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Effects of Phenethyl Alcohol on *Bacillus* and *Streptococcus*

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The activity of phenethyl alcohol (PEA) on *Bacillus cereus*, *B. megaterium*, and *Streptococcus faecalis* was studied by electron microscopy of thin sections and by the assay of intracellular K^+ leakage. *S. faecalis* was unaffected by PEA at concentrations up to 0.5%, *B. cereus* was severely damaged by 0.5% PEA, and *B. megaterium* behaved intermediately. Important membrane ultrastructural alterations were observed in *B. cereus* cells treated with 0.5% PEA, namely the change in the geometry of the membrane profile from asymmetric to symmetric, the occurrence of prominent, complex mesosome-like structures, and membrane fracturing and solubilization. Protoplasts from *B. megaterium* were found to be quickly lysed by 0.5% PEA due to the disruption of the cytoplasmic membrane. The electron microscopic observations, together with the results of the study of the K^+ efflux from *B. cereus* and *B. megaterium*, indicate that PEA primarily and directly damages the cytoplasmic membrane of sensitive bacteria. The breakdown of the permeability barrier probably is responsible for the observed bactericidal action of 0.5% PEA on *B. cereus*.

Phenethyl alcohol (PEA) is known to inhibit the growth of several bacteria, particularly gram-negative organisms (1, 7). The mechanism of action of PEA was initially considered to be primarily due to the inhibition of deoxyribonucleic acid (DNA) synthesis (1). Silver and Wendt (17) showed, however, that PEA primarily affects the cytoplasmic membrane of *Escherichia coli*, the DNA synthesis and other cellular functions being inhibited as a secondary effect. As part of a study on the alterations induced by membrane-damaging treatments on gram-positive bacteria, we report here the results concerning the effects of PEA on *Bacillus cereus*, *Bacillus megaterium*, and *Streptococcus faecalis*.

MATERIALS AND METHODS

B. cereus (strain NCTC 7587) and *B. megaterium* (strain KM CCM 2037, kindly supplied by M. Kocur) were grown in tryptone broth (1.0% tryptone [Difco], 0.5% NaCl, pH 7.2) at 30°C with aeration by shaking to about 3×10^8 to 5×10^8 cells/ml. *S. faecalis* (strain ATCC 9790) was grown to late exponential phase as previously described (culture *a* in reference 9). PEA (Sigma Chemical Co.) was added to the cultures to final concentrations of 0.25, 0.35, 0.5, and 1.0% (vol/vol). Incubation was continued for several hours under the same conditions as indicated above. The absorbancies of control and PEA-treated *B. cereus* cultures were determined at 520 nm (A_{520}) with a Baush & Lomb Spectronic 70. The number of viable cells in the control and treated *B. cereus* cultures was determined by serial dilution plate counting. The efflux of K^+ from control and treated bacteria was determined by the assay of K^+ in filtrates obtained at intervals with membrane filters (Millipore Corp., type HA, pore size 0.45 μm). The values are given as percentages. A 100% value corresponds to the K^+ leaked from bacteria boiled for 30 min (*B. cereus* and *B. megaterium*) or treated with 10% HNO_3 (*S. faecalis*). K^+ was measured with an EEL flame photometer, model 150. For the K^+ efflux experiments, *B. cereus* and *B. megaterium* were grown in the medium used for *S. faecalis* (9), which contained 0.5% dipotassium phosphate. The cells from these cultures were washed twice with 0.1% peptone to remove extracellular K^+. The final suspensions of washed bacteria were made in 0.1% peptone.

For electron microscopy, samples were collected by centrifugation before adding PEA (control) and 5, 15, 30, 40, 60, 80, and 240 min thereafter. The sedimented bacteria were fixed by the following procedures: (i) Ryter-Kellenberger (R-K) OsO_4 (8), for 16 h at 20°C, followed by uranyl acetate (8, 15) (the prefixation step of the R-K procedure was not used [13]); (ii) glutaraldehyde (TAAB, London) at 2.5% in 0.1 M cacodylate buffer, pH 7.0, for 1 h at 20°C, followed by OsO_4 and uranyl as in procedure (i); and (iii) uranyl acetate at 0.1 to 0.2% in R-K veronal acetate buffer, final pH 5.0, or in the same buffer with the pH adjusted to 6.5 with 0.2 M sodium bicarbonate, for 30 min at 20°C, followed by R-K OsO_4 as in procedure (i) (14). The fixed specimens were processed for electron microscopy as described elsewhere (15). All electron micrographs presented in this paper are of sections contrasted with lead citrate (20) for 5 min.

Protoplasts from *B. megaterium* (3) and *S. faecalis* (9) were exposed to 0.5 and 1.0% PEA for 5, 30, and 45 min. Control and treated protoplasts were fixed with glutaraldehyde (final concentration,

2.5%) followed by OsO_4 and uranyl acetate as described (9).

RESULTS

Effects of PEA on *B. cereus*. The effect of 0.25 and 0.5% PEA on the absorbance of *B. cereus* cultures is shown in Fig. 1. Soon after the addition of 0.5% PEA, the A_{520} started to decrease, reaching a minimum value after about 3.5 h. PEA at 0.25% had a very slight effect on the A_{520} of *B. cereus* cultures. The reduction in the number of viable cells in cultures treated with 0.25, 0.35, and 0.5% PEA is shown in Fig. 2. After 15 min with 0.5% PEA, about 99% of the cells were nonviable. PEA at 0.25% did not produce a significant reduction in the number of viable cells during the period of time studied. Figure 3 shows K^+ efflux induced in *B. cereus* by 0.5% PEA. After 5 min about 80% of the intracellular K^+ had leaked from *B. cereus* exposed to 0.5% PEA.

Aspects of control *B. cereus* cells fixed by procedure (i) are shown in Fig. 4a and 7e. More pictures of the same strain fixed by the three procedures used in the present study can be seen in previous publications (13–16). Several ultrastructural alterations were observed in *B. cereus* treated with PEA at concentrations ranging from 0.35 to 0.5% and fixed by the three procedures described in Materials and Methods.

