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# STUDIES ON THE PHOSPHOLIPIDS AND MORPHOLOGY OF PROTOPLASTS OF *BACILLUS MEGATERIUM*

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SUMMARY

1. The phospholipids of the membrane fraction of cells of *Bacillus megaterium* (MK 10D) cultured at pH 7.0 were found to consist of cardiolipin (5%), phosphatidyl ethanolamine (40%), phosphatidyl glycerol (40%) and *O*-lysyl phosphatidyl glycerol (15%). The content of phosphatidyl glycerol was decreased to 8% in cells harvested at pH 5.0, whereas glucosaminyl phosphatidyl glycerol represented 32% of the total phospholipids. The content of other phospholipids remained constant.

2. Protoplasts derived from cells harvested from different media displayed a different behaviour during lysis experiments in hypotonic sucrose.

3. Electron microscopy demonstrated that cells grown at pH 7.0 gave spherical protoplasts, whereas from cells exposed to pH 5.0, rod-shaped protoplasts were produced by lysozyme. In the latter protoplasts the original structure of the bacteria was maintained to a great extent even after exposure to hypotonic conditions. Similar protoplasts, when derived from cultures in which overnight the pH dropped from 7.0 to 5.0, also tend to preserve more of the original structural organisation.

4. Environmental conditions may induce differences in chemical make-up or physical properties of lipoprotein structures resulting in significant variation in the morphology of bacterial protoplasts.

## INTRODUCTION

In certain Gram-positive bacteria significant differences were found in the proportions between various phospholipids, depending on the conditions of the culture at the time of harvesting the cells[1-4]. HOUTSMULLER AND VAN DEENEN[3,4] reported that the ratio between aminoacyl phosphatidyl glycerol and phosphatidyl glycerol could be increased in *Staphylococcus aureus* and *Streptococcus faecalis* by lowering the pH of the medium, but other bacterial species did not respond in a similar way upon exposure to an acidic environment[4,5]. Alterations in the proportions of differently charged phospholipids may be a reflection of a more profound

change in the composition and properties of lipoprotein membranes. In order to investigate this possibility, experiments were carried out with *Bacillus megaterium*. Cultivation in two different media (with and without glucose and $(NH_4)_2SO_4$ as an additional supply of nitrogen) caused a significant difference with respect to the content of phosphatidyl glycerol and a new phospholipid which was identified as glucosaminyl phosphatidyl glycerol[6,7]. In the present paper further details are given on the phospholipids, the morphology, and some properties of protoplasts of cells of *B. megaterium* grown under different conditions.

## EXPERIMENTAL

*B. megaterium* MK 10D (Rijksinstituut voor de Volksgezondheid, Utrecht) was cultivated at 37° under strong aeration in 1-l portions of one of the following media. Medium A contained: 10 g of pepton, 10 g of yeast extract, 5 g of NaCl, 400 mg of sodium phosphate and 300 $\mu$C of [$^{32}$P]orthophosphate per l of water (pH 7.0). Medium B contained, in addition: 20 g of glucose and 2 g of $(NH_4)_2SO_4$. The cells, harvested at various times were washed with distilled water which was acidified to pH 5.0 to prevent degradation of alkali-labile phospholipids. The cells were lyophilized, weighed and extracted with chloroform–methanol buffer mixtures as described by HOUTSMULLER[4]. Lipid extracts were dried *in vacuo*, weighed and analysed for their phosphorus content[8]. Separation of the phospholipids was achieved by chromatography on silica-impregnated paper using the solvent system of MARINETTI, ERBLAND AND KOCHEN[9]. The relative amount of each phospholipid was measured by scanning the chromatograms in front of an end-window Geiger–Müller tube.

### *Preparation of protoplasts*

*B. megaterium* cells were converted into protoplasts in 0.06 M phosphate buffer (pH 6.2) containing 0.3 M sucrose after the addition of 1 mg *N*-acetylmuramide glucanohydrolase (lysozyme, EC 3.2.1.17) per ml of suspension. From these protoplasts, a membrane fraction was isolated after suspending the protoplasts in a hypotonic phosphate buffer (0.06 M, pH 6.2), ultrasonication for 30 sec, and centrifugation at 26 000 $\times$ *g* for 30 min. The osmotic behaviour of protoplasts was measured after diluting a concentrated protoplast suspension with a sucrose-containing phosphate buffer (0.06 M, pH 6.2) until an absorbance of 0.500 was reached. The decrease in absorbance was measured in a Unicam spectrophotometer at 550 m$\mu$. The amount of 260 m$\mu$ absorbing material released into the medium was measured in the supernatant after centrifugation at 30 000 $\times$ *g* for 15 min. Diaminopimelic acid was determined after acid hydrolysis of isolated protoplasts according to RHULAND *et al.*[10].

### *Electron microscopy*

For the purpose of studying sectioned protoplasts, three different approaches were followed: (1) Cells of *B. megaterium* were grown in fresh medium A for 4, 5 or 18 h and then converted into protoplasts. (2) Cells in medium A from a 5-h culture were incubated, prior to conversion into protoplasts, for an additional 1 h at pH 5 adjusted with 4 M HCl. (3) Cells grown in glucose-rich medium B, in which the pH

had dropped naturally to about 5, were converted into protoplasts immediately after 18 h growth (*i.e.* in the early stationary phase). Cells were converted into protoplasts at 37° with lysozyme in the acetate–veronal buffer[11] with 0.3 M sucrose at pH 6. After the conversion the protoplasts were spun down and fixed overnight. In all cases the acetate–veronal buffer was supplemented with tryptone and 0.01 M magnesium acetate.

After trying various fixation methods, we found that the usual fixation in $OsO_4$ in acetate–veronal buffer, as suggested by RYTER AND KELLENBERGER[11], but supplemented with 0.3 M sucrose, was suitable for preservation of fine structure in protoplasts. In one set of experiments, protoplasts were fixed and prepared in a hypotonic medium, *i.e.* by omitting sucrose in the buffers (Figs. 16–18).

Protoplasts were also made under conditions of protection by agar. To this end, the bacilli were embedded in agar made up with acetate–veronal buffer and 0.3 M sucrose. The agar was chopped up into minute blocks, which were then submerged for 1 h in lysozyme dissolved in acetate–veronal buffer, after which the protoplasts were fixed through the agar and treated as usual with uranyl acetate (Figs. 19–21)*. Cells and protoplasts were embedded either in Vestopal W or in Epon. Sections were cut on an LKB ultratome, stained with lead according to REYNOLDS[12] and photographed with a Philips EM 200.

## RESULTS

### *Phospholipid composition*

The qualitative differences in the phospholipid composition of cells of *B. megaterium* grown in media A and B are illustrated by the autoradiograms in Fig. 1. The cells harvested from medium A (without glucose) were found to contain at least four phospholipids. The compound with the lowest mobility already has been reported to be identical to lysyl phosphatidyl glycerol[6]. Chromatographic comparison with a chemically synthesized substance[13] confirmed this conclusion. The two other major phospholipids were demonstrated to be identical with phosphatidyl glycerol and phosphatidyl ethanolamine. A minor spot exhibited chromatographic properties similar to those of synthetic diphosphatidyl glycerol. Because this fraction was not thoroughly investigated it is possible that other anionic phospholipids are present in small quantities as well. The cells grown in medium B (containing glucose) were found to contain, in addition to these phospholipids, a fifth component, which was isolated by a combination of column and thin-layer silicic acid chromatography[6,7]. On the basis of data from alkaline and acid hydrolysis experiments, and a glucosamine:nitrogen:phosphorus:glycerol ratio of 1.0:1.1:1.1:2.0, it has already been concluded that this phospholipid is a glucosamine derivative of phosphatidyl glycerol[6,7]. A similar phospholipid was described by PHIZACKERLEY, MAC DOUGALL AND FRANCIS[14] in *Pseudomonas ovalis*. Determination of the amino nitrogen content ($NH_2$:P, 1:1) confirmed that no *N*-acetylglucosamine was present. Extension of earlier reported hydrolysis experiments with phospholipase C confirmed that this enzyme catalyses the release of glucosaminyl glycerophosphate[15]. Further degradation of this product by a phosphomonoesterase of wheat germ (Sigma Chemical Co.,

* This method resembles that of RYTER AND LANDMAN[21], but was developed independently.

