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Effects of Local Anesthetics on Bacterial Cells

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The membrane effects of chlorpromazin, nupercain, tetracain, and procain were studied using *Bacillus cereus, B. megaterium, B. subtilis,* and *Streptococcus faecalis,* protoplasts from *S. faecalis,* and isolated membranes from *B. subtilis.* Chlorpromazin, nupercain, and tetracain produced characteristic micromorphological alterations after treatment for 5 to 30 min at pH 7.0 and 20°C; the membrane staining pattern changed from asymmetric to symmetric, complex mesosome-like structures appeared, and membrane fractures and solubilization occurred. Procain at concentrations up to 100 mM did not induce detectable alterations. Protoplasts were quickly lysed by 10 mM tetracain. A rapid and extensive leakage of K^+ was induced by chlorpromazin, nupercain, and tetracain. Procain (100 mM) induced a slight K^+ leakage. The membrane respiratory activity of intact *B. cereus* cells (as measured by the triphenyl tetrazolium reduction) and the succinic dehydrogenase activity of *B. subtilis* isolated membranes were found to be inhibited by the four local anesthetics. The concentrations that produced 50% inhibition of those activities are correlated with the hydrophobicities of the anesthetic molecules.

Previous studies from several laboratories have shown that the interaction between local anesthetics and membranes results in interesting effects. These include binding of the anesthetic molecules to membrane components, displacement of Ca^{2+} from the membranes, anesthetic action on excitable membranes, and modification of several membrane physical properties (11, 20). So far, the interaction of local anesthetics with membranes has been studied using natural eucaryotic membranes and artificial membranes. Here we report on the results of the study of the effects of four local anesthetics on bacterial cells. These results confirm and extend previous observations from this laboratory (24, 26, 27) and show that bacterial cells offer important advantages over eucaryotic cells as tools for the study of the effects of membrane-directed treatments. Such advantages derive from the following points: (i) permeability changes can be easily assayed in intact bacteria; (ii) characteristic ultrastructural alterations occur when gram-positive bacteria are subjected to membrane-active treatments; (iii) the chemical composition of bacterial membranes can be varied within large limits in a given strain by modifying the growth conditions, as discussed elsewhere (24); and (iv) the membrane organization in gram-positive *Eubacteria* is extremely simple (28), and it is easy and quick to prepare isolated membranes from these microorganisms (6). The relevance of studies in this area is evident since they may contribute to the advance in our knowledge of both the membrane structure and the mechanism of action of membrane-directed molecules with biological or pharmacological interest.

MATERIALS AND METHODS

Strains and growth conditions. *Bacillus cereus* NCTC 7587 and *B. megaterium* CCM 2037, kindly supplied by M. Kocur, were grown in tryptone broth (1.0% tryptone [Difco] and 0.5% NaCl, pH 7.2) at 30°C, with aeration by shaking, to 3×10^8 to 5×10^8 cells per ml. *B. subtilis* 168 was grown in 1.0% tryptone, 0.5% yeast extract (Difco), and 0.5% dipotassium phosphate (pH 7.2) at 37°C, with aeration by shaking, to 3×10^8 to 5×10^8 cells per ml. *Streptococcus faecalis* ATCC 9790 was grown to late exponential phase as described (culture *a* in ref. 19). Protoplasts of *S. faecalis* were prepared as reported elsewhere (19).

Treatment with anesthetics. Treatment with anesthetics was carried out by suspending the bacteria collected by centrifugation in 10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.0) plus the anesthetic. The treatments were done at 20°C for 5 to 60 min. Protoplasts were treated with 10 mM tetracain by adding the anesthetic to the protoplast suspension in the stabilization mixture (0.1 M Tris-hydrochloride buffer [pH 7.0] and 0.6 M sucrose).

Effects of tetracain on growth and viability of *B. cereus*. The absorbancies of control and tetracain-treated *B. cereus* cultures were determined at 520 nm

with a Bausch and Lomb Spectronic 70 spectrophotometer. The number of viable cells in the control and tetracain suspensions was determined by serial dilution plate counting.

Assay of K^+ efflux. The efflux of K^+ from control and anesthetic-treated *B. cereus* was determined as described elsewhere (27, 28), except that the final bacterial suspensions were made in 0.1% peptone plus 50 mM Tris-hydrochloride (pH 7.0).

