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Bacteria-shaped Gymnoplasts (Protoplasts) of *Bacillus subtilis*

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Addition of glucose to the medium in which *Bacillus subtilis* was grown lowered the *p*H and increased the amount of lysylphosphatidylglycerol relative to the phosphatidylglycerol content of the membrane fraction. This change in phospholipid composition was accompanied by changes in the shape and behavior of the gymnoplasts obtained by cell wall removal with lysozyme. These gymnoplasts appeared to retain most of their original cell shape and internal organization, often with preservation of the mesosomes. Cells harvested from neutral growth medium gave the usual spherical gymnoplasts. In a hypotonic medium, the spherical gymnoplasts lysed rapidly, whereas the rod-like gymnoplasts lost only part of their cell content while showing a tendency to preserve the original shape. This type of gymnoplast could not be produced from cells grown in neutral medium by simply raising the magnesium concentration. When this was done the gymnoplasts assumed bizarre shapes; they became compact and susceptible to the tonicity of the medium. Gymnoplasts or protoplasts, produced from bacilli exposed to low *p*H values, were found not to conform to the formulations on "protoplasts" given in 1958 by 13 authors. Cells exposed to a low environmental *p*H during growth seemed to possess a more rigid membrane structure than cells grown at neutral *p*H.

The enzyme lysozyme is known to hydrolyze linkages in the murein sacculus (i.e., the rigid layer) of bacterial walls (15) and in gram-positive species such that the cytoplasmic membrane is freed completely from cell wall materials. The classic example is the treatment of *Bacillus megaterium* with lysozyme in a hypertonic medium, as a result of which the rod-shaped cells are converted into spheres (13, 14) which are very sensitive to osmotic shock. Such cell wall-free forms have been called "protoplasts." In 1958, 13 specialists (1) agreed to use this term for structures in which the cell wall is known to be absent. If this definition is accepted, there are, according to these 13 authors, certain properties which isolated protoplasts will exhibit. The first of these is described as follows. "The internal osmotic pressure of a bacterial cell is normally resisted by the cell wall. If the wall is removed, the protoplast will be lysed unless a medium of suitable composition is present to balance this pressure. In a suitable medium, the resulting form is usually a sphere, irrespective of the shape of the cell from which it comes."

Frey-Wyssling (3) pointed out that in bacteriology the term "protoplast" is used incorrectly and stated that the word "gymnoplast" is preferable. The word gymnoplast is therefore used to denote bacterial cells which are devoid of cell wall material.

It has been shown that, when *B. megaterium* is grown in a culture medium in which the *p*H remains more or less neutral, the gymnoplasts which are produced on treatment with lysozyme are spherical and sensitive to lysis in hypertonic medium. However, exposure of cells to *p*H 5 produces gymnoplasts which more or less preserve their original cell shape and are less sensitive to lysis (5); these elongated gymnoplasts even tend to preserve their original cellular organization. Concomitant with the changes in shape and behavior of the gymnoplasts, the phospholipids of the membrane fraction of *B. megaterium* were found to vary. In cells harvested at *p*H 5, the phosphatidylglycerol content, which was high in cells grown at *p*H 7, decreased considerably, and there now appeared a phospholipid, namely, glucosaminyl phosphatidylglycerol, that was absent in cells from neutral medium (4, 5). The content of other phospholipids remained the same under both conditions.

Not all bacteria respond in a similar way to acidic conditions in their growth medium. It was of interest to investigate the effect of a slight

lowering of the *p*H of the culture medium on *B. subtilis*. In a paper dealing with the phospholipid composition of *B. subtilis* (6), it was shown that lowering the *p*H from 7 to 5.5 (by glucose fermentation during growth) results in an increase of the amount of lysylphosphatidylglycerol and a decrease in the phosphatidylglycerol content, whereas the amount of the other phospholipids and the total amount of phospholipids remain the same under the two different conditions of acidity of the culture medium. The relationship between environmental conditions, phospholipid composition, and properties of the gymnoplasts found in *B. megaterium* has also been found in *B. subtilis* and is the subject of this communication. We were also able to verify that, in *B. subtilis*, the change in the phospholipid composition found by Op den Kamp et al. (6) was accompanied by the preservation of the bacillary shape of the gymnoplasts. In view of observations of Rogers et al. (7), we decided to study the effect of the magnesium concentration in the suspending fluids on the morphology of the gymnoplasts.

MATERIALS AND METHODS

Cultivation of the organism and production of gymnoplasts. *B. subtilis* strain Marburg was grown at 35 C in shaking cultures in media of the following composition. Medium A contained 10 g of peptone (Difco), 10 g of yeast extract (Difco), 5 g of NaCl, and 400 mg of Na_2HPO_4 per liter of water (*p*H 7.0). Medium B was the same as medium A but contained, in addition, 20 g of glucose and 2 g of $(NH_4)_2SO_4$. Growth overnight in medium B caused a drop in the *p*H from 7.0 to 5.5

The cells were harvested by centrifugation, washed, and suspended in Ryter-Kellenberger acetate-Veronal buffer (*p*H 6); they were then treated with 25 mg of lysozyme (muramidase; Worthington Biochemical Corp.) per 10 ml of buffer until protoplasts were formed, as checked in the light microscope with cells harvested at *p*H 7. The acetate-Veronal buffer was, except for special experiments, routinely supplemented with 0.01 M $MgCl_2$. In parallel experiments, the effect of the presence and absence in the buffer of 0.25 M sucrose as well as the effect of 0, 0.01, 0.04, and 0.1 M $MgCl_2$ were studied.

In preliminary experiments, the *p*H was lowered to 5 with 1 N HCl after 3 hr of growth in medium A, whereupon the bacteria were grown for an additional hour.

Electron microscopy. Two procedures were followed for electron microscopy. (i) After centrifugation, the sedimented gymnoplasts were fixed with 1% OsO_4 in the Ryter-Kellenberger acetate-Veronal buffer which, with respect to magnesium and sucrose concentrations, was the same as that in which gymnoplasts had been produced by lysozyme treatment. The gymnoplasts were then washed with the same buffer, enmeshed in 1% agar of similar composition, treated for 1 hr with 0.05% uranyl acetate (8), dehydrated with acetone, and embedded in Vestopal W. (ii) After centrifugation, the cells were at once enmeshed in agar, whereupon the gymnoplasts were produced by diffusion of lysozyme through the agar. The gymnoplasts were then fixed and further treated by diffusion of the various solutions through the agar, similar to the treatment described above.

Thin sections were cut on an LKB ultratome, usually with glass knives. The sections were stained with lead citrate by the method of Reynolds. Electron micrographs were taken with a Philips EM 300 operated at 80 kv and equipped with an objective aperture of 40 μm and a cooling device.

RESULTS

Morphology of the intact cells. The drop in *p*H from 7.0 to 5.5 during growth overnight in medium B hardly affected the morphology of the cells or their vitality as seen in the electron microscope. When cells grown overnight in media A and B were reinoculated into fresh medium A, the lag period was longer in the case of the cells from medium B, but the growth curves of both types of cells in the exponential phase were parallel.