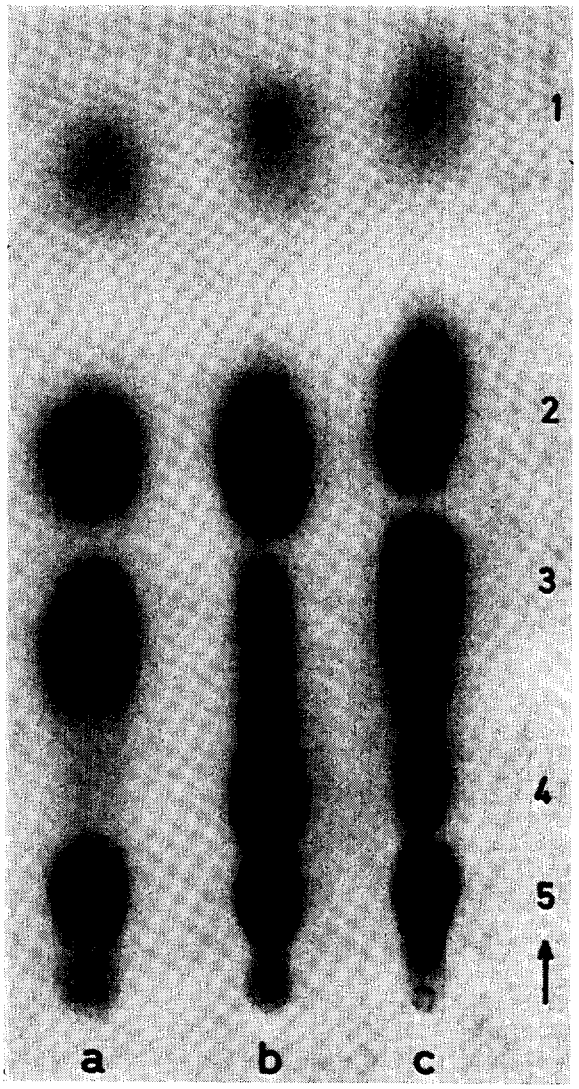
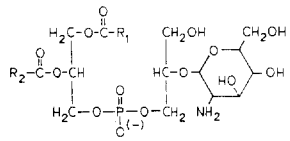



Fig. 1. Autoradiograms of phospholipids from *B. megaterium*. Paper chromatograms were developed on silica-impregnated paper with diisobutyl ketone–acetic acid–water (40:25:5, v/v/v)[9]. a, phospholipids of cells grown in medium A for 5 h. A similar pattern was obtained from 18-h cultures. b, phospholipids of cells grown in medium B for 18 h. c, phospholipids of cells grown in medium A for 5 h. Thereafter the pH of the culture was brought to 5.0 with the aid of HCl and the incubation was prolonged 1 h. The compounds are 1, polyglycerol phospholipid presumably cardiolipin; 2, phosphatidyl ethanolamine; 3, phosphatidyl glycerol; 4, glucosaminyl phosphatidyl glycerol; 5, lysyl phosphatidyl glycerol.

St. Louis, Mo.) yielded $P_i$ and glucosaminyl glycerol. A negative reaction of the latter product with the periodate–Schiff reagent indicated that the glucosamine is bound to the 2-position of glycerol. Measurement of the periodate consumption of the intact phospholipid demonstrated that the *trans* hydroxyl groups of the glucosamine constituent are free, while the compound did not react with the alkaline

$AgNO_3$ reagent[16]. The stereochemical configuration of the two glycerol moieties was found to be identical to that found in phosphatidyl glycerol[17]. Hence it seems likely that this phospholipid is identical to 1,2-diacyl-glycerol-3-phosphoryl-1′-glycerol-*O*-(2′→1)glucosamine (I). The configuration of the glycosidic bond is under in-



Glucosaminyl phosphatidyl glycerol (I)

vestigation. When the pH of a culture growing in medium A was artificially lowered to 5.0 by the addition of HCl, the glucosamine-containing phospholipid could be detected as well (Fig. 1). Quantitative data on the lipids of *B. megaterium* grown in media A and B are compiled in Table I. The presence of glucose in the medium

TABLE I

LIPID CONTENT OF *B. megaterium*

Values are the means of 40 experiments and are expressed as weight percentages followed by the standard deviation. Cells were harvested from 18-h cultures.

| | *Medium A* | *Medium B* |
|---|---|---|
| Total lipid | 2.1 ± 0.3 | 4.0 ± 0.5 |
| Total phospholipid | 0.9 ± 0.3 | 0.9 ± 0.3 |
| Cardiolipin | 0.05 ± 0.01 | 0.05 ± 0.01 |
| Phosphatidyl ethanolamine | 0.39 ± 0.05 | 0.37 ± 0.05 |
| Phosphatidyl glycerol | 0.40 ± 0.05 | 0.08 ± 0.05 |
| Glucosaminyl phosphatidyl glycerol | — | 0.30 ± 0.05 |
| *O*-Lysyl phosphatidyl glycerol | 0.14 ± 0.03 | 0.13 ± 0.03 |

doubled the amount of total lipid per g dry weight of cells, whereas the quantity of phospholipids was the same in both cases. The increase of total lipids is probably due to an increase in the formation of β-hydroxybutyric acid as demonstrated by LEMOIGNE[18] and confirmed by the electron micrographs showing the presence of more granules in cells from medium B (Figs. 10 and 13). The present data do not allow conclusions to be made about the amount of phospholipid per cell. The values of weight percentages on a dry weight basis revealed that the quantities of cardiolipin, phosphatidyl ethanolamine, and lysyl phosphatidyl glycerol are not influenced to any appreciable extent by the different growth conditions used (Table I). However, a significant difference can be noted with respect to the amount of phosphatidyl glycerol which is decreased in cells of *B. megaterium* grown in medium B when compared with those harvested from medium A. This decrease is counterbalanced nearly quantitatively by the occurrence of glucosaminyl phosphatidyl glycerol. This phenomenon was quite reproducible as it was demonstrated in 40 experiments which gave only relatively small differences in phospholipid composition between the batches harvested from either medium A or B (compare also Table II). That the occurrence of the glucosaminyl phosphoglyceride in glucose-grown cells is at least

TABLE II

PHOSPHOLIPID COMPOSITION OF *B. megaterium*

Values are expressed as percentages of total phospholipid followed by the standard deviation.

| *pH at harvesting* | *Medium A pH 7.2* | *Medium B pH 4.8–5.2* | *Medium A pH 5.0* | *Medium B pH 7.2* |
|---|---|---|---|---|
| Cardiolipin | 5 ± 0.5 | 5 ± 0.5 | 5 ± 0.5 | 5 ± 0.5 |
| Phosphatidyl ethanolamine | 40 ± 2 | 40 ± 2 | 35 ± 3 | 40 ± 3 |
| Phosphatidyl glycerol | 40 ± 2 | 8 ± 2 | 29 ± 3 | 40 ± 2 |
| Glucosaminyl phosphatidyl glycerol | — | 32 ± 4 | 15 ± 3 | — |
| Lysyl phosphatidyl glycerol | 15 ± 1 | 15 ± 1 | 16 ± 1 | 15 ± 1 |
| Number of experiments | 40 | 40 | 5 | 5 |

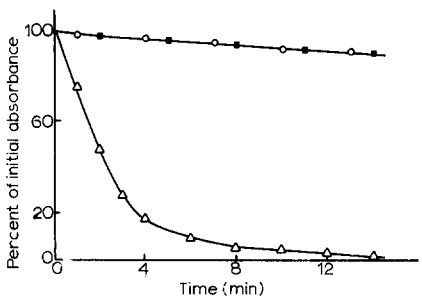
partly a function of the pH, which in medium B attains a lower value than in medium A, is borne out by the following experiments.