(i) **Membranes.** Important alterations were visible in the membranes of most cells treated

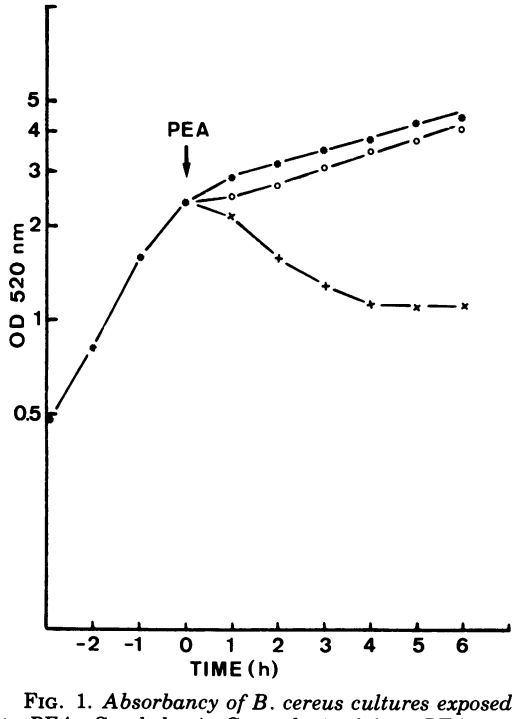


FIG. 1. *Absorbancy of B. cereus cultures exposed to PEA. Symbols: *, Control; ○, 0.25% PEA; +, 0.5% PEA.*

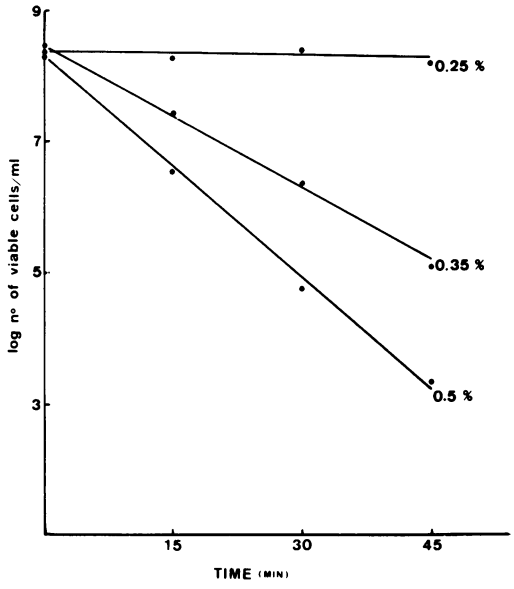


FIG. 2. *Number of viable cells in cultures exposed to PEA.*

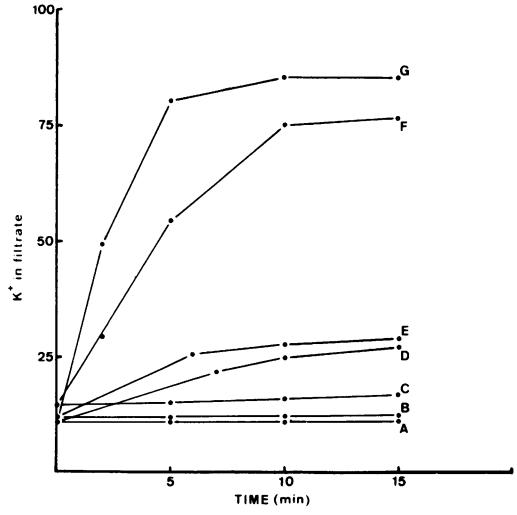


FIG. 3. *K^+ efflux from control and PEA-treated bacteria. (A) Control B. cereus; (B) control B. megaterium; (C) control S. faecalis; (D) B. cereus treated with 10 mM sodium azide; (E) S. faecalis treated with 0.5% PEA; (F) B. megaterium treated with 0.5% PEA; (G) B. cereus treated with 0.5% PEA. All experiments were carried out at 20°C. Ordinate gives filtrate K^+ as percentage of total culture K^+.*