The strong resemblance between the morphology of cells from medium A and from medium B is shown in Fig. 1 and 2. Figure 1 shows a section of *B. subtilis* grown overnight in medium A in which the *p*H remained neutral. The cell has a thick wall from which the plasma membrane (PM) has retracted somewhat and a compact cytoplasm in which ribosomal structures can be recognized by their electron opacity, which is greater than that of the surrounding material. In addition, there is one nuclear area in which the fibrillar pattern of the deoxyribonucleic acid can be recognized, and, in this section (arrows), at least four mesosomes can be distinguished. In Fig. 2, showing *B. subtilis* harvested at lower *p*H, essentially the same features can be seen. In this more or less median section from a longer series of sections, there is also apparent a single nucleoid of ordered fibrillar structure close to a mesosome (M). At the arrows, there are small involuted membrane structures which, in this cell, are quite numerous.

Gymnoplast formation. Cell wall removal in *B. subtilis*, harvested at neutral *p*H from medium A and suspended in buffer with 0.25 M sucrose, resulted in spherical gymnoplasts (Fig. 3 and 4). Cell wall removal in cells harvested at a *p*H of about 5, on the other hand, resulted in gymnoplasts which have more or less retained their original cell shape (Fig. 9), even when no sucrose had been added to the buffer (Fig. 5, 10, and 11). In Fig. 3 and 4, gymnoplasts of 4.5-hr-old cells grown in neutral medium are shown. Although

both suspension and fixation media contained 0.01 M Mg^{2+}, the cytoplasm in such gymnoplasts appeared dilated, and the ribosomal structures could not be easily recognized. In these rounded structures, the nucleoplasm is swollen and it appears to have lost its original structural organization. In at least one area (large arrows in Fig. 3 and 4) and perhaps in several others (see small arrows), the fibrillar material of the nucleoplasm may be in contact with the plasma membrane. The evidence is strongest for this at site M in Fig. 4, where presumably a mesosomal vesicle has been expelled.

When gymnoplasts from cells harvested at neutral *p*H were sustained by agar, i.e., when they were produced by diffusion of lysozyme through the agar (Fig. 6) in which they were suspended, the swelling of the gymnoplasts and the alteration of their fine structure were less marked than when the gymnoplasts were produced directly in liquid medium (Fig. 3 and 4). Although the cytoplasm tended to preserve its normal granularity and the nucleoplasm tended to preserve its normal location and organization, mesosomes were no longer present in the cell: apparently they moved outwards and were expelled (Fig. 6; 2, 5, 8, 10, 11). Figure 5 shows a gymnoplast produced from a cell which was grown in medium B. This gymnoplast was prepared in a similar manner to those in Fig. 3 and 4, in buffer with 0.01 M $MgCl_2$ although without sucrose; it is evident that here the original shape of the cell is well preserved. Even the fine structure of these cells appears to have altered surprisingly little, even to the extent that the organization of the cytoplasm and nucleoplasm resembles that of the intact cells shown in Fig. 1 and 2.

Mesosomes were preserved in the rod-shaped gymnoplasts from cells from the naturally acidified medium B, regardless of whether 0.25 M sucrose was (Fig. 9) or was not (Fig. 10) added to the Ryter-Kellenberger buffer in which the cells were converted. In Fig. 10, as well as Fig. 5 and 11, the stability of the gymnoplasts produced from cells exposed to a low *p*H is evident, even in a hypotonic milieu.

Influence of HCl on the morphology of gymnoplasts. In preliminary experiments, the drop in *p*H to 5.5 was rapidly produced by adding 1 N HCl (Fig. 7 and 8). On treatment of these cells with lysozyme, the bacillary shape was well preserved both in hypertonic and in hypotonic medium. The cytoplasm, however, lost its normal granularity, even in the presence of 0.01 M $MgCl_2$ (Fig. 5), and now appeared structureless (Fig. 7). The nucleoplasm became swollen and contained numerous dispersed vesicles of the mesosomes (arrows, Fig. 7). This structural alteration induced by exposure of the cells to dilute HCl, however,

FIG. 1. *B. subtilis grown overnight in medium A and thus harvested at a neutral pH. The fine structure of the cell is similar to that in the cell harvested at low pH in Fig. 2. There is one nucleoid with a mesosome (M) close to it. At the arrows, three more mesosomes can be seen. Ribosomes can be recognized in the compact cytoplasm by their high electron opacity. PM, plasma membrane receded from the cell wall.* × *84,000. Bar indicates 0.5 μm.*

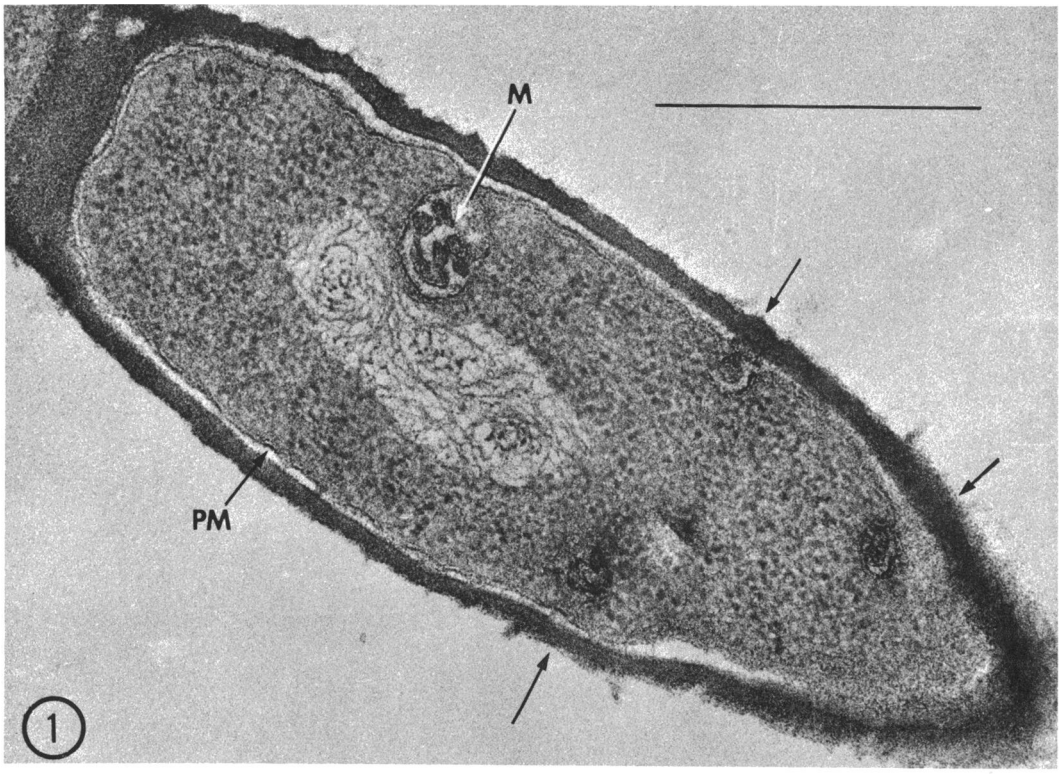
FIG. 2. *B. subtilis grown overnight in medium B which became acidified to pH 5.5. No morphological differences are seen in these cells compared to those in Fig. 1. Again, there is one nucleoid with a mesosome close to it. At the arrows, small membrane involutions can be distinguished, i.e., small mesosomes.* × *78,000. Bar indicates 0.5 μm.*