Cells were grown in the glucose-free medium A at pH 7.2, which was adjusted to a value of 5.2 1 h before harvesting the cells. This procedure caused a shift in the phospholipid composition (see Fig. 1) resulting in a decrease of the relative quantity of phosphatidyl glycerol compensated by the occurrence of glucosaminyl phosphatidyl glycerol (Table II). The maximal alteration was found to be reached in about 1 h, after changing the pH. Alternatively, cells were grown in the glucose-containing medium B, but the pH, which normally drops to about 5.0, was maintained at its initial value of 7.2. Under these conditions, the phospholipid composition turned out to be the same as that of cells grown in medium A, namely a high content of phosphatidyl glycerol, while the glucosaminyl derivative was not detectable (Table II). Some differences are to be noted between *B. megaterium* and *S. aureus* with respect to the alteration in their phospholipid composition as a function of the pH of the medium. HOUTSMULLER AND VAN DEENEN[3,4] observed that in an acidic medium the quantity of phosphatidyl glycerol of *S. aureus* was decreased, this being only partially counterbalanced by an increased level of lysyl phosphatidyl glycerol. In *B. megaterium*, the lysyl phosphatidyl glycerol content remained constant. Furthermore, in experiments on *S. aureus*, about the same ratio between lysyl phosphatidyl glycerol and phosphatidyl glycerol could be obtained, independent of the way the acidic pH was induced. However, in the present experiments, the content of the glucosamine-containing phosphoglyceride was always significantly higher in cells harvested from medium B, when compared with cells from medium A where the pH was abruptly lowered. This alteration was found to inhibit the growth immediately. For that reason the pH of a culture growing in medium A was more gradually altered by the addition of lactic acid in such a way that the pH decreased at a rate similar to that found to occur in cultures in medium B. Under such conditions the content of glucosaminyl phosphatidyl glycerol was found to be the same in lipid extracts from both cultures.

*Properties of protoplasts*

Protoplasts of cells grown in media A and B were lysed in a hypotonic sucrose solution, or by sonication, and a membrane-containing fraction was isolated by centrifugation at 30 000 × *g* for 30 min. This fraction was found to contain 95%

of the phospholipids which revealed the differences in composition discussed above. During the preparation of these protoplasts, it was observed that their shapes were quite different. Under the phase-contrast microscope, protoplasts prepared from cells grown in medium A exhibited the typical spherical form, whereas lysozyme treatment of cells grown in medium B produced structures having the same rod-like form of the intact cells. Cells from medium A, the pH of which was altered by the addition of HCl, also gave protoplasts which possessed this peculiar form. Both types of structures were found to be devoid of diaminopimelic acid. Furthermore, all protoplasts were Gram-negative and had lost their ability to divide. The behaviour of these protoplasts in hypotonic sucrose solutions was quite different, at least when absorbance measurements were made at 550 mμ (Fig. 2). Protoplasts of cells cultured in medium A gave a rapid decrease in absorbance, whereas this characteristic did not alter when a suspension of protoplasts obtained from glucose-grown cells (medium B) was used. A similar difference was observed when the experiments were done with suspensions in sucrose solutions of different molarities (Fig. 3). The pH



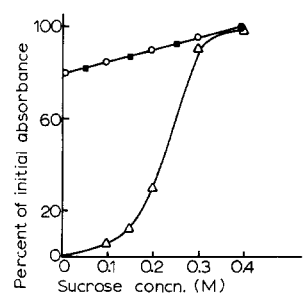




Fig. 2. Behaviour of *B. megaterium* protoplast in hypotonic medium. Protoplasts prepared from cells grown for 18 h in media A (△), B (○) and A in which culture the pH was adjusted to 5.0 with the aid of HCl 1 h before harvesting (■), were suspended in a hypotonic solution of 0.15 M sucrose in 0.06 M phosphate buffer (pH 6.2) until an absorbance of about 0.500 was reached. The decrease of the absorbance was measured at 550 mμ. Values are corrected for the absorbance due to membrane fragments.

Fig. 3. Behaviour of *B. megaterium* protoplasts in sucrose–phosphate buffer. Protoplasts prepared from cells grown (18 h) in media A (△) and B (○) were suspended in 0.06 M phosphate buffer (pH 6.2) having different sucrose concentrations. The initial absorbance was about 0.500. After 30 min the final absorbance was measured at 550 mμ and expressed as per cent of the initial value. The same experiments were carried out with protoplasts made from cells grown in medium A for 4 h, thereafter the pH of the culture was brought to 5.0 with the aid of HCl and the incubation was prolonged 1 h (■).

of the hypotonic solutions used in these experiments was 6.2, and similar results were obtained at values ranging from pH 5.0 to pH 7.0. Furthermore, protoplasts made of cells from medium A, the pH of which was adjusted to 5 1 h before harvesting, behaved like those obtained from glucose-grown cells (Figs. 2, 3). The behaviour of the rod-shaped protoplasts in the hypotonic solutions may cast some doubt on the designation of these structures as protoplasts. On the other hand, these experiments may be misleading in the sense that the measurement at 550 mμ is not a correct indication for the occurrence or non-occurrence of lysis, because the absorption at this wavelength is to be attributed to the presence of particles. For that

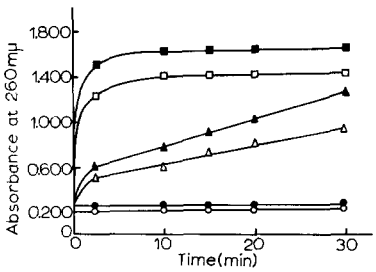



Fig. 4. Release of 260-mμ absorbing material from *B. megaterium* protoplasts. *B. megaterium* cells grown in medium A (18 h) were converted to protoplasts in the usual manner. 1-ml portions of this protoplast suspension were diluted with respectively 10 ml 0.3 M sucrose containing phosphate buffer (●), 10 ml 0.15 M sucrose containing phosphate buffer (▲) and 10 ml buffer (■). After incubation the protoplasts and membranes were centrifuged at 26 000 × *g* for 5 min and the absorbance from the supernatant was measured at 260 mμ. The same experiments were carried out with protoplasts derived from *B. megaterium* grown in medium B (18 h), which were diluted with 0.3 M sucrose buffer (○), 0.15 M sucrose buffer (△) and buffer (□).

reason the experiments in hypotonic sucrose solution were repeated, and after removal of the remaining structures by centrifugation, the absorbance of the supernatant was measured at 260 mμ (Fig. 4). It was found that from both types of protoplasts nucleic acid-containing material was released. Hence, it appears likely that the rod-shaped protoplast, even after lysis, maintains the original form of the bacterium. Conclusive evidence for this view was obtained by electron microscopy.

*Electron microscopy*

*Intact cells (Figs. 5–8).* It was found necessary to fix the intact bacilli with omission of sucrose in the buffer, since at a level of 0.3 M sucrose the hypertonicity of the buffer proved to be so high that the cytoplasm retracted from the cell walls. Such plasmolysis has been referred to in many previous cases[19–23].

*B. megaterium* cells grown overnight in media A (Fig. 5) and B (Figs. 6 and 7) differ in some aspects of their fine structure. In these cells from the early stationary phase the cell walls are thick and straight and in smooth contact with the plasma membrane as is usual in Gram-positive bacteria[24]. The bacilli developed more capsular material in the glucose-rich medium B than in medium A (*cf.* Figs. 6 and 7 with Fig. 5). The cytoplasm in cells from both media is compact. It contains invaginations of the plasma membrane which have been described variously as 'membranous organelles'[19,25] 'chondrioids'[26,27] or mesosomes[28]. In the sections of cells grown in medium A (Fig. 5), the nuclear material is often seen to be organized in a rather fragmentary fashion between rounded spaces. With the light microscope, *B. megaterium* can be observed to accumulate bright refractile granules which long have been known to be a lipid[29] and identified as a polymer of β-hydroxybutyrate[18]. ROBINOW[30] stressed that 'the chromatin bodies seem to be softer than the lipid globules and are moulded by them into a variety of different shapes'. This seems also to be the case with the nuclear material in the cells of Fig. 5. Empty rounded areas to be seen in the sections are considered to be caused by the extraction of poly-β-hydroxybutyrate accumulations during the preparation procedure. Cells grown in the glucose-rich medium B possess irregularly shaped areas of greater elec-