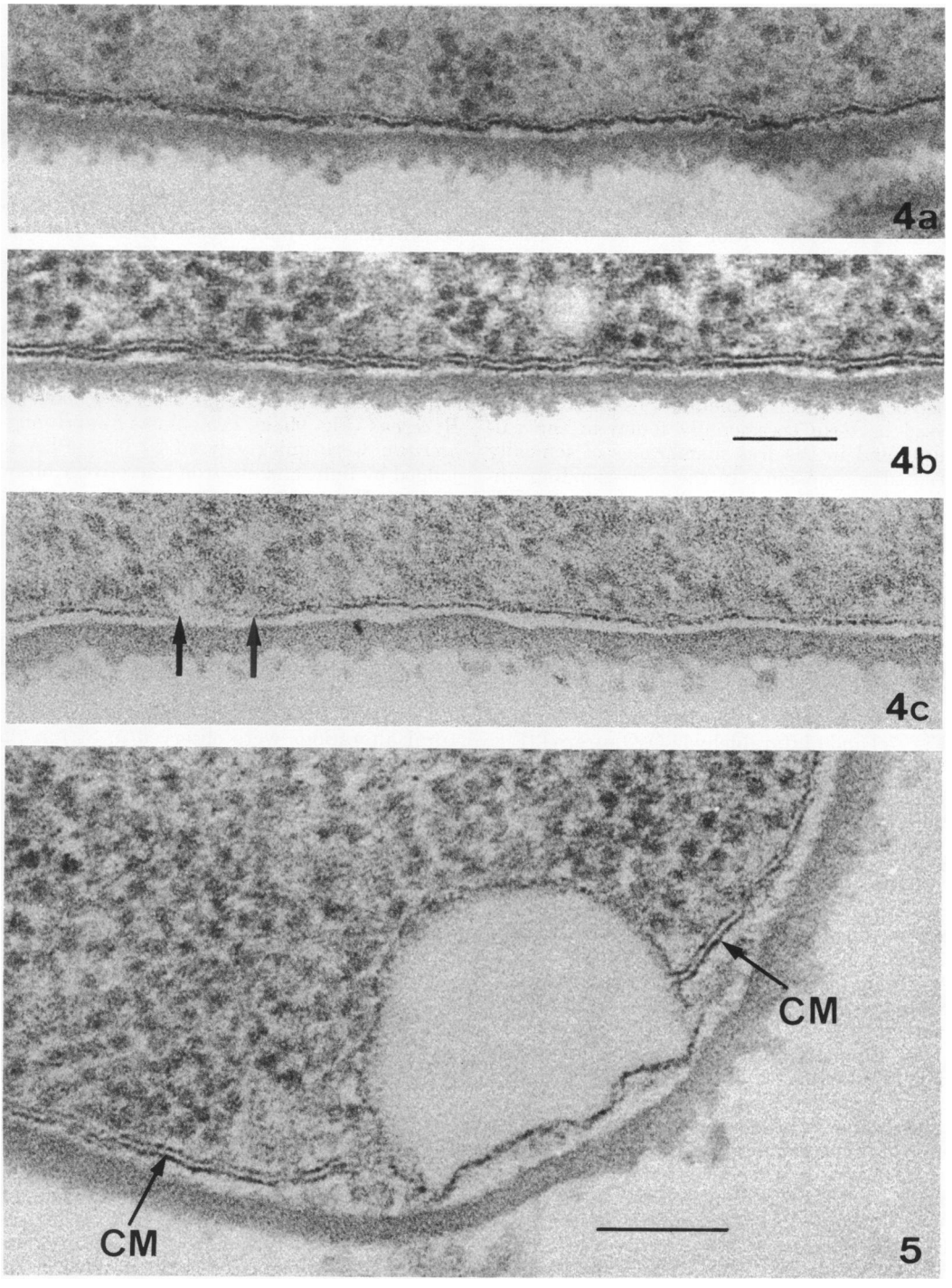


FIG. 4. *Profiles of the cytoplasmic membrane of B. cereus. Bar indicates 0.1 μm. (a) Control cell, showing the asymmetric geometry typical of gram-positive bacteria. Fixation with OsO_4-uranyl acetate (procedure i). (b) Symmetric profile in a cell treated with 0.5% PEA for 30 min. Fixation with glutaraldehyde-OsO_4-uranyl acetate (procedure ii). (c) Cell treated with 1.0% PEA for 40 min. Fixation as in (a). The cytoplasmic membrane is almost completely solubilized in several regions, mainly in the portion between the arrows.*

FIG. 5. *Vesicle formed by the splitting of the cytoplasmic membrane of a B. cereus cell treated with 0.5% PEA for 40 min. The unsplit regions of the membrane exhibit an almost symmetric profile (CM). Fixation with OsO_4-uranyl acetate (procedure i). The bar indicates 0.1 μm.*

with 0.5% PEA and in many cells treated with 0.35% PEA. One of the first detectable alterations was the change in the profile of the membranes from asymmetric to symmetric (Fig. 4b, 5, 7d, and 8). Such an alteration was visible in many cells treated with 0.5% PEA for only 5 min. Membrane fractures (Fig. 6b and 8b) and membrane solubilization in large extensions (Fig. 4c and 8b) occurred after long treatments with 0.5% PEA and short treatments with 1.0% PEA. One striking alteration was the localized splitting of the membranes, with the formation of vesicles bounded by a single-layered "membrane" (Fig. 5, 7d, and 8a). This splitting was also observed in free membranes derived from lysed bacteria occasionally found in the cultures and in the free membranes of sonically treated suspensions. In the corresponding untreated suspensions, the free membranes always had a continuous triple-layered profile. Prominent, complex mesosome-like structures were frequently found in treated samples, regardless of the fixation used in the preparation of the specimens (Fig. 6 and 7). The configuration of these membranous structures varied from vesicular (Fig. 6a, 7a, and 7b) to lamellar (Fig. 6b). Myelin-like figures were also observed in samples fixed with both OsO_4-uranyl (Fig. 7c) and glutaraldehyde-OsO_4-uranyl (Fig. 7d). In control, untreated cells fixed by the three procedures indicated in Materials and Methods, such mesosome-like structures were not present (Fig. 7e; see reference 14 and M. T. Silva et al., Biochim. Biophys. Acta, in press).

(ii) Nucleoids. The conspicuous DNA areas present in control cells (Fig. 7e) became inapparent in most treated cells, even in samples taken after 5 min of exposure to 0.5% PEA. Abundant DNA-like fibrils were, however, readily seen in cells that had lost intracellular material due to lysis (Fig. 8).

(iii) Lipid droplets. In *B. cereus* cells showing membranes affected by PEA, the β-hydroxybutyrate inclusions exhibited a limiting single "membrane." This finding, also observed with several other membrane-damaging treatments in *B. cereus* and *B. megaterium*, was previously reported and discussed (M. T. Silva and A. M. Parente, Proc. IX Annu. Meet. Port. Soc. Electron Microscopy, 1974, Abstr. 25).

(iv) Cytoplasm. The cytoplasmic matrix showed areas of increased compactness as compared with the controls. Such areas were clearly seen in the spaces without clusters of ribosomes (Fig. 6 and 8).

(v) Lytic alterations. Most cells present in samples collected after long treatments with 0.5% PEA appeared with signs of extensive lysis (Fig. 8), with fractures in both the cell wall and the cytoplasmic membrane, and with loss of intracellular material.

