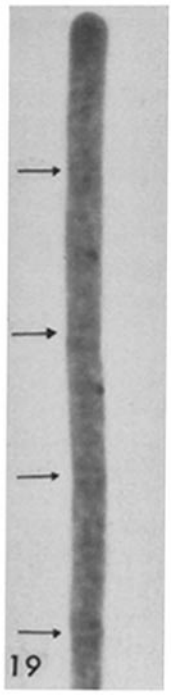
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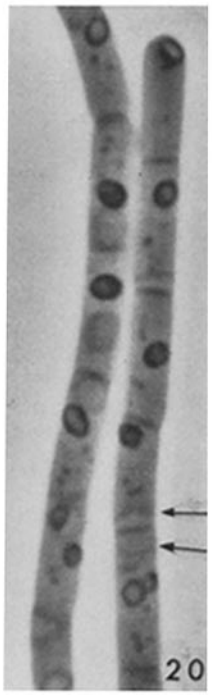
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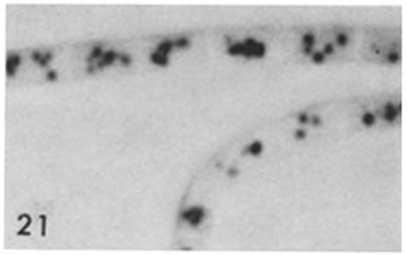
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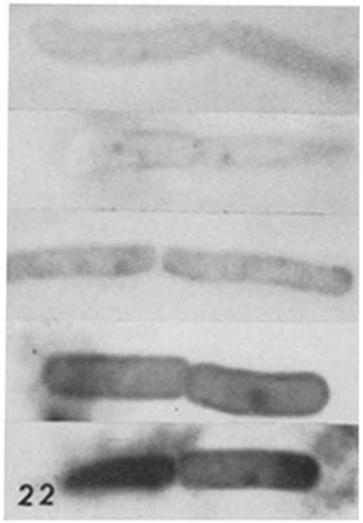
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hence the electron-opaque "lipid lines." Conceivably, the fine, wavy, single line found consistently in the bacillus plasma membrane *in situ* and which, when sharply resolved, has a width of ~25 A may represent a single lipid line which, Stoeckenius predicts, should mark the center of a single bimolecular layer of phospholipid. Further, the borders of the light wings could then correspond to the sites of protein—certainly they are more prominent after uranyl acetate treatment. However, the proper interpretation of morphological structure in terms of molecular arrangements demands further work on the morphology and chemistry of bacterial membrane systems.

The Possible Functions of Mesosomes: The continuity of the outer layers of the mesosomes with the plasma membrane at the edge of the transverse septum and the spiral arrangement found in some of these layers in the organelle suggest a special function of these bodies both in the formation of transverse septa and the laying down of membrane at these sites. In this connection, it is particularly interesting to recall the suggestion of Salton (18) that cell wall synthesizing mechanisms may be located in the peripheral bodies and his speculation that these structures may be "outside" the limiting plasma membrane. In this study the interiors of many of the mesosomes of rapidly dividing cells, as shown in Fig. 23, were clearly extracytoplasmic, their outer limits being the cell wall.

However, it is also conceivable that this location at the site of rapid cross-wall formation simply reflects a more general role in the provision of energy at this point. Thus these bodies like the plasma membrane with which they are continuous, may be the site of energy-yielding enzyme systems and in this respect like the mitochondria of higher forms. Hence they should be found at any site of rapid synthesis. Such might explain their intimate relation with the chromatin bodies which was particularly apparent during early stages of growth from germinating spores, while desoxyribonucleic acid was being rapidly synthesized and before any cross-wall formation had begun (unpublished observations). However, the true function of these organelles is only suggested by these observations and analyses of their composition and biochemical capabilities are demanding problems for the future.