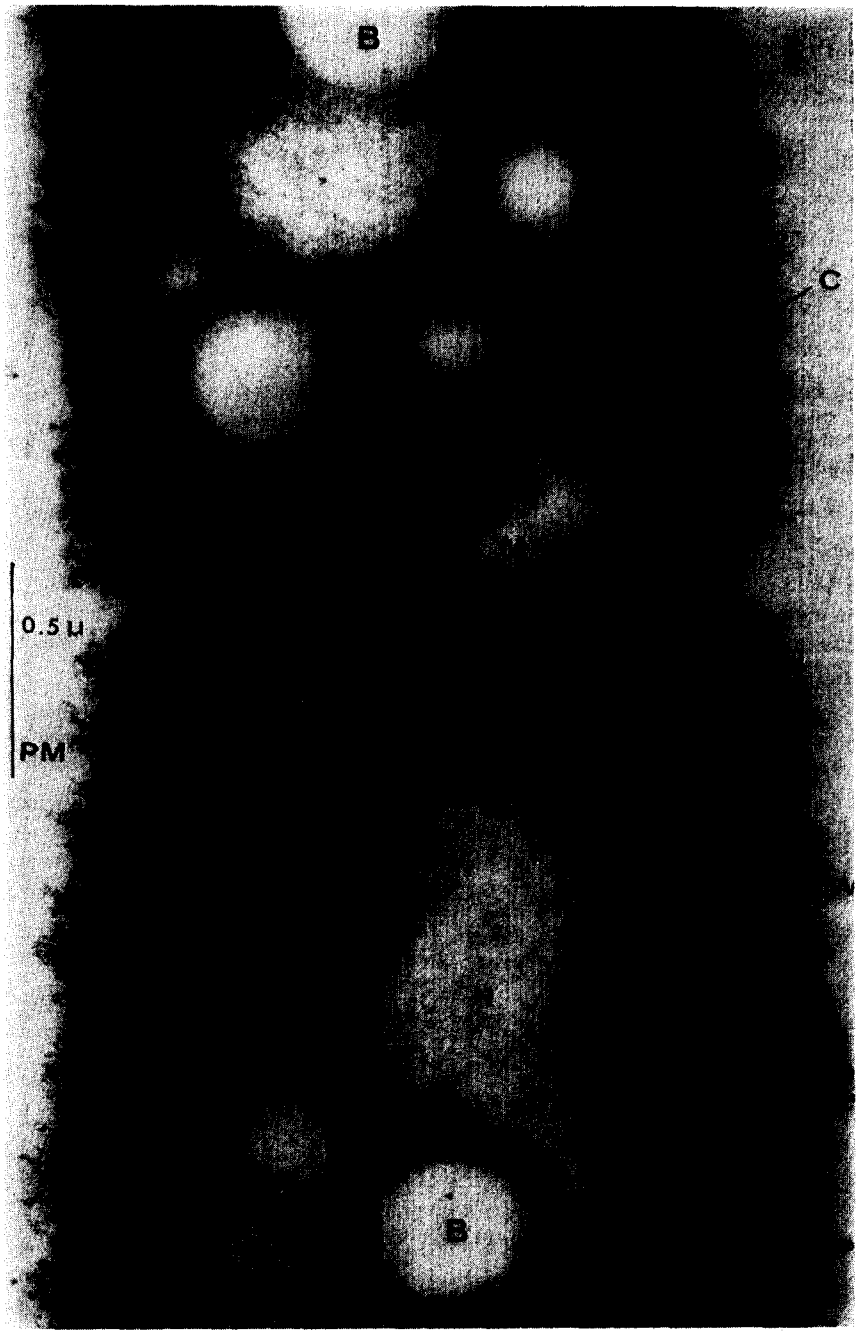



Fig. 5. Part of two adjacent cells grown overnight at neutral pH (medium A). Note that the nucleoplasm is molded by reserve material inclusions (poly-β-hydroxybutyrate) which are dissolved during the specimen preparation. Abbreviations: C, capsular material; N, nucleoplasm; W, cell wall; B, poly-β-hydroxybutyrate inclusions; PM, plasma membrane; P, inclusions, presumably of polysaccharide; M, membranous material (mesosome).


Fig. 6. Cell grown overnight in medium B during which the pH dropped to 5. Note the strong development of capsular material (C), and centrally an inclusion presumably of glycogen (P).

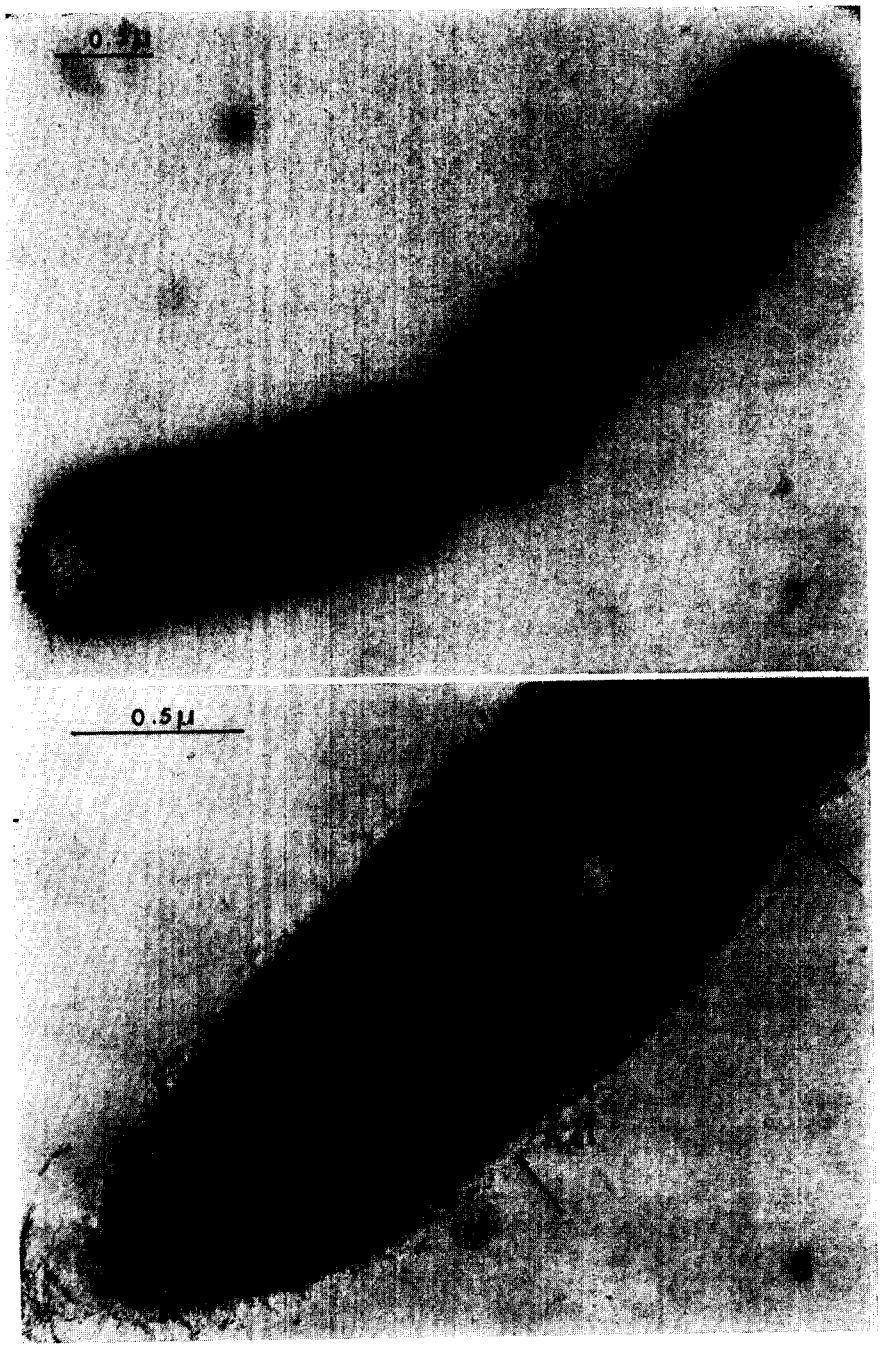



Fig. 7. Cells grown overnight in medium B. The inclusions of polysaccharides are at both poles. Nucleoplasm in narrow areas scattered in the cytoplasm.

Fig. 8. Cell in early exponential phase of growth in medium A exposed to pH 5 adjusted with HCl. Note at arrows the effect of the treatment on the cell wall and capsular material.

tron transparency than the surrounding cytoplasm (Figs. 6 and 7). In the lead-stained sections such areas remind one of the stored glycogen observed in sections of liver cells[31]. ELLAR AND LUNDGREN[32] interpreted with reservation in *B. cereus* similar areas as glycogen, whereas HOLME AND CEDERGREN[33] found areas much like these in *Escherichia coli* which had stored glycogen. It therefore seems reasonable to assume that the transparent areas in *B. megaterium* grown in glucose-rich medium B are likewise accumulations of this polysaccharide. In Fig. 6 the location of this reserve material is more or less in the cell centre, whereas it is also frequently found at both cell poles (Fig. 7).

Fig. 8 represents part of a cell grown for 4 h in fresh medium A, followed by another hour of incubation in the same medium after its pH was lowered with HCl to 5. It can be seen that this acid treatment affects the structure of the cell wall and of the capsular material. Sometimes there are in the periphery of such cells extensive areas of membranous structure. Accumulations of reserve material are scarce in the young cells.