FIG. 3. *Gymnoplasts of B. subtilis harvested from medium A after 4.5 hr of growth at neutral pH and converted with lysozyme in the presence of 0.01 M $MgCl_2$ and 0.25 M sucrose in the buffer. The nuclear material is fused. The mesosomes are extruded (small arrows). At the large arrows, the nucleoplasm appears to reach the cytoplasmic membrane. In hypotonic medium such spherical gymnoplasts are seen to burst.* × *27,500. Bar indicates 0.5 μm.*

FIG. 4. *Gymnoplast from a cell converted after 4.5 hr of growth at neutral pH. Although the media were supplemented with 0.25 M sucrose and 0.01 M $MgCl_2$, ribosomal structures cannot be observed in the cytoplasm, which appears dilated. The nucleoplasm has lost also its original fine structure and location in the cell. At the large arrow (near M), but perhaps also at other sites (small arrows), fibrils from the nucleoplasm may reach the cytoplasmic membrane. The small vesicle at M could be from a mesosome (12).* × *96,500. Bar indicates 0.5 μm.*

FIG. 5. *Gymnoplast of B. subtilis harvested at low pH after one night of growth in medium B. Note the difference with the gymnoplasts in Fig. 3 and 4 from cells grown at neutral pH. Although the cell wall materials have been removed, much is preserved of the original shape of the cells. The media were supplemented with 0.01 M $MgCl_2$, and consequently the fine structure of both nucleoplasm and cytoplasm is very well preserved. No sugar had been added to the media, but these cells have not been emptied by burst.* × *60,000. Bar indicates 0.5 μm.*