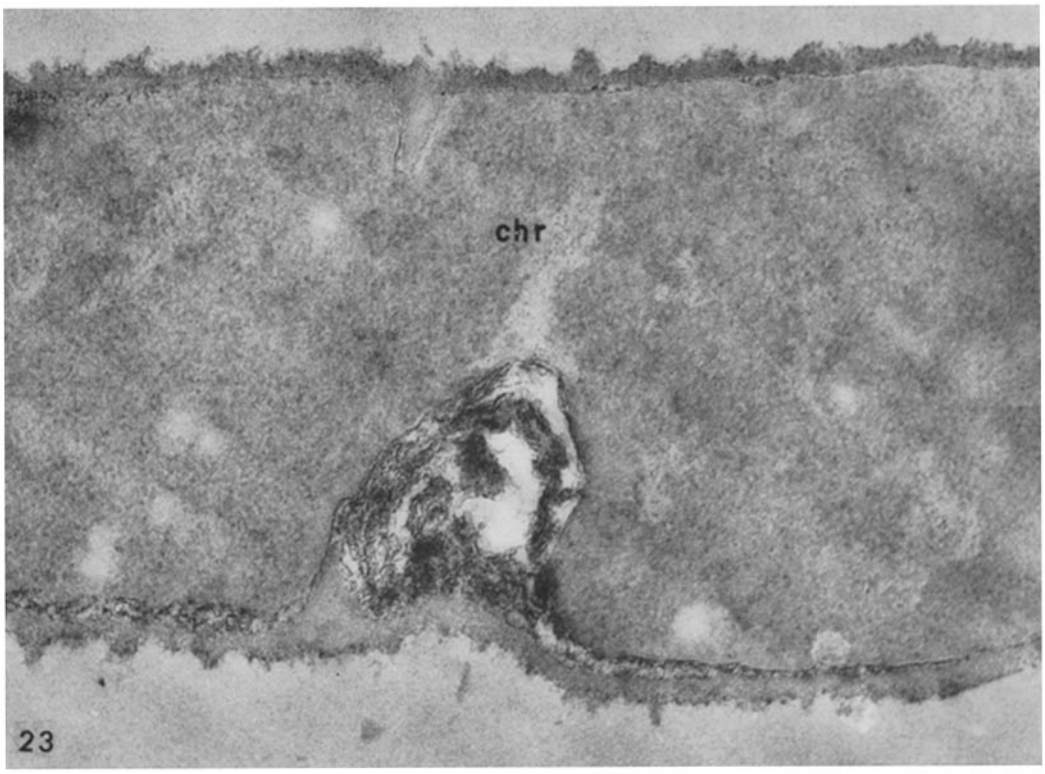
The fate of those mesosomes involved in the final cell division and their activity during the 30 to 60 minutes interval before the beginning of the spore septum has yet to be elucidated. Although at least one similar structure is enclosed with the chromatin into the forespore, the micrographs suggest that the perisporal mesosomes arise *de novo* as indicated in Fig. 9 and are not those of the previous cell division.

The size and structural associations of the organelles described above in growing cells may possibly explain some of the peculiarities associated with the cytology of the nuclear bodies of bacteria. Acting as a framework onto which the chromatin becomes plated during hydrolysis, such structures could account for the rather beaded appearance of chromatin observed in Feulgen-type stains of rapidly dividing bacilli. It is also likely that after condensation of chromatin by cold or salt treatments, such organelles would be exposed and so account for the appearance of "accessory chromatin granules" or "centrioles" in stained preparations (7, 16). Possibly also, the mesosomes or remnants of them could give rise to the chromatin-attached cores found associated with nuclear bodies isolated from protoplasts (21).