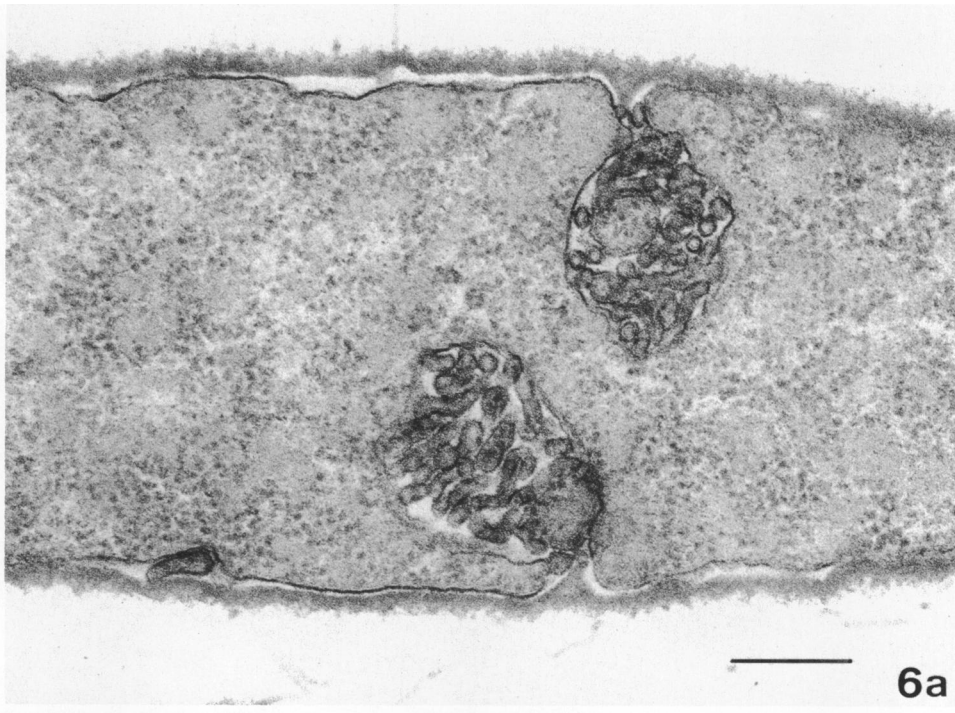
Effects of PEA on *B. megaterium*. Figure 9a shows the ultrastructural appearance of the cytoplasmic membrane of a control *B. megaterium*. Other pictures of *B. megaterium* can be seen in previous publications (11, 12). The cytoplasmic membrane of *B. megaterium* was also affected by PEA, although to a lesser extent than with *B. cereus*. With 0.5% PEA, only some cells appeared altered after 30 min of treatment. The membrane alterations included the change in the profile and disorganization of the triple-layered structure, as described above for *B. cereus* (Fig. 9b, c). Protoplasts from *B. megaterium* were quickly lysed by 0.5% PEA as judged by light and electron microscopic observations. Figure 10a shows a control protoplast, and Fig. 10b depicts the ultrastructural alterations observed in PEA-treated protoplasts. The membranes of affected protoplasts exhibited a symmetrical profile and had fractures (Fig. 10b). Lysed and almost completely empty ghosts were frequent in treated samples (Fig. 10b). Figure 3 shows the K^+ efflux induced by 0.5% PEA in *B. megaterium*.

Effects of PEA on *S. faecalis*. No ultrastructural alterations were observed in *S. faecalis* cells treated with PEA at the concentrations studied for periods of time up to 60 min. Protoplasts from this bacterium were not lysed under the same conditions, as deduced from light and electron microscopic observations. The K^+ efflux from *S. faecalis* treated with 0.5% PEA was rather slight (Fig. 3).

DISCUSSION

PEA is known to preferentially inhibit the growth of gram-negative organisms (1, 7), and *E. coli* has been the most used microorganism in studies on the mechanism of action of that alcohol. Among gram-positive organisms, *B. cereus* is particularly sensitive to PEA-induced growth inhibition (1). *S. faecalis* is rather resistant, and *B. megaterium* behaves intermediately (1). The results of the present study agree with those observations. *B. cereus* was found to be drastically affected by 0.5% PEA, as deduced from the loss of turbidity of the cultures and from the quick reduction in the number of viable cells.

Several effects observed in PEA-treated bacteria, namely the leakage of intracellular K^+ from *B. cereus* and *B. megaterium*, the membrane ultrastructural alterations in *B. cereus* and *B. megaterium*, and the lysis *B. megaterium* protoplasts, clearly indicate that PEA af-



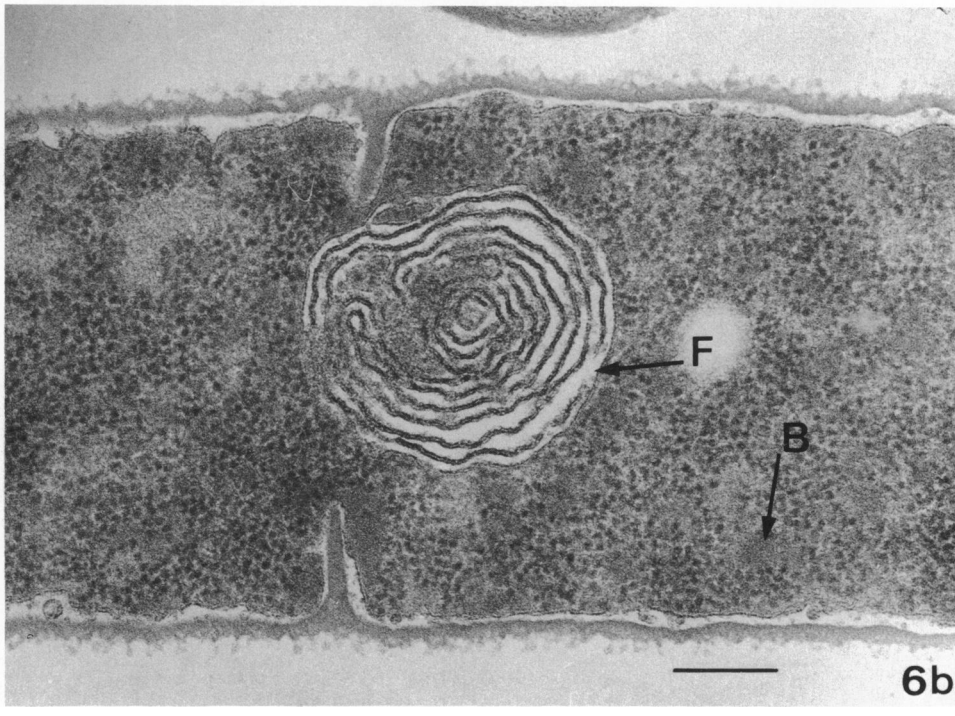


FIG. 6 and 7. *Aspects of complex, prominent mesosome-like structures present in B. cereus treated with PEA and fixed by three different procedures. The bar indicates 0.2 μm.*