FIG. 6. *Gymnoplast comparable to the ones in Fig. 3 and 4 but made of a cell, enmeshed in agar, by diffusion of the lysozyme through the agar. The gymnoplast has not become spherical, and it has to a large extent been restrained from swelling through the mechanical stabilization by the agar. Nucleoplasm and cytoplasm have preserved much of their fine structure, but the mesosomes have disappeared from the gymnoplast.* × *78,000. Bar indicates 0.5 μm.*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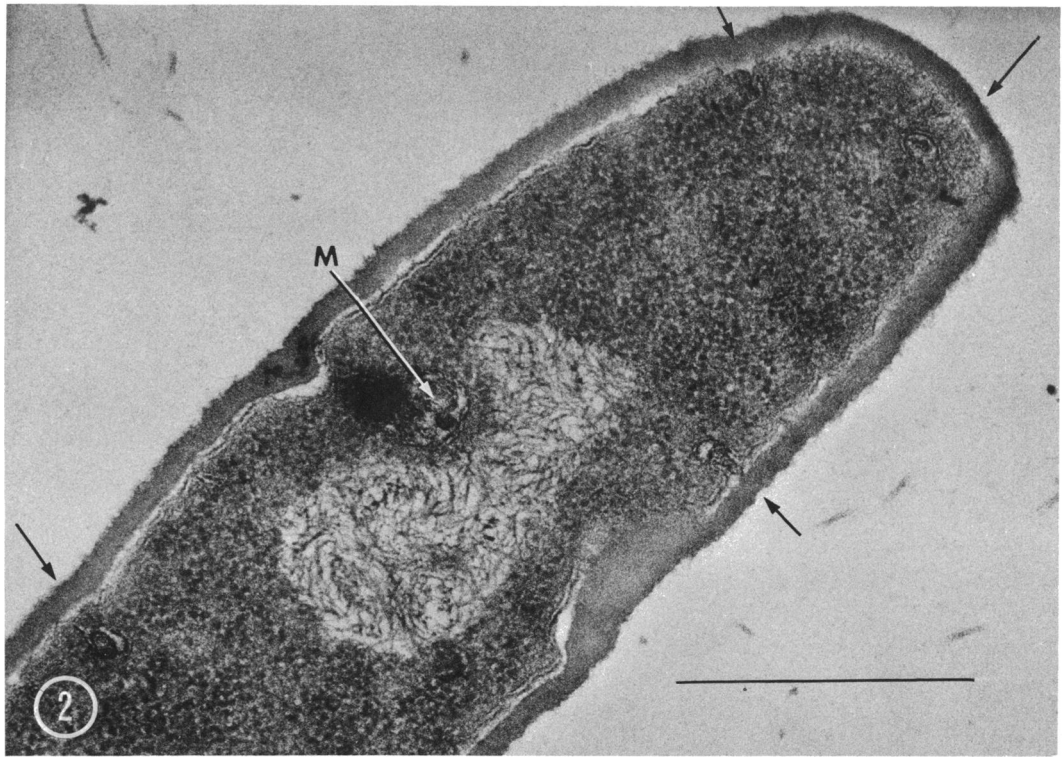
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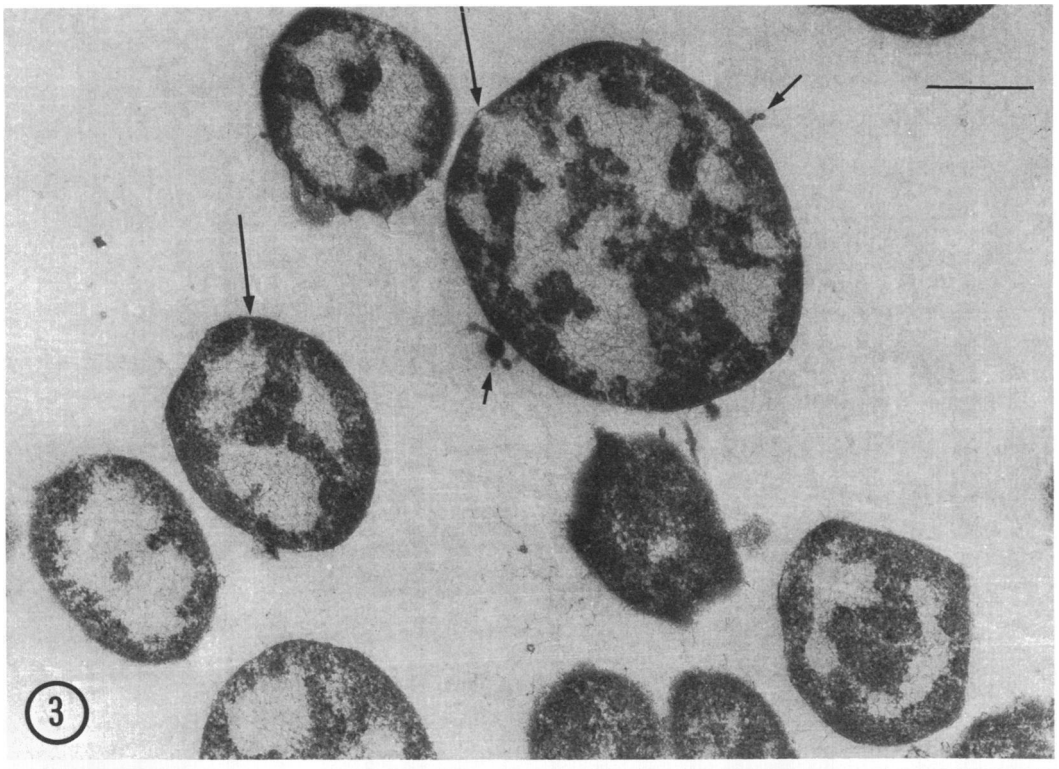
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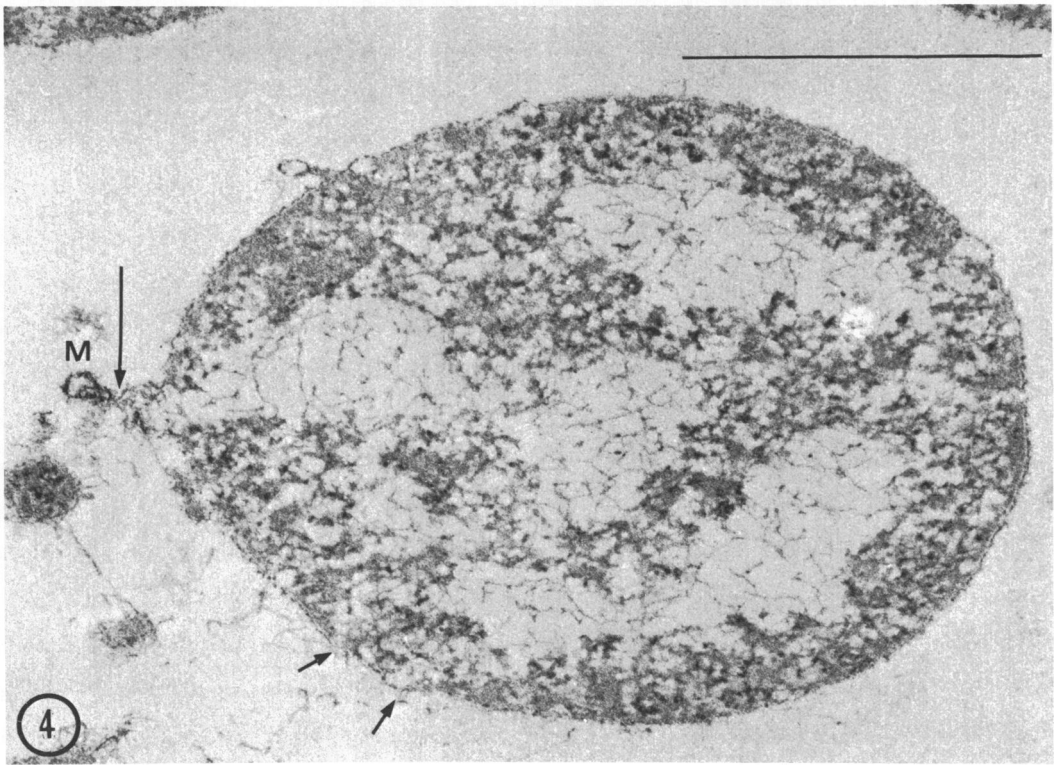
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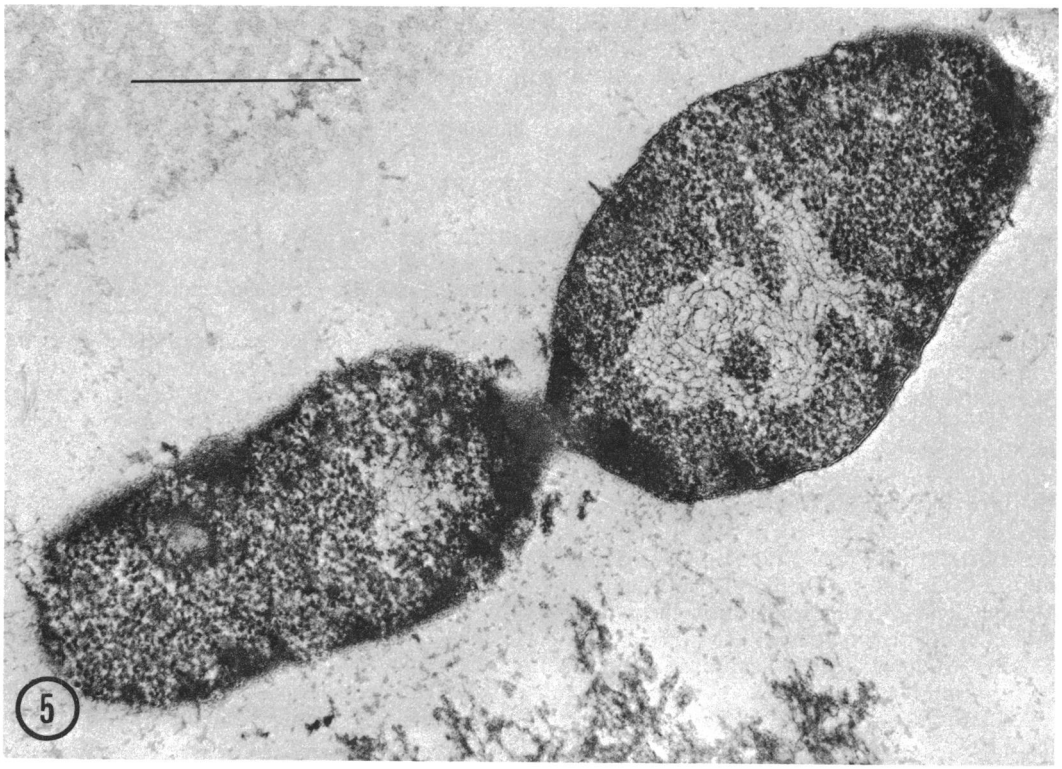

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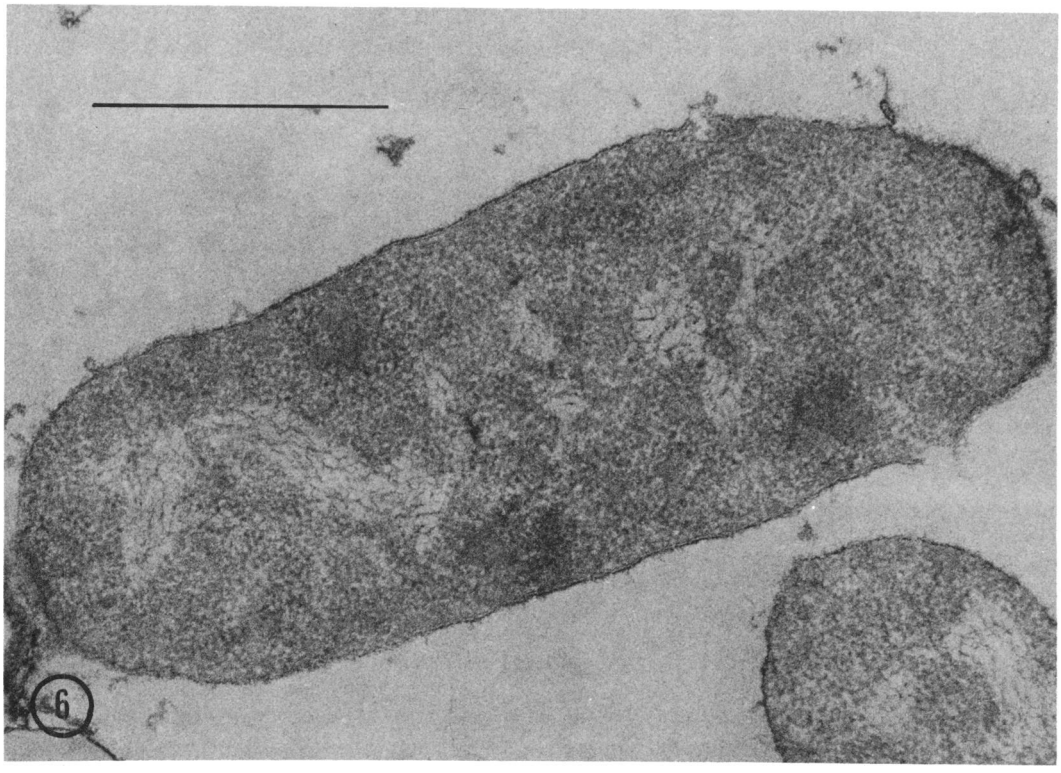