Attempts to isolate mesosomes from protoplasts

FIGURES 23 and 24

Sections of parts of rapidly dividing cells of *B. megaterium* strain KM showing the prominent osmophilic mesosomes. In Fig. 23 the interior of the body appears to be outside the limiting plasma membrane between it and the growing cell wall. Some chromatin (*chr*) is seen adjacent to the apex of the organelle. Extensive proliferation of the plasma membrane is also seen in the region surrounding the point of cell division. × 62,000. In Fig. 24 the connection of the mesosome to the surface membrane is not revealed. The close contact between the chromatin (*chr*) of the cell and the mesosome is marked by an arrow. Some damage to the interior of the organelle has occurred but the membranous tubules are well resolved in some places. The vacuole-like area (*v*) is probably part of a lipid granule. × 107,000.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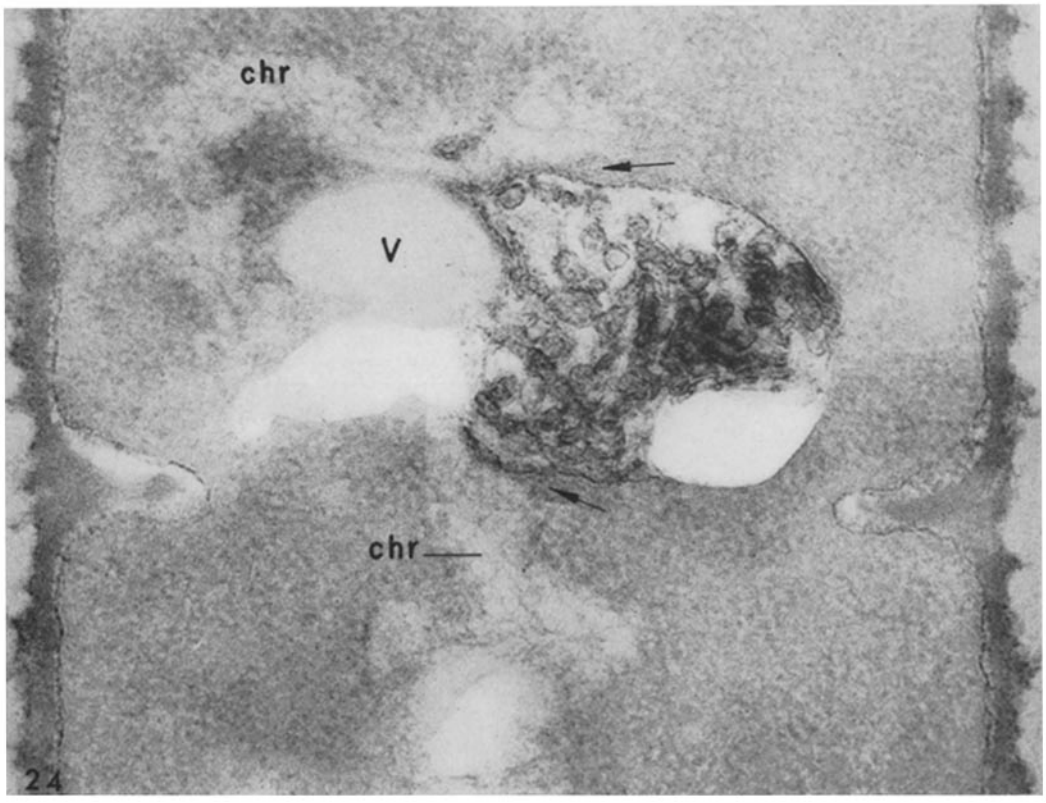
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are now underway; already it has been found that because of their location (Fig. 23) some can, by remaining attached to the cell wall, be readily lost from the protoplast, depending on the stage of division when lysozyme is added.

Note Added in Proof: A paper by Giesbrecht (*Zentr. Bakt. I Abt., Orig.*, 1960, **179,** 538) has just been published describing these membranous organelles in *Bacillus megaterium*. Similarly a paper by Hagedorn (*Deutsch. Bot. Ges.*, 1960, **73,** 211) presents electron micrographs of membranous inclusions in a variety of microorganisms.