Electron microscopy. For electron microscopy, three fixation procedures were used: OsO_4-uranyl acetate, glutaraldehyde-OsO_4-uranyl acetate, and uranyl acetate-OsO_4. Details of these methods as well as of embedding and electron microscopic observation procedures are found in previous publications (24, 25, 27, 28). All the micrographs presented in this paper are of sections contrasted with lead citrate (33) for 5 min.

Assay of membrane-bound enzymatic activities. The reduction of tetrazolium by *B. cereus* was used as a membrane-bound respiratory activity (10, 32) and was measured as follows. The bacteria were incubated in the presence of 50 mM Tris-hydrochloride buffer (pH 7.0), 50 mM sodium succinate, and 0.6 mM triphenyl tetrazolium chloride. After 10 min of incubation, glutaraldehyde was added to a final concentration of 2.5%. After 60 min at room temperature, the fixed bacteria were pelleted by centrifugation, and the red formazan was extracted with methanol. The amount of extracted formazan was measured by reading the absorbancies of the methanolic extracts at 485 nm in a Spectronic 70 spectrophotometer. To determine the effects of the local anesthetics on the tetrazolium-reducing activity, the bacteria were incubated in the presence of the drugs for 10 min at room temperature before the addition of succinate and tetrazolium. The activity of succinic dehydrogenase of *B. subtilis* membranes was measured as follows. The membranes of exponentially growing cells were isolated by the procedure of Konings et al. (6) without using ethylenediaminetetraacetic acid. Enzyme activity was assayed at 37°C by the dichlorophenolindophenol-phenazine methosulfate method (13) using a Perkin-Elmer recording spectrophotometer, model 420, fitted with a temperature-controlled automatic Multi-sampler. The assay mixture contained: 0.2 ml of 0.2 M Tris-hydrochloride buffer (pH 7.0), 10 μl of 0.2 M sodium succinate, 10 μl of 2.5 mM dichlorophenolindophenol, and 5 μl of 29.4 mM phenazine methosulfate in a final volume of 1.0 ml. Enzyme activity in the absence of added succinate was less than 5% of the activity in the complete mixture. To determine the enzyme inhibition produced by the local anesthetics, the membranes (610 to 660 μg/ml of protein) were incubated for 10 min in the presence of the drugs and Tris-hydrochloride buffer before the addition of succinate and electron acceptors. Control membranes were similarly incubated for 10 min in the presence of buffer alone. Enzyme activities were calculated on the basis of the initial rates (less than 20%) of the drop in the absorbancy (600 nm) of dichlorophenolindophenol. Protein concentrations in membrane suspensions were determined by the procedure of Lowry et al. (8) using bovine serum albumin (Sigma) as a standard.

Chemicals used. Procain hydrochloride, tetracain hydrochloride, dichlorophenolindophenol, and phenazine methosulfate were obtained from Sigma. Nupercain hydrochloride and chlorpromazin hydrochloride were gifts from Ciba-Geigy and Laboratórios Vitória (Lisbon), respectively.

RESULTS

Figure 1 shows the effect of tetracain on the growth curve of *B. cereus* and the number of survivors in cultures of *B. cereus* exposed to tetracain.

Ultrastructural aspects of control cells are shown in Fig. 2a and 3a and in several previous publications (24–27). Incubation of *B. cereus* for 60 min in the Tris-hydrochloride buffer used, did not produce any detectable ultrastructural alteration. The treatment with appropriate concentrations of tetracain, nupercain, and chlorpromazin resulted in important ultrastructural alterations in all bacteria studied. Such alterations were seen in samples fixed by any of the three fixation procedures used. Typical micromorphological alterations were observed in most *B. cereus* cells after 5 to 10 min of incubation in 10 mM tetracain. They consisted of the change in the membrane staining pattern from the normal (24) asymmetric to a symmetric geometry (Fig. 2b, 3b). Complex mesosome-like membranous structures were observed in many cells (Fig. 2b and 4). These structures sometimes exhibited a vesicular configuration; lamellar mesosomes were also observed (Fig. 2b). Sometimes, mainly after longer treatment times, the membrane alterations were more serious, with fracturing (Fig. 2b, 3b) and membrane solubilization. Membrane alterations similar to those described above were found in samples treated for 5 to 30 min with 1.0 to 2.5 mM nupercain (Fig. 3c, 3d, and 4) or with 0.25 to 1.0 mM chlorpromazin (Fig. 3e). Alterations to about the same degree were produced by 1.0 mM chlorpromazin, 2.5 mM nupercain, and 10 mM tetracain. In the cytoplasm of *B. cereus* treated with 10 mM tetracain, 2.5 mM nupercain, or 1.0 mM chlorpromazin, characteristic ultrastructural alterations were observed after 5 to 10 min of treatment: ribosomes became invisible, and blocks of an electron-dense material appeared (Fig. 2b and 3b, d and e). The well-defined nucleoids seen in the control *B. cereus* cells (Fig. 2a; see below also Fig. 1 in ref. 26) were not found in the treated specimens; dispersed areas of DNA-like fibrils were frequently observed (Fig. 2b). In samples treated with 10 mM tetracain for more than 20 min, many cells exhibited extensive lytic alterations with the characteristics already described in other situations, accompanied by bacterial cell lysis (23, 24). In samples