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appeared to be reversible. When cells from an acidified medium were embedded in agar made with acetate-Veronal buffer (*p*H 6) and were subsequently treated with lysozyme, the cytoplasm and nucleoplasm possessed a more normal appearance (Fig. 8). The mesosomes were again expelled from the cell (arrows, Fig. 8).

Effect of Mg^{2+} on the gymnoplasts. The addition of magnesium ions to the buffers in which the gymnoplasts were prepared resulted, in the first place, in a considerably better preservation of the cytoplasm and nucleoplasm. When a comparison is made between the cells in Fig. 9 and 10, which were prepared in the absence of Mg^{2+}, and the cells in Fig. 4, to which 0.01 M $MgCl_2$ was added, it is obvious that the omission of Mg^{2+} from the buffers was responsible for the structureless appearance of the cells in Fig. 9 and 10. In a preparation which was prepared in a similar manner to the one in Fig. 10 in the absence of Mg^{2+}, the cell structure appeared to be the result of cell wall removal from an incompletely divided cell, which was bent during processing so that it touches the other half (Fig. 11). In the upper part of the figure, the connection between the two division halves is still recognizable. Two mesosomes are present in the right-hand half.

Secondly, magnesium ions affect the cytoplasmic membrane, although those of *B. subtilis* gymnoplasts (Fig. 12) are affected less than those of *B. megaterium* (Fig. 13). In the case of *B. subtilis*, stabilization of the plasma membrane with magnesium ions could not be obtained to the same extent as could be produced by lowering the *p*H of the culture medium. Stabilization did, however, occur when cells from a neutral medium were converted to gymnoplasts in elevated magnesium concentrations (0.04 and 0.1 M) in combination with 0.3 M sucrose. The outlines of the *B. subtilis* gymoplasts then showed a tendency to become irregular (Fig. 12), whereas those of *B. megaterium* became particularly unusual (Fig. 13). At *p*H 7 in the absence of sugar, the mere addition of magnesium ions proved to be ineffective in influencing the shape of the gymnoplasts: they became spherical as usual. In gymnoplasts from cells at low *p*H, the only effect of elevated magnesium concentrations appeared to be a decrease in the leakage of cell material and a better preservation of the fine structure of the cytoplasm, as judged by the electron micrographs.

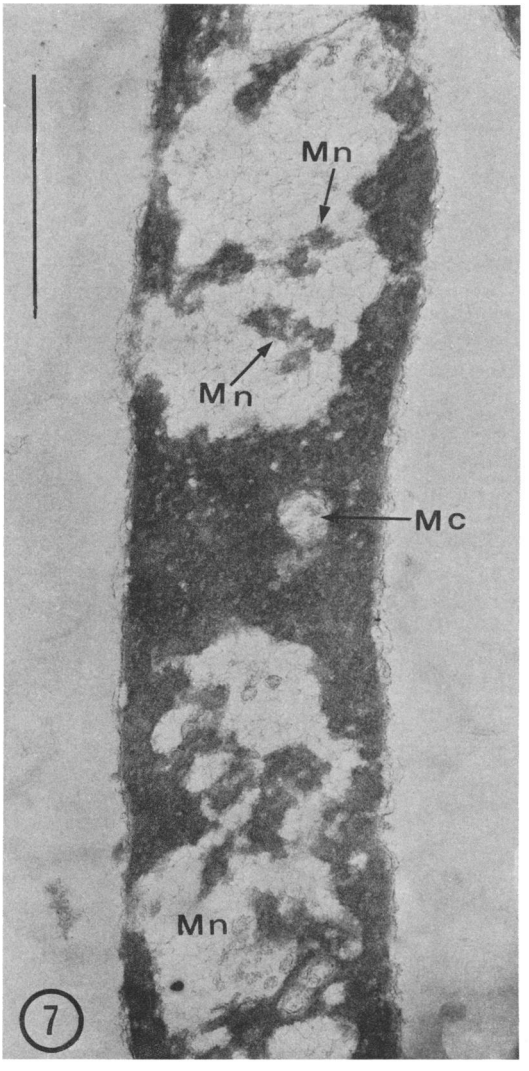
DISCUSSION

Exposure of *B. subtilis* cells to growth medium in which the *p*H is lowered from 7.0 to 5.5 affects the phospholipid composition of the membrane fraction (6). Concomitant with a decrease in the phosphatidylglycerol content and an increase in the amount of lysylphosphatidylglycerol, we observed changes in the shape and in the resistance against lysis of the gymnoplast, which was obtained by cell wall removal with lysozyme. Cells harvested from neutral growth medium A gave, in hypertonic medium, spherical gymnoplasts which lysed rapidly when the tonicity of the suspending medium was lowered by the omission of sucrose. Cells grown in acidic medium maintained their original shape to a great extent, when the gymnoplasts were produced either in buffer with sucrose or in a hypotonic environment, i.e., in the absence of sucrose in the buffer. These results are in agreement with previous experiments on *B. megaterium* (5).

In the electron microscope, no visible change in the cell morphology could be observed when cells were harvested after overnight growth in medium B instead of medium A (Fig. 1 and 2). However, when the *p*H was adjusted by means of 1 N HCl instead of by the fermentation of glucose, the cells of *B. megaterium* and of *B. subtilis* appeared to be affected unfavorably, and therefore this method was later abandoned. The cell walls of such cells looked abnormal, as did the fine structure of the cytoplasm.

As was observed with *B. megaterium*, lowering of the *p*H during growth tends to preserve the original fine structure inside the cell wall-free cells (5). When the suspension media were not supplied with magnesium ions, disturbance in the fine structure resulted; notably, the ribosomal granules were no longer distinguishable in the cytoplasm (Fig. 9 to 11). In gymnoplasts from cells harvested at *p*H 7.0, there was better preservation of fine structure when they were sustained by agar in which the bacilli had been enmeshed before their walls were lysed. At present, no satisfactory explanation can be given for this effect, except that, by a mechanical stabilization, the agar gel prevents an undue swelling of the cytoplasm and the nucleoplasm more effectively than hypertonic medium alone. Unlike the gymnoplasts from cells harvested at *p*H 5.5, the gymnoplasts made in agar, either from neutral *p*H cells (Fig. 6) or from cells harvested at low *p*H (Fig. 8), did not retain their mesosomes, although, in these treatments, the composition of the various media was the same.