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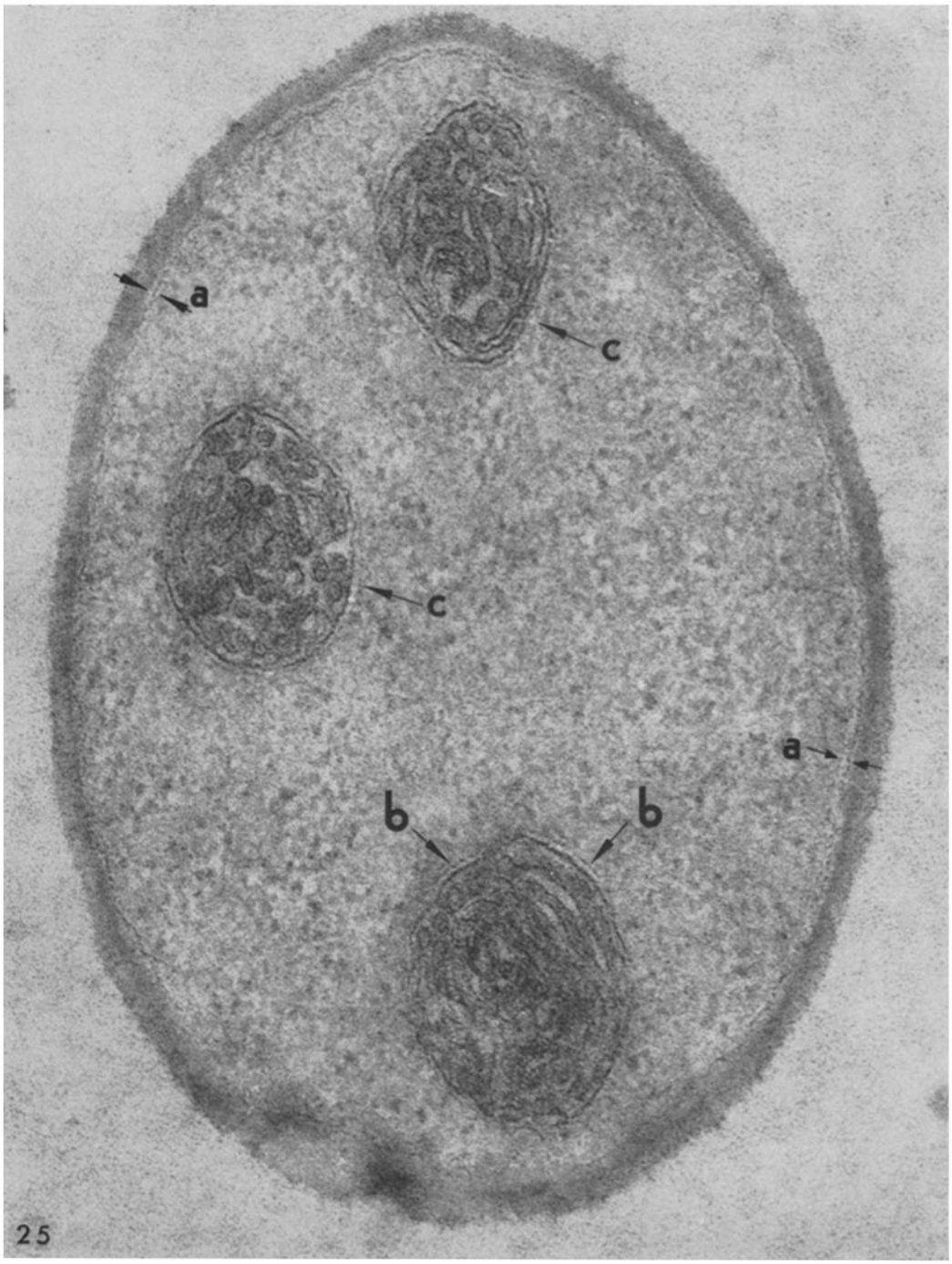
Figure 25