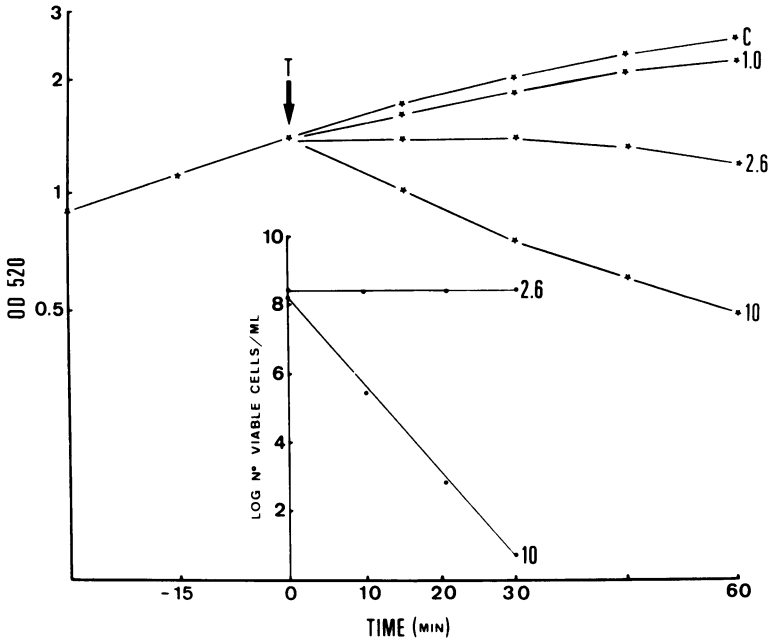

FIG. 1. *Absorbancy and number of viable cells of B. cereus cultures exposed to tetracain (1.0, 2.6, and 10 mM). C, Control.*

treated with 1 mM tetracain, 0.5 to 1.0 mM nupercain, or 0.1 mM chlorpromazin for 30 min, few cells appeared affected. In these cells alterations in the membranes and DNA areas similar to those described above for the treatment with higher concentrations for 5 to 10 min were observed. Ribosomes were, however, visible in most cells (Fig. 4). Most *B. cereus* cells treated for 5 to 30 min with the concentration of tetracain that induces a 50% inhibition of the membrane-bound enzymatic activity (2.6 mM, see Table 1) exhibited a cytoplasmic membrane with a symmetric staining pattern similar to that shown in Fig. 3b, but without fractures or solubilizations; ribosomes were visible. Procain at the concentrations studied did not produce significant ultrastructural alterations in *B. cereus.*

In *B. megaterium, B. subtilis,* and *S. faecalis* cells treated with 10 mM tetracain, 2.5 mM nupercain, or 1.0 mM chlorpromazin, ultrastructural alterations similar to those described above for *B. cereus* were observed (not shown).

Protoplasts from *S. faecalis* were quickly lysed by 10 mM tetracain. The membranes of the resulting ghosts showed a symmetric staining pattern and exhibited fractures. Dense blocks were present in the interior of the lysed protoplasts and could be seen leaking through the fractures in the membranes.

Figure 5 shows the K^+ effluxes induced in *B. cereus* by chlorpromazin, nupercain, tetracain, and procain. The order of activity concerning the permeability changes is chlorpromazin > nupercain > tetracain > procain.