On extrusion of the mesosomes, vesicles either remained attached to the outside of the plasma membrane or seemed to disappear in the medium after removal of the cell wall. Several authors (2, 5, 8, 10, 11) have already reported that digestion of the cell walls of bacilli with lysozyme is



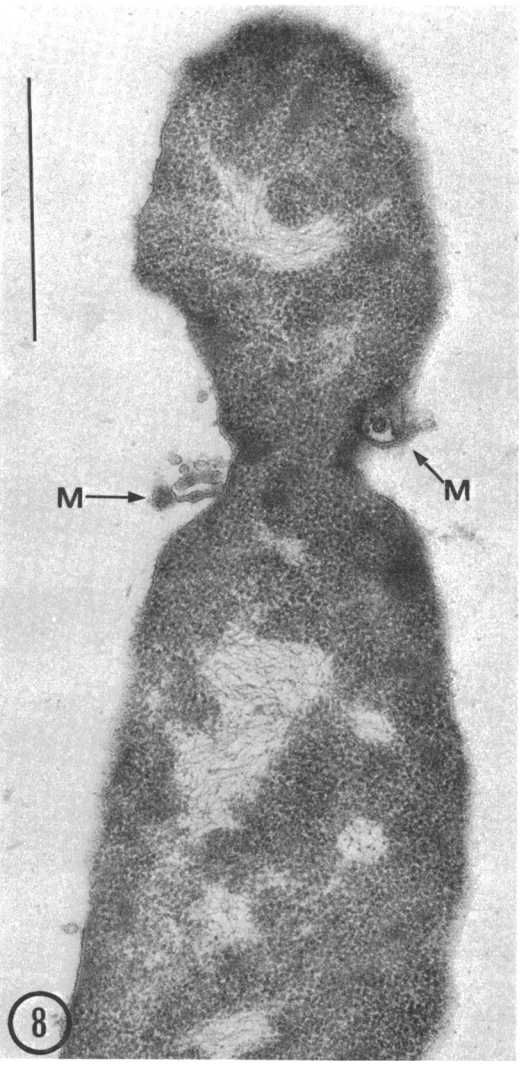
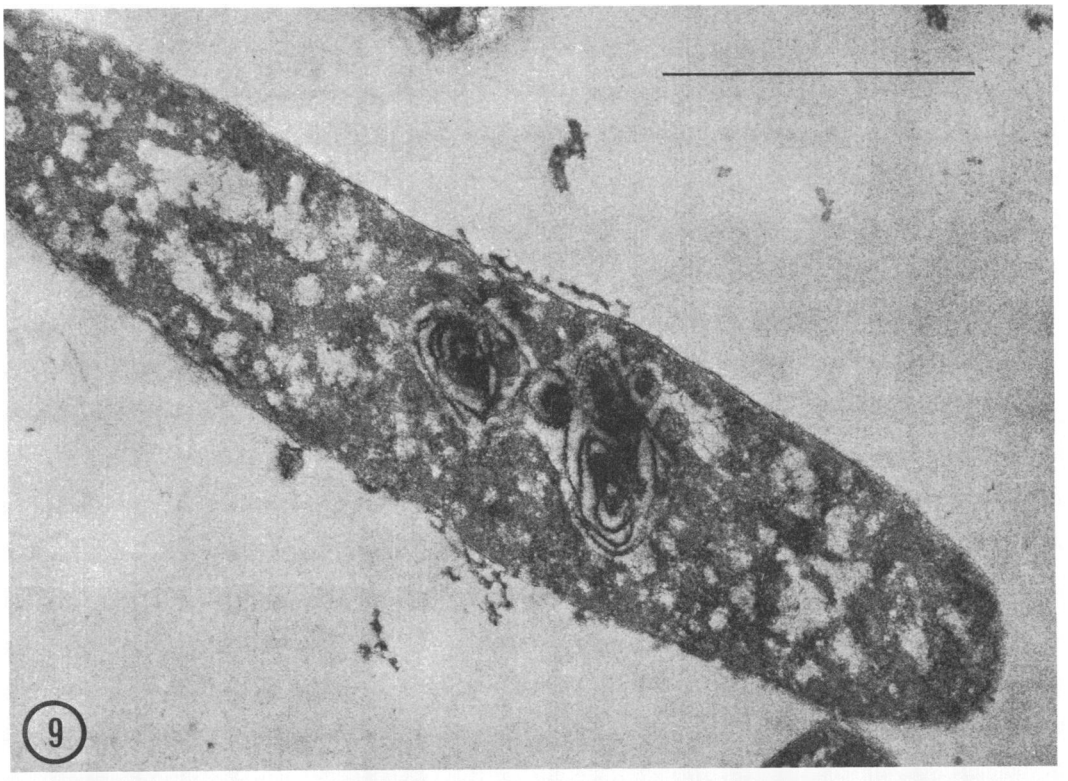


FIG. 7. *Here the drop in pH of the growth medium was effected with 1 N HCl. The bacillary shape of the gymnoplast was, under such conditions, well preserved, and the mesosomes, although somewhat disturbed, remained situated in the cytoplasm (Mc) and nucleoplasm (arrows, Mn). $MgCl_2$ was added in a concentration of 0.01 M, but the cytoplasm appears structureless. × 59,000. Bar indicates 0.5 μm.*

FIG. 8. *Same as in Fig. 7, but the gymnoplast was produced by treatment with lysozyme diffused through the agar medium. The fine structure of the cytoplasm and the nucleoplasm now looks much more normal, and the mesosomes are expelled from the cell. × 67,500. Bar indicates 0.5 μm.*

accompanied by the release of mesosomal tubules or vesicles in the surrounding medium. A most interesting illustration of this process was given by Ryter et al. (11). These authors presented electron micrographs of negatively stained preparations of gymnoplasts that were produced in hypertonic medium with 0.002 M $MgSO_4$; in these preparations, the extruded structures looked like a string of pearls attached to the surface of the gymnoplast, whereas in hypotonic medium the membranous structures fragmented (11).