A *tangentially* cut section of a cell of *B. medusa* in which sporulation was blocked by the addition of chloramphenicol (30 μg./ml.) 8 hours prior to osmium fixation. This has somewhat reduced the density of the cytoplasm and contrast has been further augmented by lead staining (25). The knife has traversed three mesosomes. The cytoplasmic membrane shows the typical pattern of a dense backbone line bordered by two lighter zones. The over-all width of this layer (gaps between arrows *a*) is 150 A. A dense line can also be seen in the limiting membrane of the mesosomes, again with a light zone and a finer line outside this marking the border of the cytoplasm (for example arrows *b*). In many places (arrows *c*) the denser outer line of the mesosomes appears to be directly involved in finger- or lamellar-like protrusions into their lumen. × 130,000.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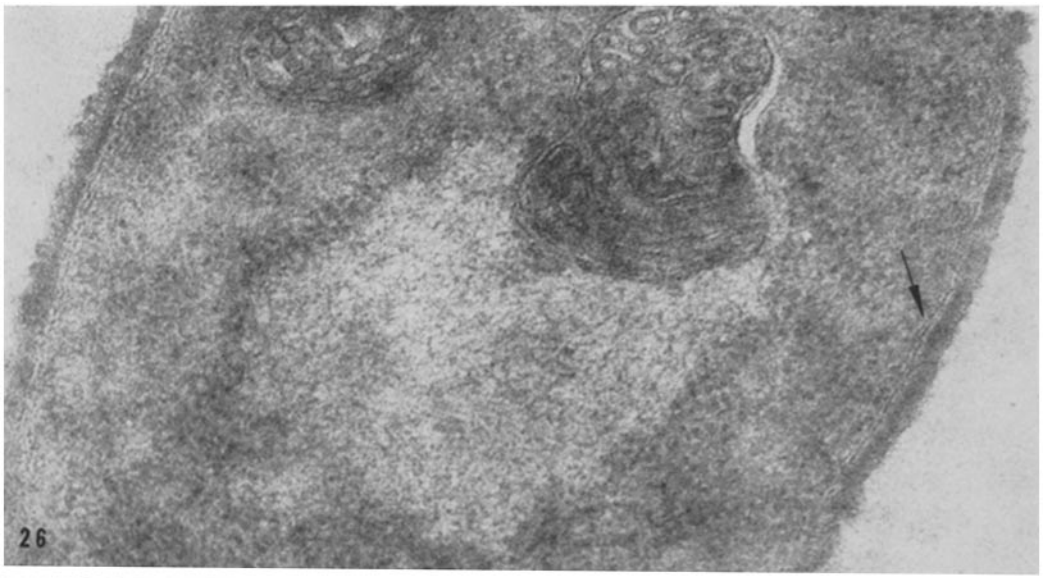
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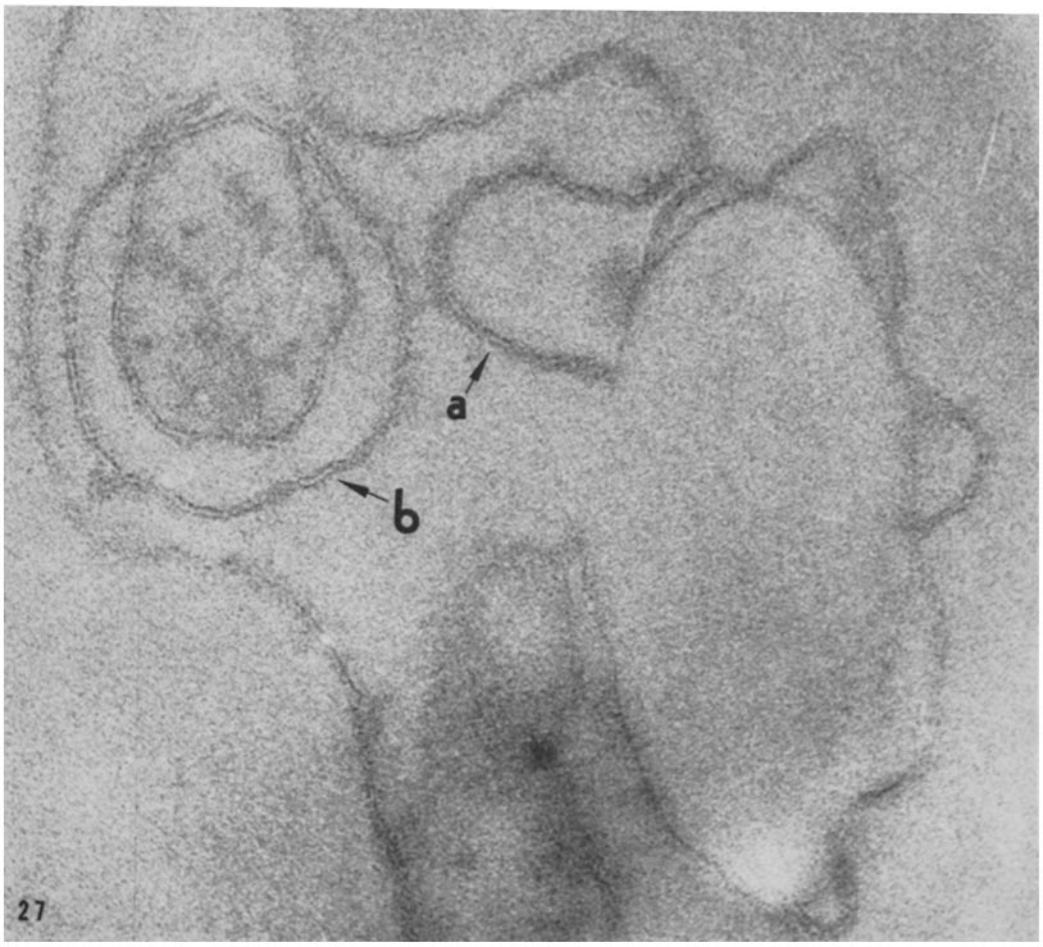
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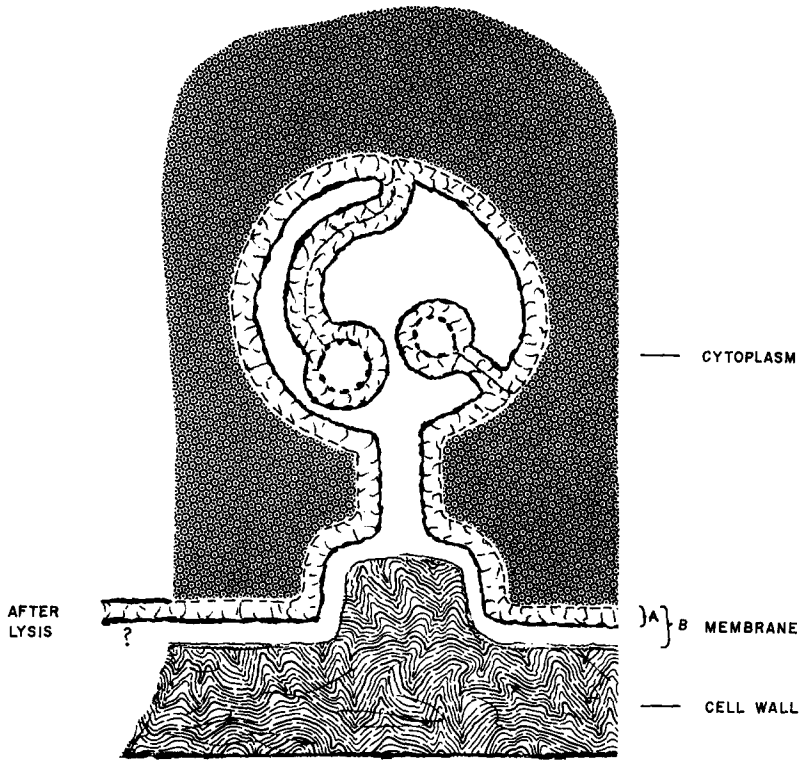