*Protoplasts in hypertonic media (buffers containing 0.3 M sucrose as stabilizing agent) (Figs. 9–15).* Interesting differences in shape and fine structure were found between the protoplasts made from cells grown at neutral pH and those grown at low pH either adjusted with HCl or more naturally by overnight growth in medium B (*cf.* at low magnification Figs. 9–11 and at higher magnification Figs. 12–15). The protoplasts in Figs. 9 and 11 were both made from cells in the early exponential

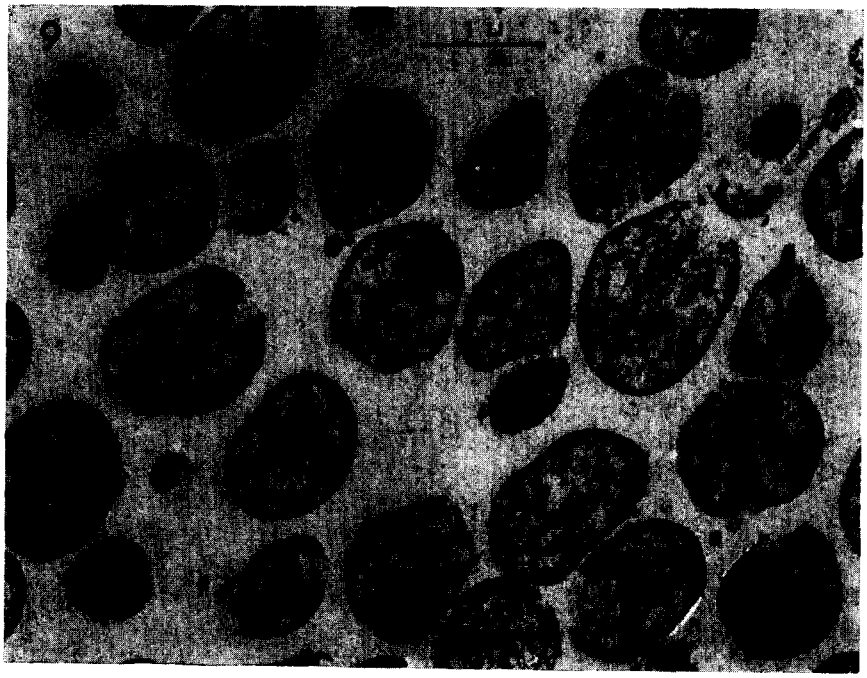


Fig. 9. Protoplasts in hypertonic medium: these protoplasts were made from cells in the exponential phase of growth from medium A (pH 7). The protoplasts are rounded and have preserved much of their content. Deviations from a sphere and breakage of the plasma membrane are supposed to be due to their processing, such as embedding in agar after formation and fixation. Note strong hydration and disarrangement of nuclear areas.

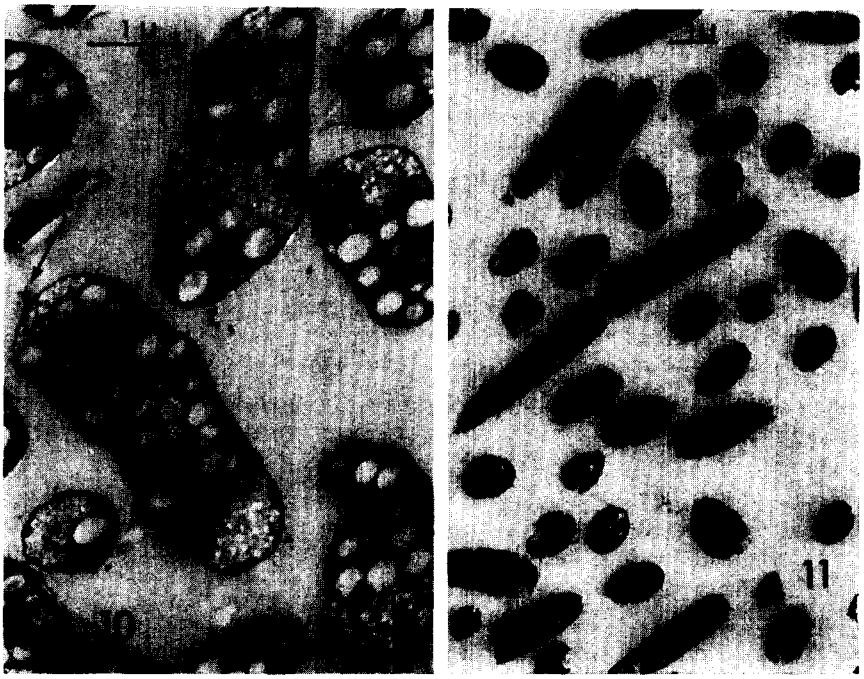


Fig. 10. Protoplasts in hypertonic medium: these protoplast were made from cells in the early stationary phase from medium B (pH 5). Such protoplasts are often elongated rods, but sometimes their shapes are somewhat distorted, presumably due to the handling and embedding in agar after their fixation. The organisation of the nucleoplasm and cytoplasm have hardly changed as compared to intact cells, but membranous vesicles are often expelled (arrow).

Fig. 11. Protoplasts in hypertonic medium: these protoplasts were made from cells like those in Fig. 9, but which have been exposed during 1 h at pH 5 adjusted with HCl. Such protoplasts keep their original shapes remarkably well.

phase of growth, the difference being in the exposure during 1 h to pH 5 in the case of Fig. 11, a treatment which caused the protoplasts strikingly to preserve their original cell shapes. The protoplasts in Fig. 10 which appear to be loaded with reserve materials, both lipids and polysaccharides, are of an overnight culture in medium B in which the pH had dropped naturally to 5.0. Under these conditions the protoplasts appear to have retained the original shape, although they are somewhat more deformed than those subjected to HCl treatment; sometimes they are elongated rods, but frequently their shapes appear to be somewhat distorted (Fig. 10), presumably due to the way they were handled. On the other hand, protoplasts made from cells of an 18-h culture in medium A appear to have the same spherical form as those shown in Fig. 9. At higher magnifications (Figs. 12–15) it can be seen that the protoplasts made from cells grown in medium B (Fig. 13) preserved their cytoplasm and nucleoplasm more naturally than those of medium A (Fig. 12). In Fig. 12 the nucleoplasm is swollen and occupies large irregular areas between the cytoplasm which has a loosened texture. The harsh treatment of the cells exposed to pH 5.0 during 1 h appears to be the most effective in this respect (Figs. 14 and 15). Under these conditions the original cell shape was found to be preserved best, and even the membrane systems (mesosomes), although somewhat disarranged, still