Figures 7 to 11 show that, contrary to all descriptions in the literature, except that of Op den Kamp et al. (5), mesosomes are retained in gymnoplasts that had been treated by lowering of the *p*H of the growth medium. In these cells, the extrusion of mesosomes is greatly reduced in both hypertonic and hypotonic medium. In a previous communication (6), it was shown that under hypotonic conditions the gymnoplasts, when derived from cells grown at low *p*H, lose most of their intracellular material. In spite of this, in



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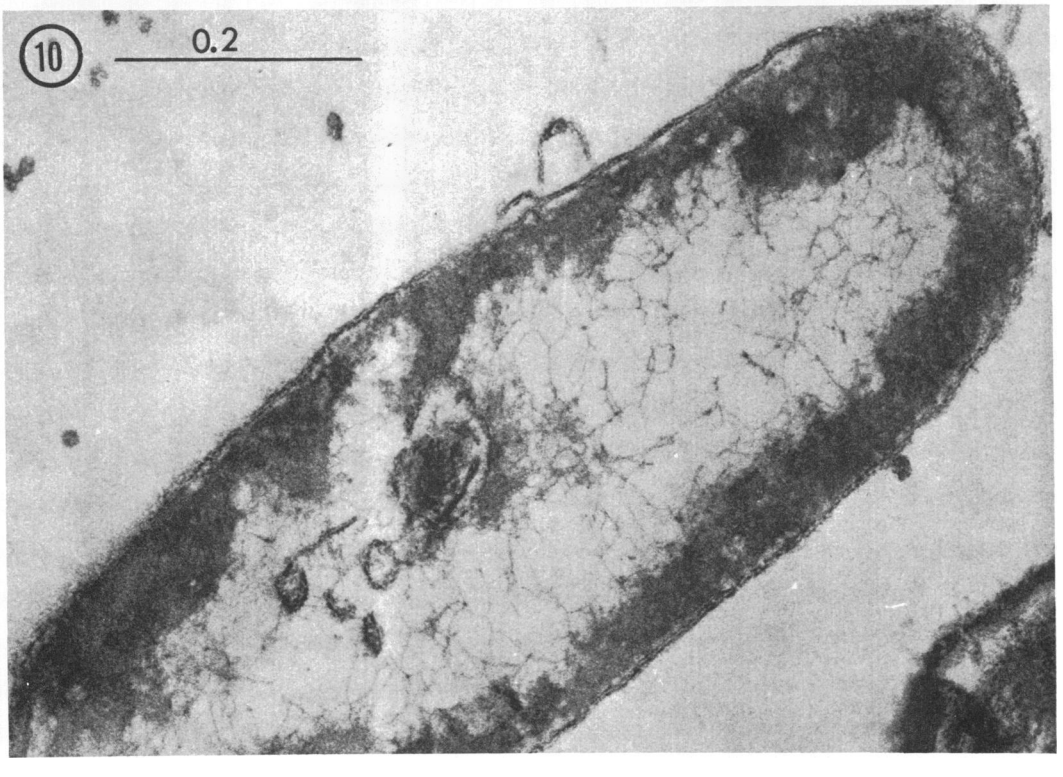


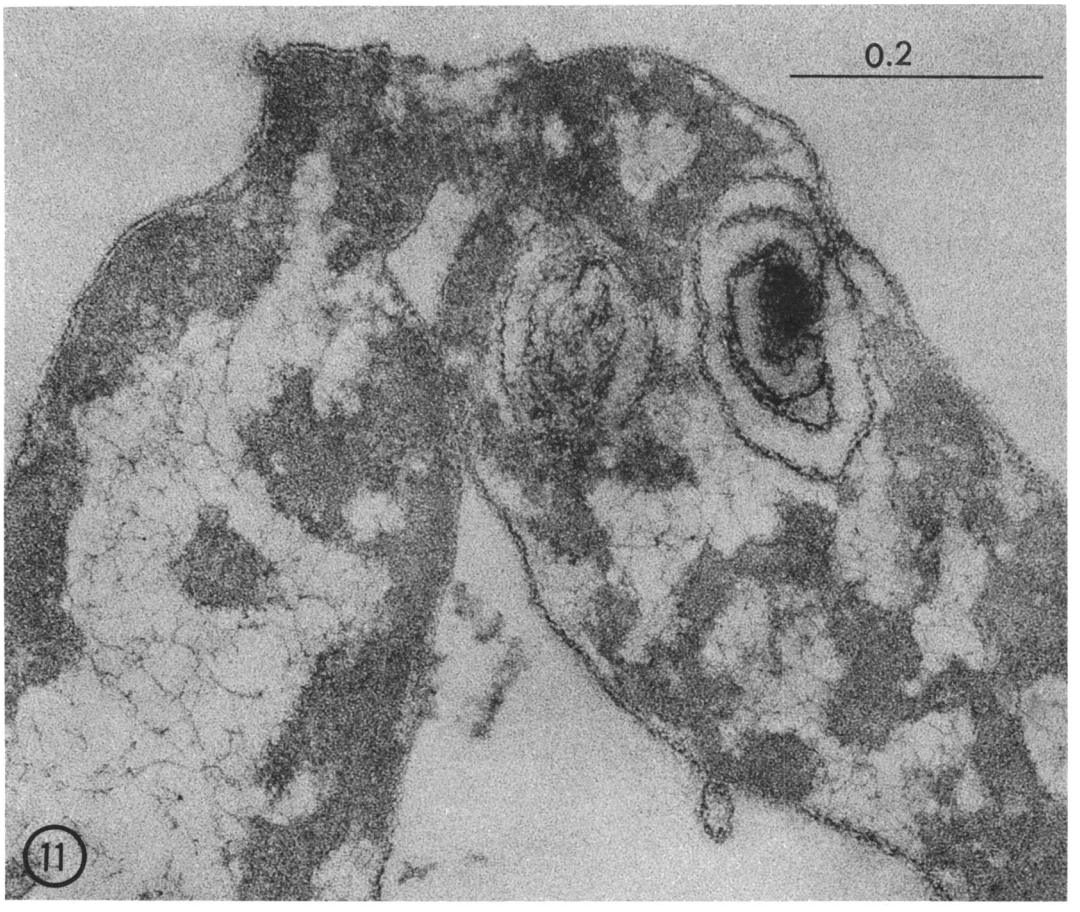


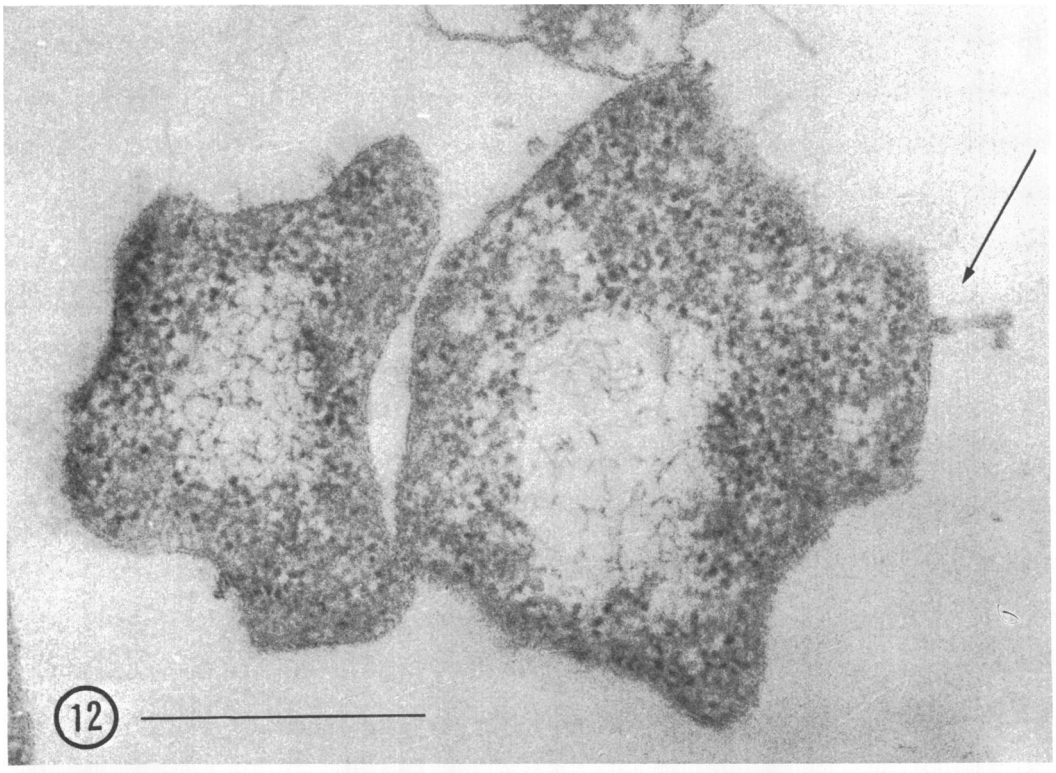
FIG. 11. *Gymnoplast of a cell grown at low pH produced in the absence of sugar and Mg^{2+} in the medium. Apparently, this gymnoplast is of an incomplete divided cell and has been bent during the processing (see top of the figure). Mesosomes are preserved in the right-hand cell half.* × *160,000. Bar indicates 0.2 μm.*