FIG. 6. *(a) Cell treated with 0.5% PEA for 30 min and fixed with glutaraldehyde-OsO_4-uranyl acetate (procedure ii). (b) A lamellar mesosome in a B. cereus cell treated with 0.5% PEA for 40 min. Notice the fracture in the membrane of the lamellar structure (F). B, Blocks of compact cytoplasmic matrix. Fixation with OsO_4-uranyl acetate (procedure i).*

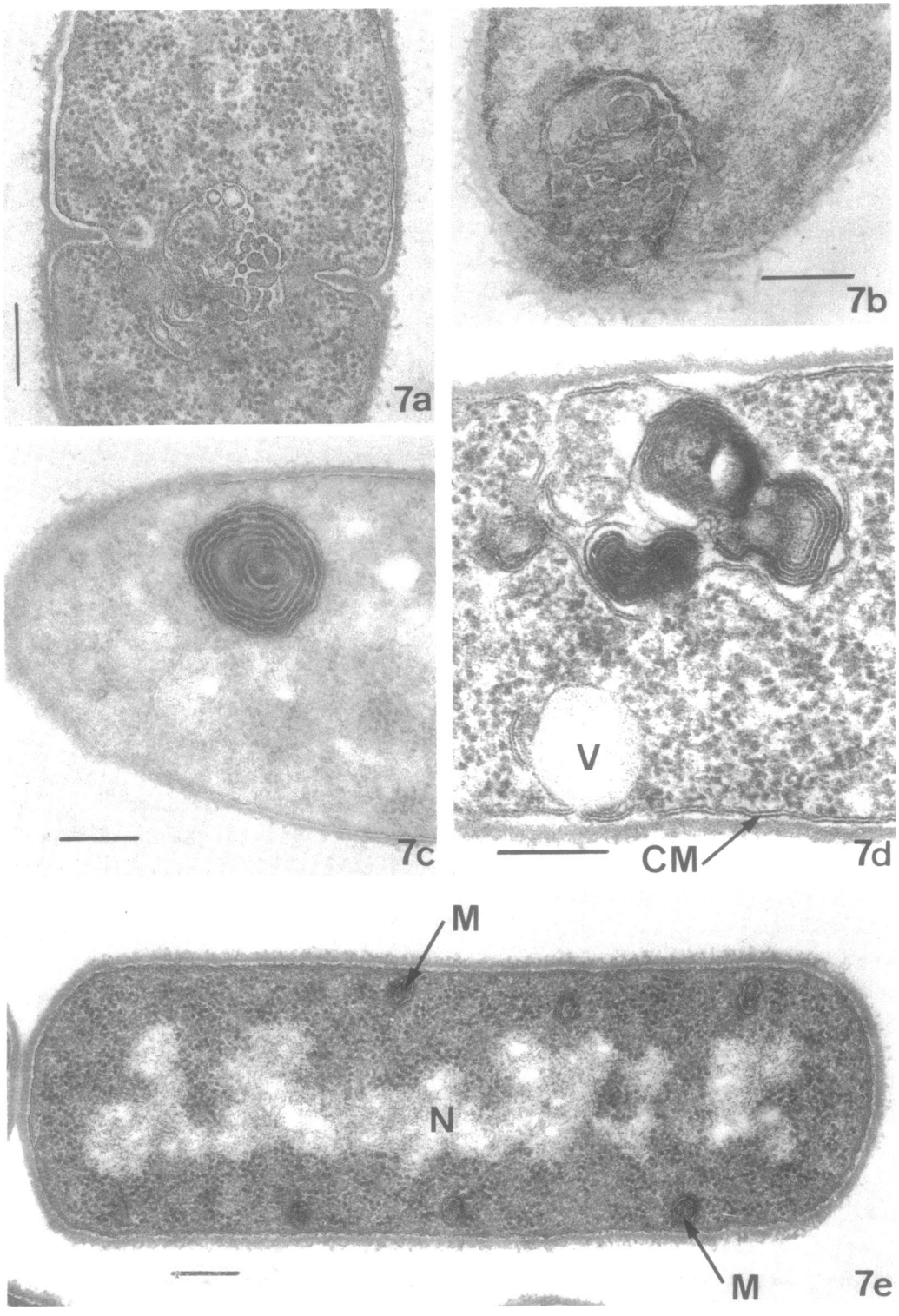


FIG. 7. *(a) Cell treated with 0.5% PEA for 40 min and fixed with OsO_4-uranyl acetate (procedure i). (b) Cell treated with 1.0% PEA for 15 min and fixed with uranyl acetate-OsO_4 (procedure iii). (c and d) Mesosomes with a "myelin-like" configuration in cells treated with 0.5% PEA for 15 and 30 min, respectively. Fixation with OsO_4-uranyl acetate (procedure i) (c) and glutaraldehyde-OsO_4-uranyl acetate (procedure ii) (d). Notice in (d) the symmetric profile of the cytoplasmic membrane (CM) and a vesicle bounded by a single-layered "membrane" (V). (e) Control B. cereus cell fixed with OsO_4-uranyl acetate (procedure i). Notice the small and simple mesosomes (M) and the fibrillar nucleoid (N).*