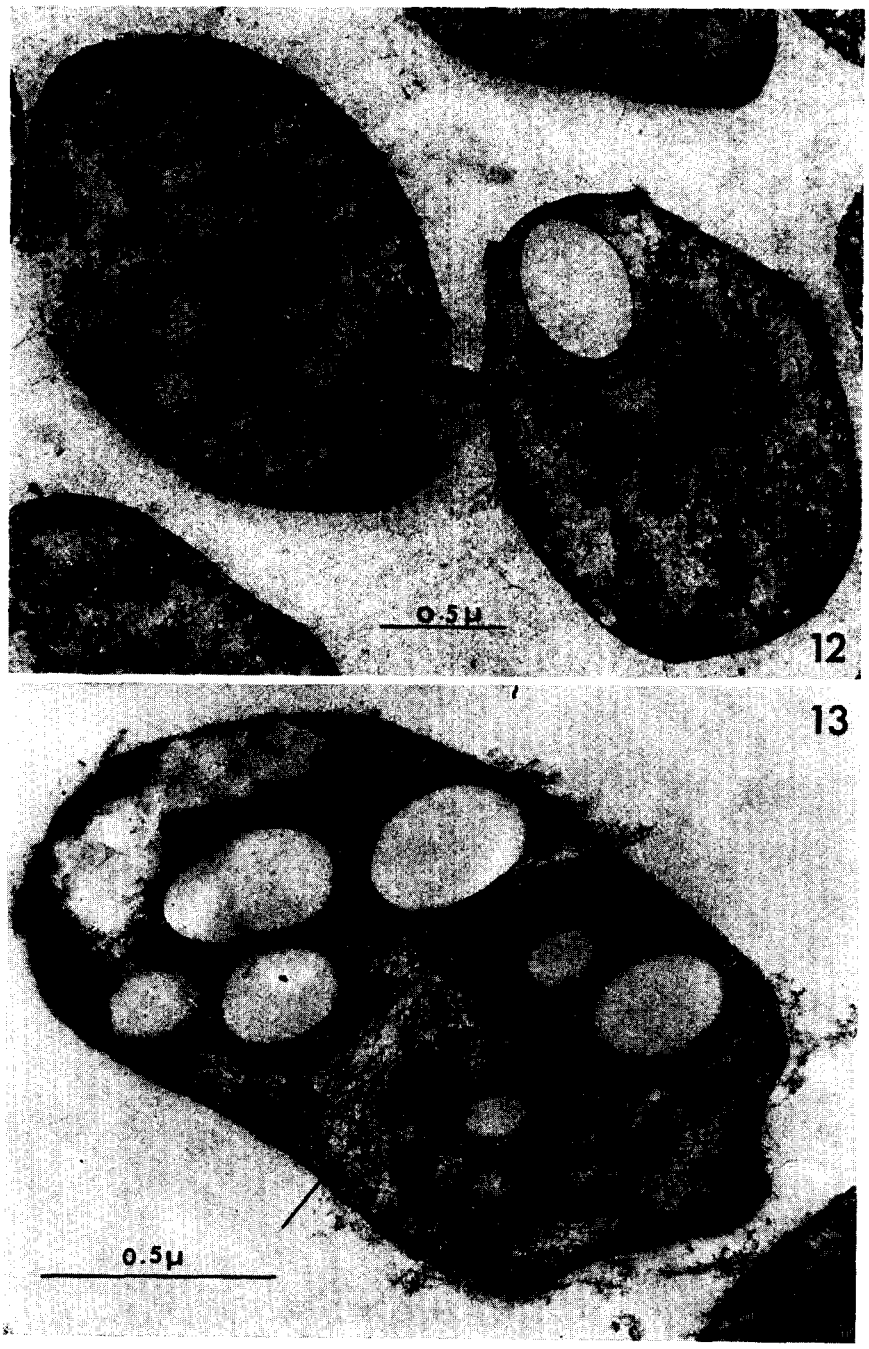
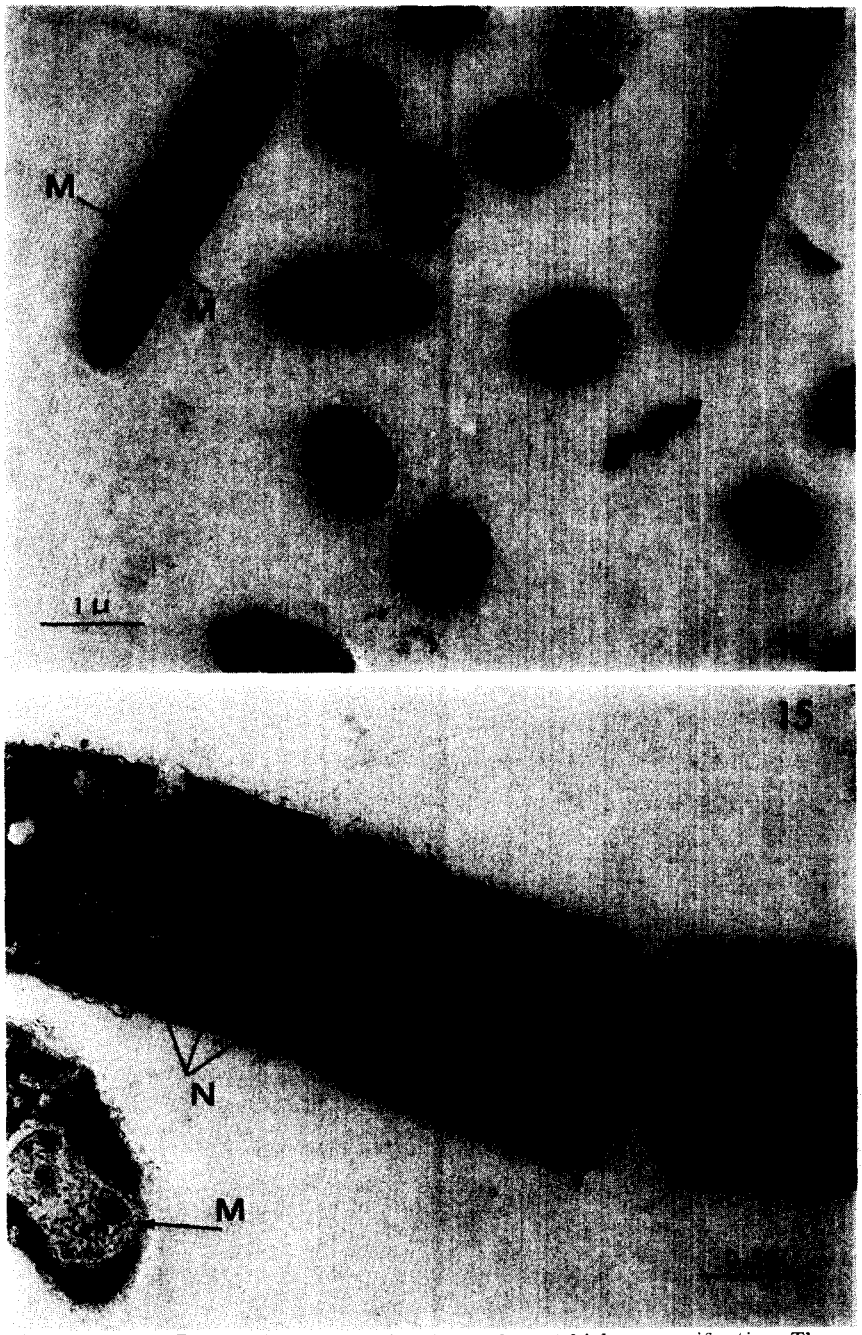



Fig. 12. Preparation as in Fig. 9, but at higher magnification. The nucleoplasm is swollen and disarranged and the cytoplasm loosened. Membrane systems are no longer present.

Fig. 13. Preparation as in Fig. 10, but at higher magnification. The shape of the protoplast of one-half of a divided cell has changed little; note at the arrow the almost normal structure of the nucleoplasm and cytoplasm; however, membranous structures are usually expelled from the protoplast.




Figs. 14 and 15. Preparation same as in Fig. 11, but at higher magnification. These protoplasts strikingly resemble normal cells; they have preserved their membrane systems (M) in a somewhat disorganized state in the normal location. However, the arrangement of the nuclear material (N) is strongly affected and is dispersed all over the cytoplasm in empty-looking areas. The cytoplasm is very compact, and the remainders of the cell envelope are not extended, and not smoothed by the turgor of the protoplasts.