Figure 6 shows the concentration-dependence plots for the inhibition induced by the treatment with the local anesthetics studied on the tetrazolium-reducing activity in *B. cereus* membranes. From this and similar plots the concentrations that produce 50% inhibition were deduced (see Table 1).

There was good correlation between hydrophobicity (as indicated by the octanol-water partition coefficients) and enzyme inhibition (correlation coefficients 0.986 and 0.985, respectively, for *B. cereus* and *B. subtilis*) (Table 1).

DISCUSSION

The results described in this paper show that some local anesthetics induce important effects in gram-positive bacterial cells. These effects include growth inhibition, reduction in the number of viable cells, lysis of protoplasts, permeability changes, characteristic ultrastructural alterations, and inhibition of membrane-bound enzymatic activities. In a recent publication (30), procain, which we found rather ineffective against *B. cereus* and *B. subtilis,* was shown to affect the synthesis of alkaline phosphatase in *Escherichia coli.* The two observations cannot be compared, since they deal with different pa-

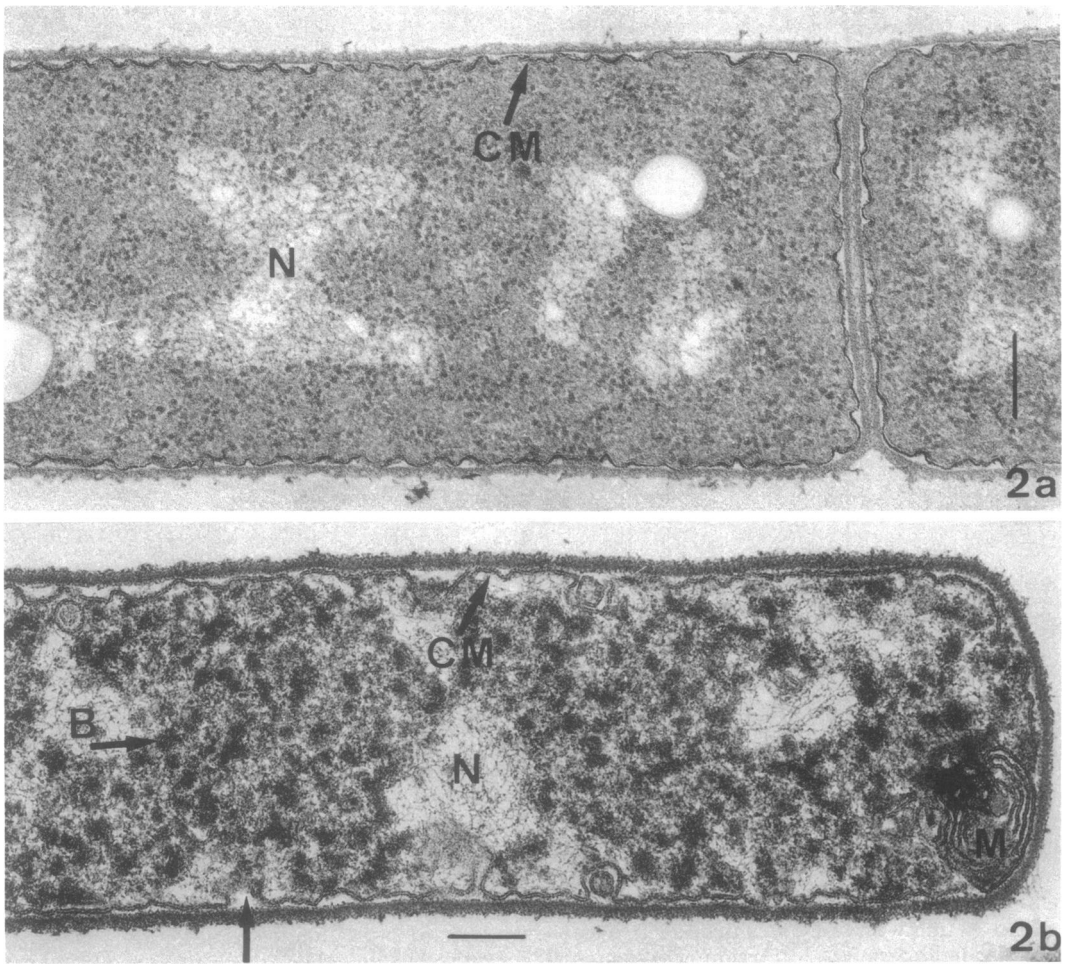

FIG. 2. *(a) Control B. cereus fixed with 0.1% uranyl acetate (28) followed by 1% OsO_4. Notice the continuous, asymmetric cytoplasmic membrane (CM), the nucleoids (N), and the numerous ribosomes. Bar, 0.2 μm. (b) B. cereus treated with 10 mM tetracain for 10 min and fixed with 1% OsO_4 (17) followed by 0.5 uranyl acetate. The cytoplasmic membrane has a symmetric profile and is fractured (unlabeled arrow). M, A lamellar mesosome-like body. In the cytoplasm, no ribosomes are visible. B, Dense blocks. Bar, 0. 2 μm.*