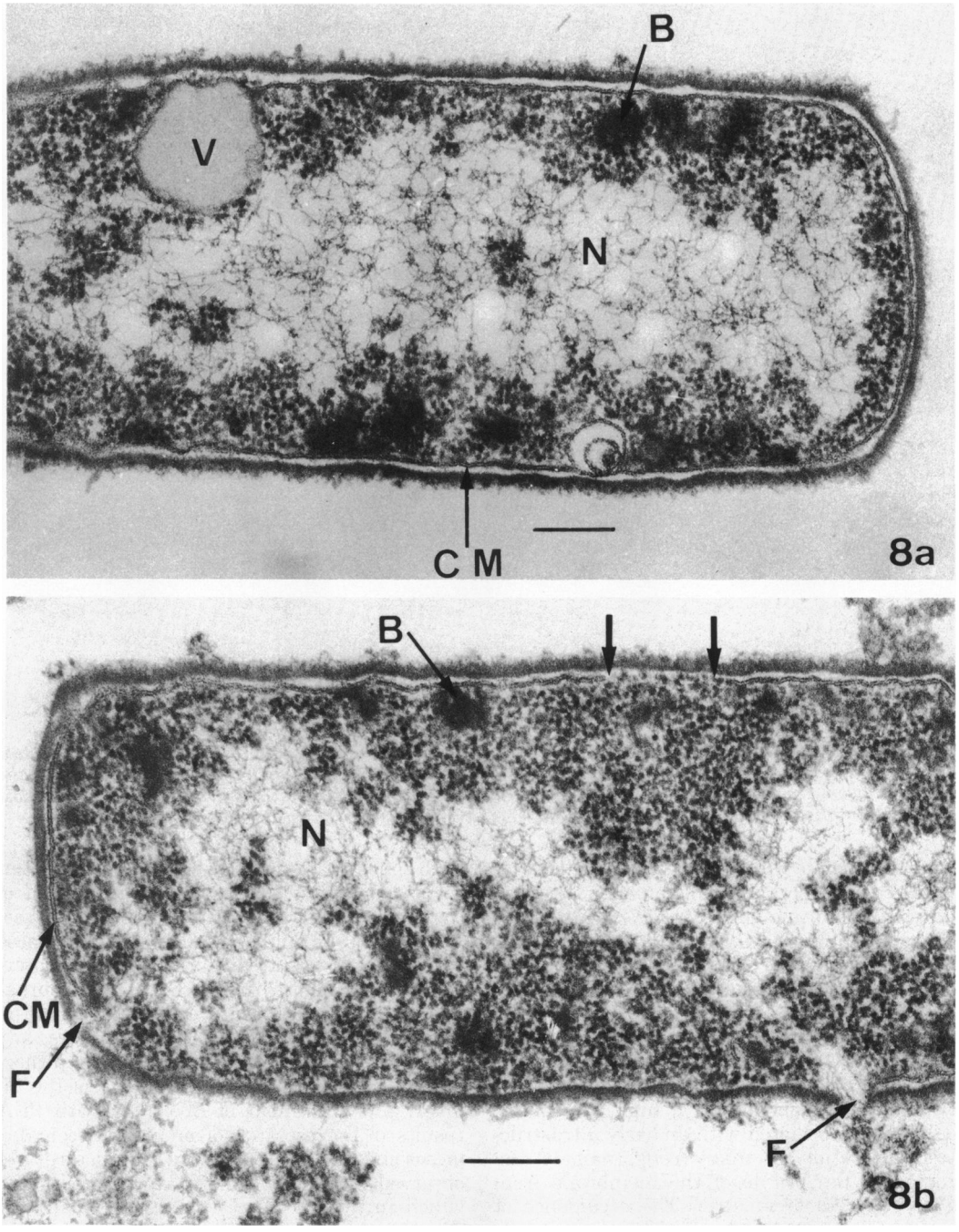


FIG. 8. *B. cereus cells treated with 0.5% PEA for 80 min and exhibiting signs of extensive lysis. The cytoplasmic membrane (CM), when present, has a symmetrical profile. In (b) the cytoplasmic membrane appears fractured (F) and solubilized in large extensions (portion between unlabeled arrows and most part of the membrane in the lower portion). The cell wall is also fractured in the cell of (b) (F). Through the pore formed by the double fracture of the cell wall and cytoplasmic membrane on the right side of the figure, DNA-like fibrils and cytoplasmic material are leaking. The nucleoids (N) exhibit dispersed DNA fibrils. V, Vesicle like those in Fig. 5 and 7d; B, blocks of compact cytoplasmic material. Fixation with OsO_4-uranyl acetate (procedure i). Bar indicates 0.2 μm.*