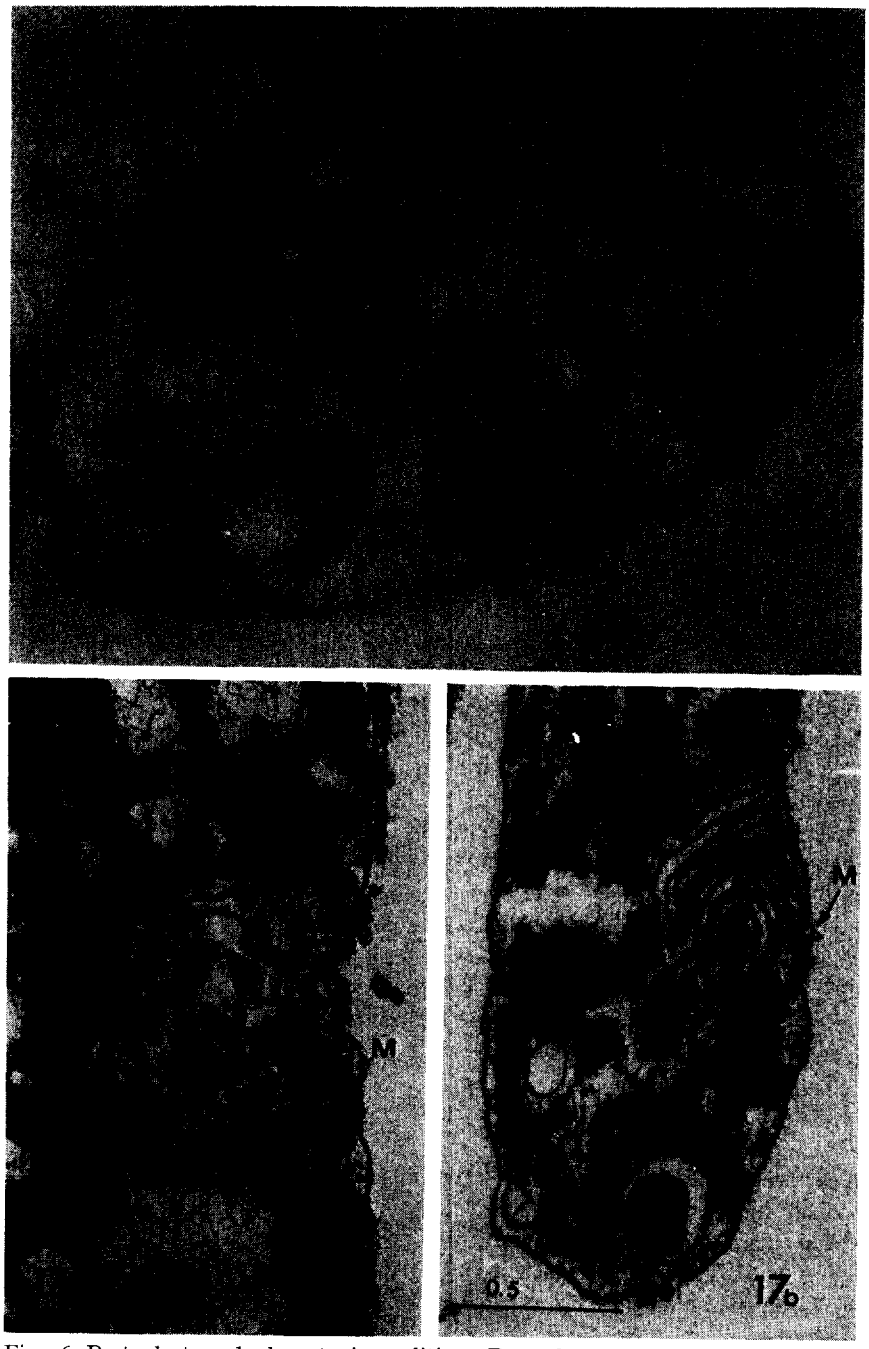



Fig. 16. Protoplasts under hypotonic conditions. Protoplasts made from cells at neutral pH, break in the absence of sucrose and shed their contents. In this protoplast from growth overnight, the circumferences of the poly-$\beta$-hydroxybutyrate globules are still preserved, as well as part of the fibrillar reticulum of the cytoplasm (arrow).

Figs. 17a and 17b. Protoplasts under hypotonic conditions. From medium B. In the hypotonic medium much of the membranous system (M) is preserved in the cell.

occupy their original sites. The nucleoplasm is unusual; it dissects the compact cytoplasm in numerous narrow areas. The plasma membrane, however, fails to cover the cytoplasm smoothly by stretching which is due to the turgor of the protoplast. The protoplasts of cells from media A and B, contrary to cells treated with HCl, shed most of their membranous structure as small vesicles in the hypertonic buffers. It is obvious that the cell wall is lacking in both the spherical and rod-shaped protoplasts (Figs. 9–15).

*Protoplasts in hypotonic medium (Figs. 16–18).* When suspended in buffer without sucrose, protoplasts from cells grown overnight in media A and B behave differently. Those from medium A (Fig. 16) become rounded, burst, and lose practically their whole cytoplasmic and nucleoplasmic content. Those from medium B, however, often remain elongated rods or short division segments in which, although some material is lost, much of the cell structure remains preserved (Figs. 17a and 17b, and 18). These observations are consistent with the different behaviour of spherical and rod-shaped protoplasts in lysis experiments (Figs. 2–4). In the empty protoplast of medium A shown in Fig. 16 little more is preserved than the thin membranes that must have surrounded the poly-$\beta$-hydroxybutyrate globules, and some fibres interconnecting them. These fibres are interpreted as having belonged to the three-dimensional network of the cytoplasm[34].

The 'protoplasts' in Figs. 17a and 17b are made from cells grown in the glucose-rich medium B. Despite the complete loss of cell wall material both types of mesosomal membrane systems (M) can be preserved in hypertonic medium.

*Protoplasts made from bacilli stabilized in 0.3 M sucrose and agar (Figs. 19–21).* The differences in shape between the two main types of protoplasts disappear when the cells are converted into protoplasts in the presence of sucrose, while mechanically protected by embedding in agar. Protoplasts made from cells grown in neutral medium (Fig. 19) are not much inflated; they are not always rounded, but sometimes elongated or somewhat angular, whereas those from acidified medium (Figs. 20 and 21) are frequently less elongated than those made without agar. The surrounding agar appears to influence the shape of the protoplasts to a fair extent. The protoplasts of cells exposed to HCl have a smooth, completely normal, plasma membrane. In all three cases (Figs. 19–21) the membranous vesicles have, however, been extruded from the protoplasts. It is interesting to note that some of the vesicles in Fig. 19 (arrows) carry some fibrillar material.

## DISCUSSION

On a dry weight basis the lipid content of cells and protoplasts of *B. megaterium* was significantly increased when the bacterium was grown overnight in the presence of glucose. This phenomenon is to be attributed to an accumulation of neutral lipid (probably $\beta$-hydroxybutyrate polymer) because with and without glucose in the medium the phospholipid content remained constant. However, the composition of the phospholipid fraction of lipid extracts of cells grown under both conditions was found to differ. Whereas the content of cardiolipin, phosphatidyl ethanolamine and *O*-lysyl phosphatidyl glycerol was not affected, it turned out that the content of phosphatidyl glycerol was decreased in glucose-grown cells (medium B). This phospholipid was in part replaced by glucosaminyl phosphatidyl glycerol, a phos-


Fig. 18. Protoplasts in hypotonic medium. Protoplasts of cells from medium B (pH 5), although losing a certain part of their contents in sucrose-free medium, are not as much affected as those from a medium of neutral pH (Fig. 16).


Fig. 19. Protoplasts stabilized with sucrose and agar. Protoplast made from a young cell comparable to those in Figs. 9 and 12 from medium A. The protoplast is now well stabilized, the nuclear areas are swollen comparatively little, but membranous vesicles have been extruded into the hypertonic milieu of the agar. At arrows, fine fibrils attached to vesicles.