FIGURE 28

A diagram showing the hypothetical relationship of the component lines of the unit plasma membrane to the surface and internal structures of the mesosomes. The approximate dimensions of the membrane are as indicated in Fig. 16. Two interpretations of the limits of the membrane are indicated. A membrane of width *A*, suggested by the effects of lysis, corresponds to that commonly described in animal cells (15) and leaves the outer zone (?) as the site of some possible gap substance which, although part of the spore septum (Fig. 16), is capable of being partly obliterated in the mesosome. A membrane of width *B* includes the entire space between cytoplasm and cell wall.

FIGURE 26

A cell of *B. medusa* like that shown in Fig. 25, in which sporulation was blocked by chloramphenicol. As a result, the inner cytoplasmic border of the membrane has become more prominent so that there now appears to be a double dense line (arrow). The prominent mesosomes show various forms of the inner membranous projections, some of which are cut transversely and show clearly a double profile. A close association of the low density chromatin mass and one mesosome is evident. × 95,000.

FIGURE 27

The remnant membrane of a cell of *B. cereus* which has undergone lysis following sporulation. The outer crumpled line (*a*) is the cytoplasmic membrane of the cell. The circular profiles (at *b*) are presumably remnants of a mesosome. Where normal to the plane of the section the membrane shows the double dense lines 60 to 75 A apart (arrows) as commonly described in sections of animal membrane. × 200,000.

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