rameters and were studied in different microorganisms. Some of the results here reported indicate that the cytoplasmic membrane is directly affected by the anesthetics studied. In fact, isolated membranes are affected by the anesthetics (Table 1). Moreover, the quick and extensive K^+ efflux induced in treated bacteria further indicates not only that the membranes are directly affected by the anesthetics but also that membrane permeability is primarily disturbed. The K^+ effluxes were much greater than would be expected if the anesthetics were acting as metabolic inhibitors, like sodium azide (Fig. 5).

The membrane activity of local anesthetics is dependent on their interaction with the phospholipid components of biomembranes (see reviews 11, 20). Although an electrostatic binding of the anesthetic to the polar groups of the surface of the membranes occurs, at least in the case of the less hydrophobic drugs (4), the anesthetic molecule ultimately penetrates the membrane bilayer and accomodates in its hydrophobic interior (1-3, 15, 22). That is, the anesthetic-membrane interaction is fundamentally of a hydrophobic character. A consequence of this type of interaction is the known influence of the lipid solubility of several neutral anesthetic molecules on their membrane activity (9, 15). In our bacterial system we found a clear correlation between the activity of the four local

anesthetics studied and their lipid solubilities (in terms of their octanol-water partition coefficients).

As already discussed in detail for bacteria subjected to several membrane-damaging treatments (24), the characteristic ultrastructural alterations we observed in the membranes of anesthetic-treated bacteria may well be interpreted in terms of increased membrane fluidity and membrane expansion. These modifications in membrane properties are known to be induced by anesthetics in eucaryotic and artificial membranes (2, 5, 20–22). It is interesting that different treatments which conceivably or demonstratedly increase membrane lipid disordering, with the consequent increase in fluidity and permeability, induce in gram-positive bacteria common ultrastructural alterations. These in-