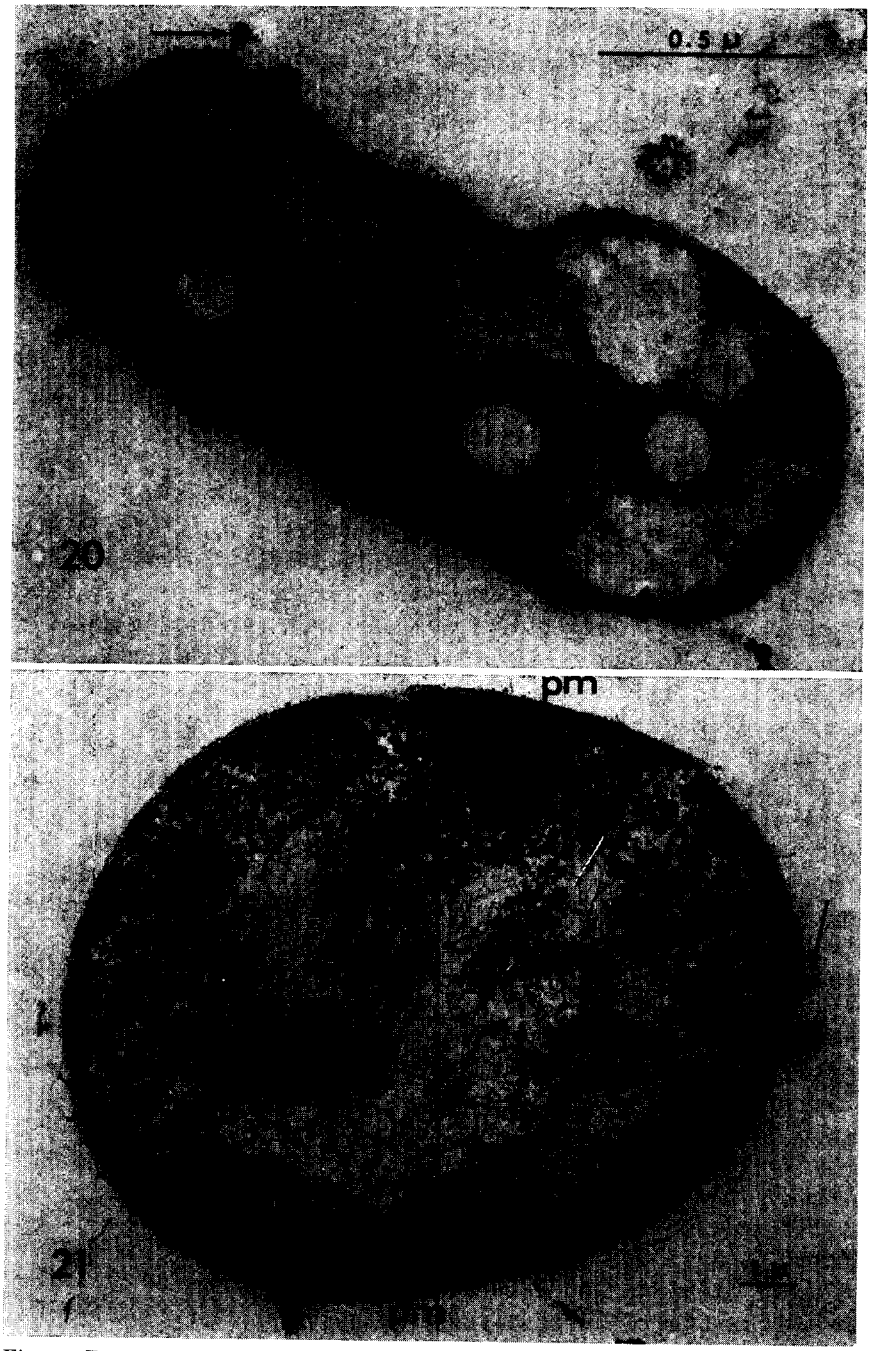



Fig. 20. Protoplasts stabilized with sucrose and agar. Protoplast of cell grown overnight in medium B. The shape of the protoplast may have been influenced by the pressure of the agar. Vesicles have been extruded.

Fig. 21. Protoplast comparable to those in Figs. 11, 14 and 15 grown at pH 5 (HCl) in medium A. Differences with the protoplasts made from cells from medium A at neutral pH (Fig. 19) and from medium B at pH 5 (Fig. 20) are not observed when the protoplasts are made in agar with acetate–veronal buffer at pH 6. Contrary to Figs. 11, 14 and 15 the plasma membrane (pm) is here smooth, and vesicles have been extruded.

pholipid which was not detectable in lipid extracts of cells grown without glucose (medium A). Taking into account that at the time of harvesting the pH of media A and B were 7.0 and 5.0 respectively, the possibility was envisaged that this shift in phospholipid composition was somehow related to this environmental difference. Cells grown in the presence of glucose, but at a constant pH of 7.0 of the medium were found to have a similar phospholipid composition to cells grown in the neutral medium not containing glucose. On the other hand, exposure of cells grown in medium A to an acidic pH by the addition of HCl resulted in a qualitatively similar shift in phospholipid composition. Although these experiments endorse the view that the pH influences the phospholipid composition, its effect on the turnover of these constituents in the bacterium remains to be investigated.

Inasmuch as the observed differences in phospholipid composition involve a change in charge of the lipid constituents it is tempting to speculate that this difference is an indication of an alteration of the lipoprotein-containing structures. Although the phospholipids are recovered in the so-called membrane fraction it is not clear whether this alteration in phospholipid composition is localized either in the cytoplasmic membrane, the intracytoplasmic membranes, or in both. Without suggesting any direct relationship with a possible variation in the phospholipid composition of the cytoplasmic membrane, it has to be emphasized that the protoplasts from cells exposed to pH 7.0 and 5.0 displayed a different behaviour in media of different hypotonicity. Although protoplasts from cells from acidic media were found to be lysed in hypotonic sucrose solutions by virtue of the release of nucleic acid material, the absorbance at 550 m$\mu$ appeared to remain practically constant*. This was in contrast with the protoplasts of cells from neutral medium which were completely disrupted under the same conditions. Protoplasts from bacteria are generally considered to be spherical structures, and such rounded protoplasts were obtained after lysozyme treatment of cells cultivated in a neutral medium (A). However, removal of the cell wall of bacteria exposed to an acidic environment surprisingly was found to give rise to structures which under the phase-contrast microscope exhibited a bacillary rod-shaped form.

Electron microscopy confirmed that exposure of cells to slightly acidic conditions influences the shape and the fine structure of their protoplasts to a considerable degree when they are suspended in hypertonic buffers, and even more so when the buffer is hypotonic. In agreement with the lysis experiments the protoplasts made from cells grown at neutral pH were observed to lyse in hypotonic media and to lose practically their full content of cytoplasm and nucleoplasm, whereas those of cells adjusted to pH 5 showed this tendency to a far lesser degree. In the latter case the intracellular membrane systems are, although in a somewhat disarranged condition, sometimes preserved in the hypotonic medium.

The fate of the mesosomes during the conversion of bacilli into protoplasts has been described by FITZ-JAMES[35] and by RYTER AND LANDMAN[21]. The first cytological change that becomes apparent in the cells appears not to be due primarily to the effect of the lysozyme, but to the transferring of the cells from a normal growth

* EDEBO[40] observed that an environmental pH below 5.0–5.5 can prevent lysis of protoplasts. The pH of 6.2 applied in our experiments eliminates this pH effect, as is shown by the release of intracellular material.

medium to one of higher tonicity; this we have been able to confirm. We found that intact cells, when transferred to buffer with 0.3 M sucrose, always resulted in preparations in which the plasma membrane had receded from the cell wall. Electron micrographs of plasmolysed bacilli have already been described by several authors[19–23]. The first cytological change in medium of higher tonicity is a displacement of the intracytoplasmic and intranuclear membrane systems towards the cell periphery, and in a later stage numerous small rounded vesicles can be observed in the empty space between the cell wall and the retracted plasma membrane. When the cell wall is removed by lysozyme the vesicles are extruded into the medium; and this we could confirm in most cases, but sometimes not in the protoplasts of cells grown at pH 5.0 when kept in hypotonic buffer. In regard to their mesosomes, the protoplasts made after the pH was lowered by means of HCl proved even to be resistant to hypertonic medium. This different behaviour from protoplasts made from cells kept at low pH is proof of an intrinsic alteration of the properties of their plasma membrane as compared to those from the cultures at neutral pH. Such an alteration has to be correlated with a change in the chemical make-up of the lipoproteins concerned. At the moment, the observed shift in phospholipid composition is the only indication available, and it is not our intention to imply that this change is responsible for these morphological deviations. In this respect it will be necessary to study whether the composition or physical state of structural proteins of the membranes are affected by the different growth conditions.

The effect of the changed condition of the plasma membrane is apparent not only in the shape of the protoplast and the behaviour of the mesosomes, but also in the appearance of the nucleoplasm and the cytoplasm. According to FITZ-JAMES[36], adjustment of the cell or the protoplast to a phosphate buffer containing 0.3 M sucrose affects the arrangement of the nuclear material in that it causes increased dispersion of the nuclear fibres due to greater hydration. The present study shows that this effect occurs in the protoplasts in acetate–veronal buffer with 0.3 M sucrose when derived from cells grown at neutral pH (Figs. 5–8), but hardly at all when, during formation of protoplasts, they are mechanically stabilized by agar (Fig. 19). However, the protoplasts made from cells grown in medium B preserve their nuclear material in an arrangement considered more or less normal after RYTER–KELLENBERGER fixation[11], and this may be taken for another proof of the altered condition of the plasma membrane changing its permeability. No explanation can as yet be offered for the appearance of the nucleoplasm in material which had been exposed to pH 5.0 adjusted with HCl. This treatment must have been rather drastic, also in view of the structure of the protoplast envelope. Although the presence of some scattered remnants of the cell wall cannot altogether be excluded, it would seem unlikely that these affect the shape of the protoplast. It is noteworthy that this strong effect was not apparent on the nucleoplasm, nor on the structure of the plasma membrane when the lysozyme treatment was applied by diffusion through agar.

A consideration of the functions of the intracellular membranes in respect of respiratory activities[24], cell wall synthesis[20,25,28,37], and replication and separation of the chromatin[38], is outside the scope of this paper. The electron microscopy of protoplasts of cultures at pH 7 and pH 5 confirms the observations made in phase-contrast with the light microscope, and also corroborates the chemical analysis which points to altered composition of the lipoprotein membranes. Our observations on the isola-

tion of rod-shaped protoplasts are in conflict with the qualification of protoplasts agreed upon by thirteen workers in 1958 (ref. 39).

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