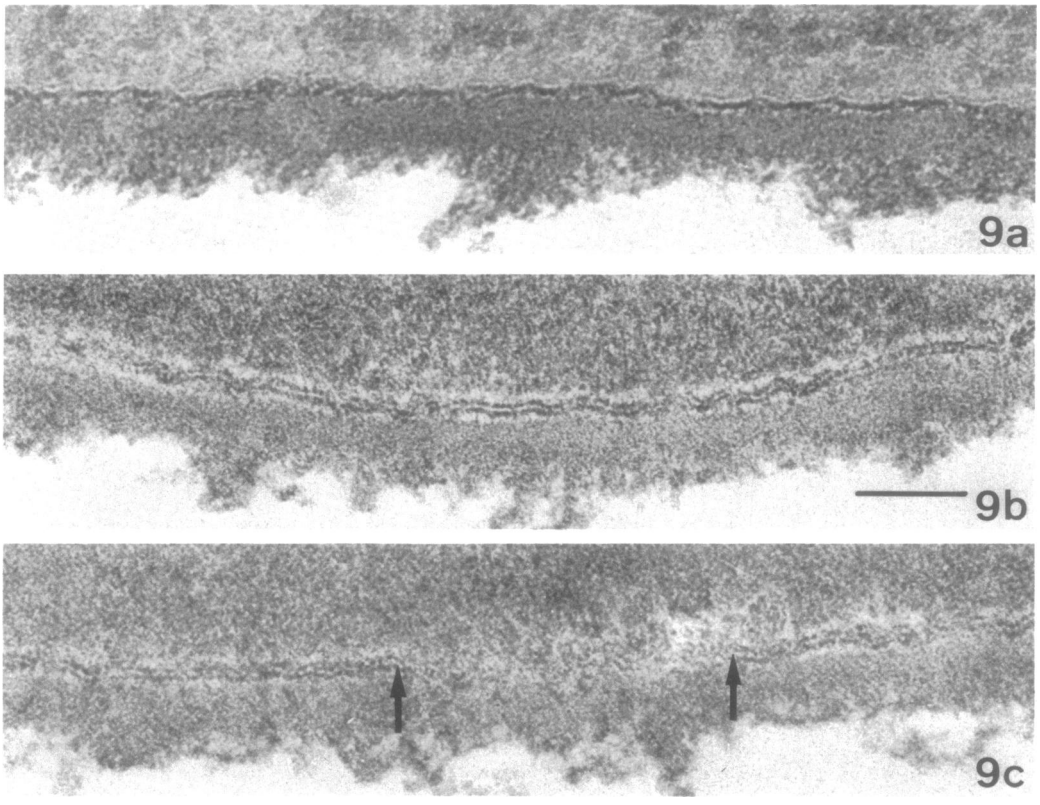
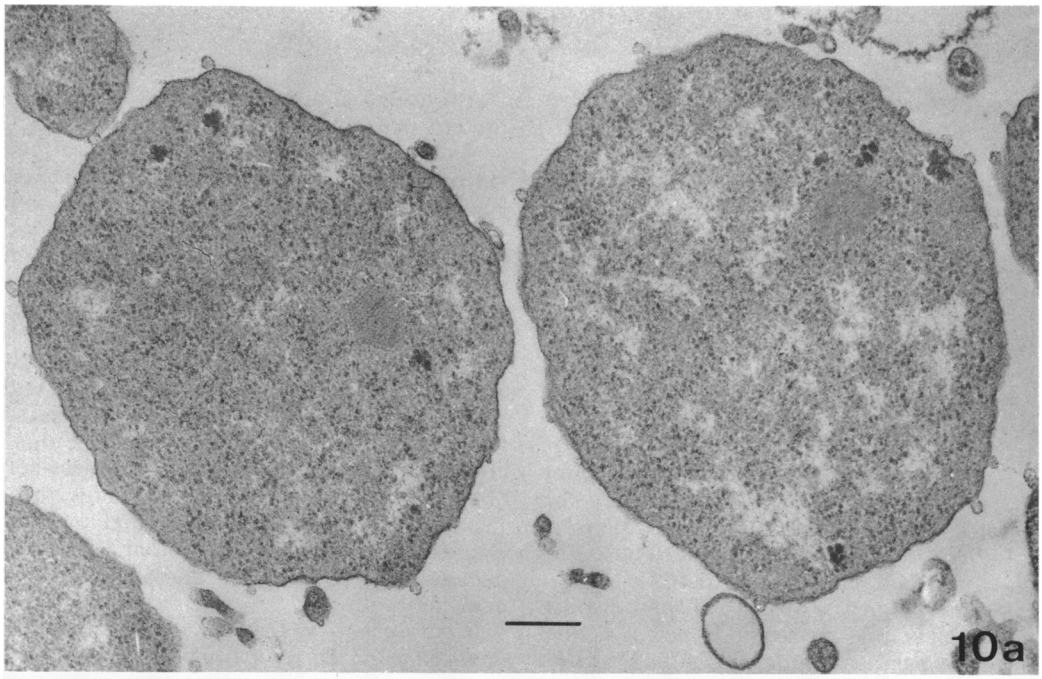


FIG. 9. *Aspects of the cytoplasmic membrane of B. megaterium. Fixation with OsO_4-uranyl acetate (procedure i). Bar indicates 0.1 μm. (a) Control cell, showing a very asymmetric membrane profile; (b) the symmetric geometry in a cell treated with 0.5% PEA for 30 min; (c) cell treated as in (b), showing an almost complete solubilization of the cytoplasmic membrane in the portion between the arrows.*

fects the membranes of sensitive bacteria. It has been shown that PEA damages the cytoplasmic membrane of *E. coli* (17). Since these effects are produced in *B. cereus* by PEA concentrations that are close to the minimal lethal concentration, it is likely that the damage inflicted to the cytoplasmic membrane is an important factor in the killing of sensitive bacteria by PEA. As already reported in other situations (14), PEA-induced membrane permeability damage, as judged by a high rate of K^+ leakage, is associated with an early ultrastructural alteration in many cells, namely the change in the profile of the membrane from asymmetric to symmetric. The occurrence of fractures in the membranes of PEA-treated *B. cereus* reflects a more drastic disturbance in the membrane structure. Other membrane-damaging treatments have also been shown to induce such an alteration (11, 14). The disappearance of the profile of the membranes in some PEA-treated *B. cereus* and *B. megaterium* cells has already been reported for *E. coli* treated with high concentrations of PEA (21). Such an alteration reflects an extensive disorganization of the molecular arrangement of the membranes. The membrane splitting found in PEA-treated *B. cereus* represents an ultrastructural observation that supports the concept of bilayer organization of biomembranes, as postulated in the Singer-Nicolson model (18) among others, and shows that the two monolayers can keep their planar continuity after the splitting of the bilayer. The treatment of *B. cereus* with PEA results in the presence of prominent, complex mesosome-like structures. These structures are observed in treated cells fixed by procedures which in normal *B. cereus* reveal small and simple invaginations of the cytoplasmic membrane, as is the case with the R-K method without prefixation (13, 14) and the glutaraldehyde-OsO_4-uranyl technique (14), or no mesosomes, as after the uranyl-OsO_4 fixation (14). As discussed in more detail elsewhere (14; Silva et al., in press), this finding can be interpreted as an indication that complex and prominent