thin sections in the electron microscope (Fig. 10 and 11), a considerable amount of cell material was found to remain. It should be mentioned in this connection, however, that in the same preparation there was always a certain difference in the reaction of the individual cells as seen in the electron microscope.

The presence of magnesium ions in the suspending fluids of the gymnoplasts is known to stimulate the adherence of the pearl strings of mesosomal vesicles (11). In *B. licheniformis*, Rogers et al. (7) found that, up to a concentration of about 0.02 M Mg^{2+}, few mesosomes remain attached to the surface of the gymnoplasts, but that above 0.02 M Mg^{2+} increasing numbers of vesicles adhere to them. This could not be confirmed in our experiments with *B. subtilis*, in which more extensive adherence of mesosomes was found in preparations made with 0.01 M Mg^{2+} than in those made with 0.04 and 0.1 M Mg^{2+}. In agreement with the descriptions of Rogers et al. for *B. licheniformis*, both *B. subtilis* and *B. megaterium* failed, at a high Mg^{2+} concentration, to form spherical gymnoplasts from cells from neutral

FIG. 9. *Rod-shaped gymnoplast made from a cell grown overnight in medium B in which the pH dropped. Sucrose (0.25 M) was added to the conversion medium, but no extra $MgCl_2$. Due to the absence of Mg^{2+}, the cytoplasm has here become structureless. Note the large mesosome in the center of the cell.* × *81,700. Bar indicates 0.5 μm.*

FIG. 10. *Gymnoplast prepared similarly to the one in Fig. 9, but here sugar was omitted from the conversion medium. Notwithstanding the low tonicity of the medium, the original cell shape has been preserved as well as the mesosomal structures in it.* × *156,000. Bar indicates 0.2 μm.*



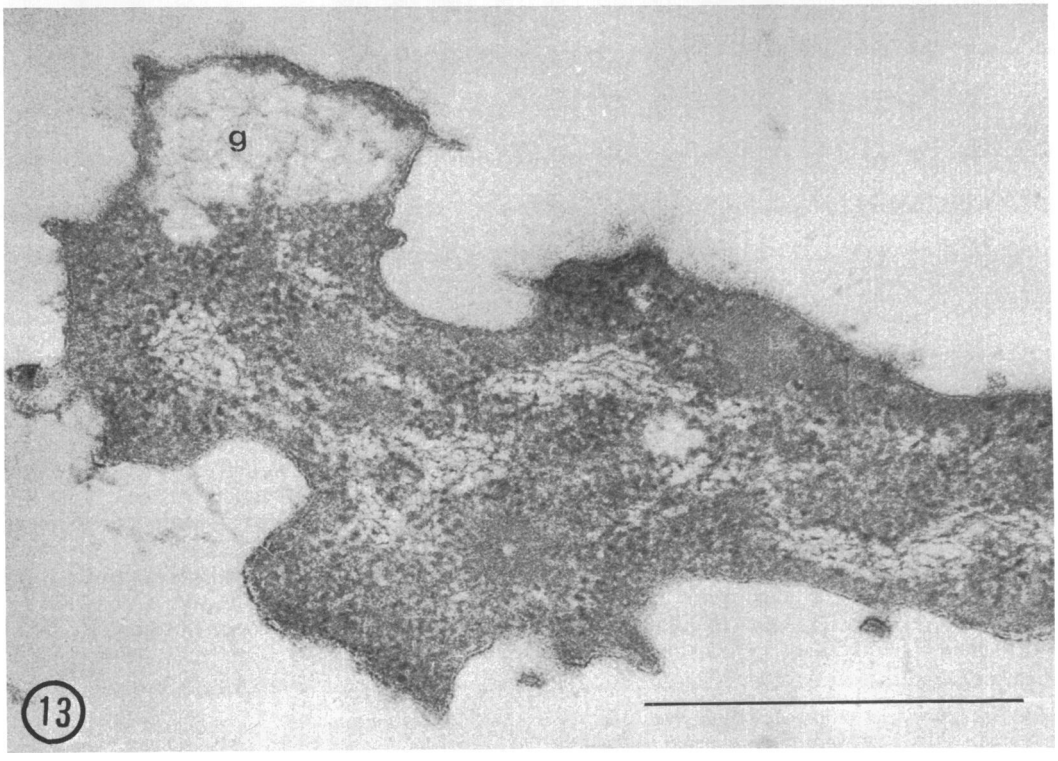


FIG. 12. *Gymnoplast of B. subtilis made in buffers with high Mg^{2+} concentrations (0.1 M) and 0.3 M sucrose. At elevated Mg^{2+} concentration, the gymnoplasts sometimes assumed shapes they did not possess before the conversion. Note the extruded mesosomes which tend to adhere to the cytoplasmic membrane (arrows).* × *74,000. Bar indicates 0.5 μm.*

FIG. 13. *Gymnoplast of B. megaterium made in a comparable manner to that of B. subtilis in Fig. 12, in a buffer with 0.04 M Mg^{2+} and 0.3 M sucrose. Through the influence of the high Mg^{2+} concentration, the gymnoplast has a bizarre outline. The nucleoplasm and cytoplasm are comparatively condensed.* × *96,500. Bar indicates 0.5 μm.*

medium. The shapes of such gymnoplasts, in particular of *B. megaterium*, in buffer with 0.25 M sucrose became quite bizarre; this effect did not occur with the same concentration of Mg^{2+} in buffer without sucrose. From this it may be concluded that the stabilizing effect of magnesium on the plasma membrane of these bacilli is intrinsically different from that brought about by lowering the *p*H of the culture medium.

The gymnoplasts, or naked protoplasts, from cells exposed to a *p*H of about 5 possess several properties which keep them from conforming to the description given by the 13 investigators (1) mentioned in the introduction.

The preservation of the original rod shape and the partial retention of the mesosomal membranes inside the gymnoplasts from cells exposed to a low *p*H is paralleled by a change in the phospholipid composition of the membrane fraction. This finding suggests that certain bacilli, after exposure to a low environmental *p*H during growth, possess membranes of a more rigid structure than those in cells from a more neutral culture medium.

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