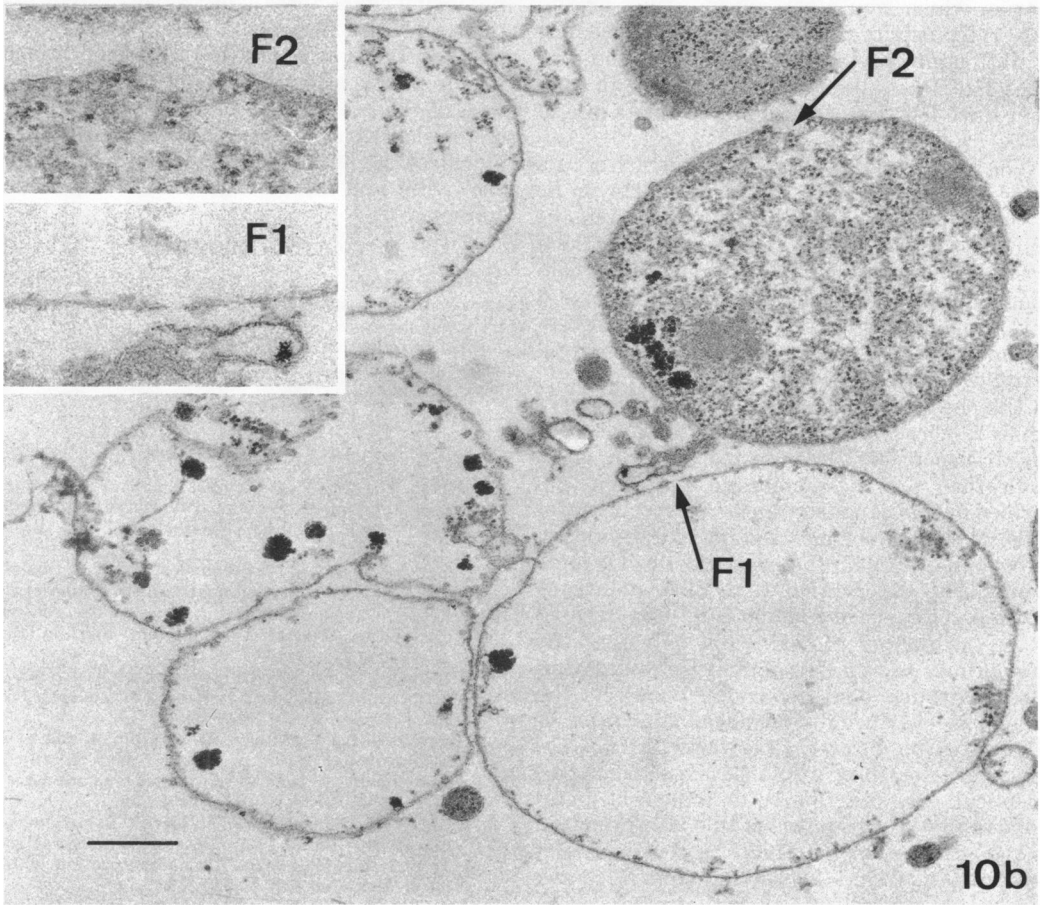


FIG. 10. *Protoplasts from B. megaterium. Fixation with glutaraldehyde-OsO_4-uranyl acetate. (a) Control. Bar indicates 0.3 μm. (b) Protoplasts treated with 0.5% PEA for 45 min. Notice the empty ghosts with fractures in the membranes (F1 and inset). The protoplast in the upper right side corner of the figure also exhibits a fracture in the membrane (F2 and inset). The bar indicates 0.4 μm.*

mesosomes can be produced by PEA. Other membrane-damaging treatments have also been found to result in the presence of mesosome-like structures, as is the case with the treatment of *B. cereus* and *S. faecalis* with moist heat (14, 16, 19; Silva et al., in press).

It is not clear why the nucleoids are not visible in most *B. cereus* cells treated with PEA. A quantitative study is necessary to determine whether this nucleic acid is degraded and/or leaked. In cultures of Ehrlich II B and L cells, PEA was found to induce both inhibition of DNA replication and loss of DNA from the cells (5), but it was later realized that these effects of PEA on DNA are not direct but are mediated by release of lysosomal enzymes (4). In *E. coli* subjected to bacteriostatic concentrations of PEA, DNA synthesis was stopped, but no decrease in DNA content was detected (1). It seems likely that the absence of the conspicuous nucleoids in PEA-treated *B. cereus* is due, at least in part, to the dispersion of the DNA fibrils, which would become indistinct among the cytoplasmic components. In accordance with this interpretation is the observation that abundant DNA-like material is visible in cells that have lost intracellular components due to lysis.

The marked fall in the turbidity which occurs after a few hours of action of 0.5% PEA on *B. cereus* cultures is explained by the extensive cell lysis shown by electron microscopy. Such lytic alterations probably are an unspecific consequence of the bactericidal action of PEA, and reveal general micromorphological aspects common to other situations acompaned by bacterial cell lysis (11, 14).

The observation that both the membranes of intact *B. cereus* cells and free membranes from lysed suspensions are affected by PEA is an indication that the membrane alterations described in this paper are due to a direct action of PEA on the membranes and not to a secondary effect of a damage inflicted to other cell components. The observation that PEA induces a quick and extensive leakage of K^+ from treated *B. cereus* further indicates not only that the membranes are directly damaged by PEA but also that such damage primarily affects the membrane permeability. Indeed, the rates of K^+ efflux from *B. cereus* treated with PEA are much greater than would be expected if this chemical were functioning as a general metabolic inhibitor. In support of this interpretation is the observation that high concentrations of sodium azide induce K^+ leakage from *B. cereus* at a much reduced rate than that produced by PEA (Fig. 3).

Alterations similar to those described in the present paper have been observed in bacteria subjected to treatments that, like treatment with PEA, result in a primary damage of bacterial cytoplasmic membrane with an increase in membrane permeability. This is the case with the treatment with several hydrophobic chemicals, including local anesthetics (14; Silva et al., in press) and with moist heat (16, 19; Silva et al., in press). The described effects induced by the hydrophobic (6) PEA molecule on *B. cereus* and *B. megaterium* are compatible with a mechanism of action similar to that reported for other membrane-active hydrophobic molecules. As discussed in more detail elsewhere (14), such effects are likely to be due to an increase in membrane fluidity and to membrane expansion, alterations that have been demonstrated in membranes treated with local anesthetics (2, 10).

In conclusion, we found that PEA affects the membranes of *B. cereus* and *B. megaterium*, producing characteristic ultrastructural alterations, lysis of protoplasts, and extensive K^+ leakage. It seems likely that PEA acts through a hydrophobic interaction with the hydrophobic core of the membrane, but the application of biophysical techniques is necessary to clearly elucidate the basic mechanism of PEA interaction with, and the damage to, bacterial membranes.

ACKNOWLEDGMENTS

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