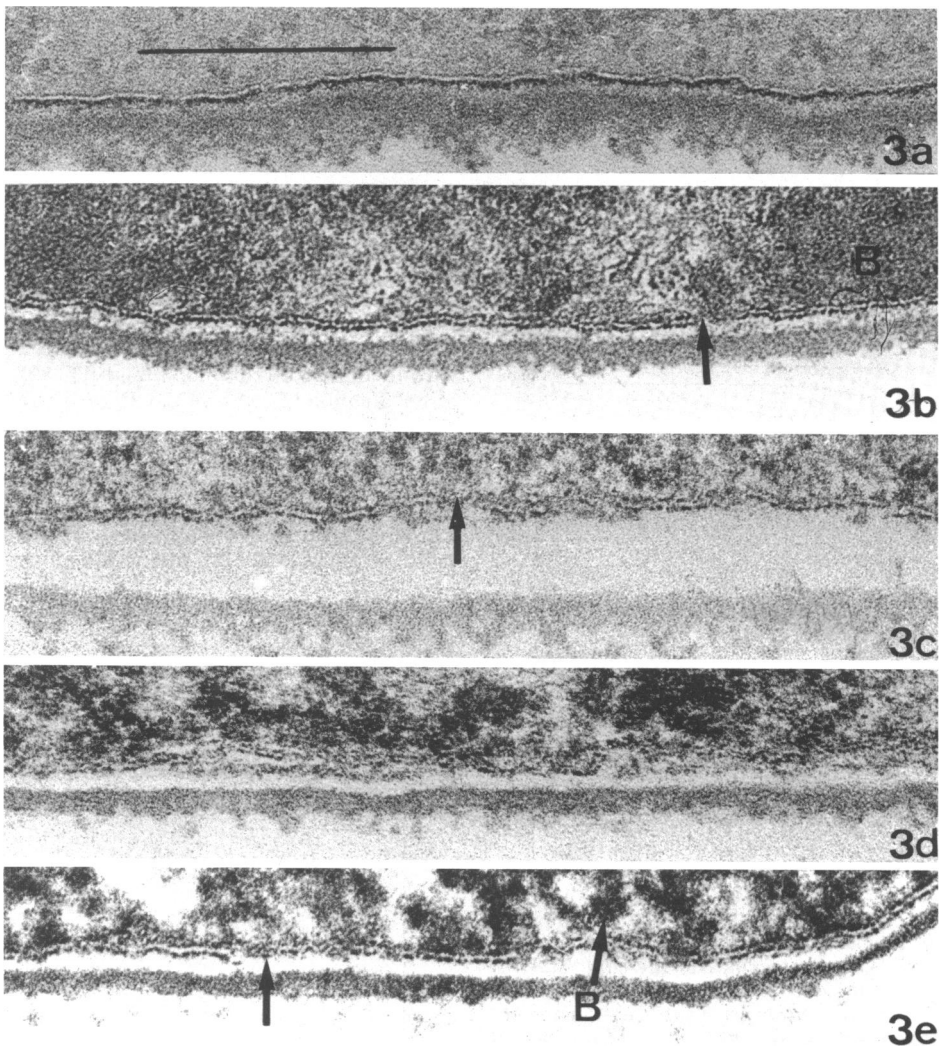

FIG. 3. *Aspects of the cytoplasmic membrane of control and treated B. cereus cells fixed with 1% OsO_4 (17) followed by 0.5% uranyl acetate. Notice the asymmetric profile of the cytoplasmic membrane and the presence of ribosomes in the cytoplasm. (a) Control cell. (b) Cell treated with 10 mM tetracain for 15 min. Notice the symmetric profile of the membrane and the membrane discontinuities (arrow). No ribosomes are visible in the cytoplasm. B, Dense blocks. (c) Cell treated with 1 mM nupercain for 30 min. The membrane has a symmetric profile and is partially solubilized (arrow). (d) Cell treated with 2.5 mM nupercain for 10 min. The cytoplasmic membrane is severely damaged, only faint remnants being left. (e) Cell treated with 1.0 mM chlorpromazin for 30 min. The membrane appears symmetric and with discontinuities (arrow). No ribosomes are visible. B, Dense blocks. Bar, 0.2 μm.*

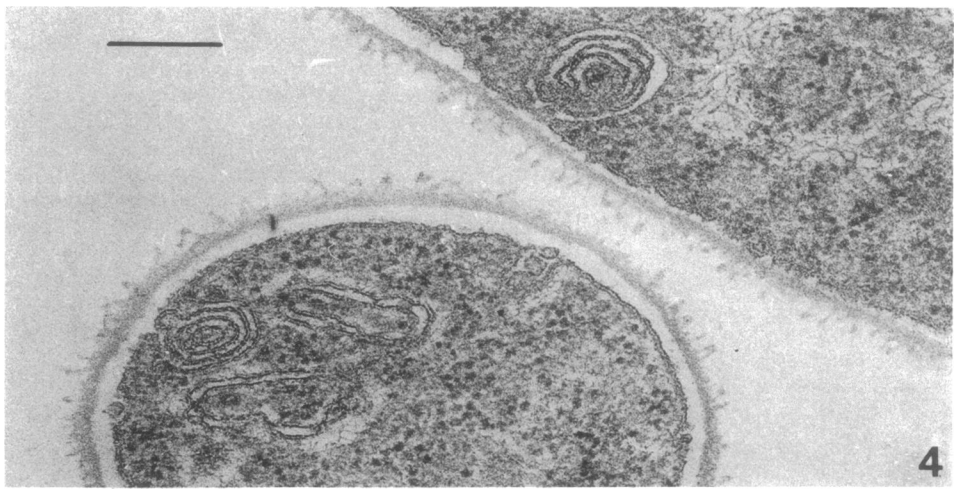

FIG. 4. *Lamellar mesosomes in cells treated with 1 mM nupercain for 30 min. The membranes are mostly symmetric. Sample was fixed with 1% OsO_4 (17) followed by 0.5% uranyl acetate. Bar, 0.2 μm.*

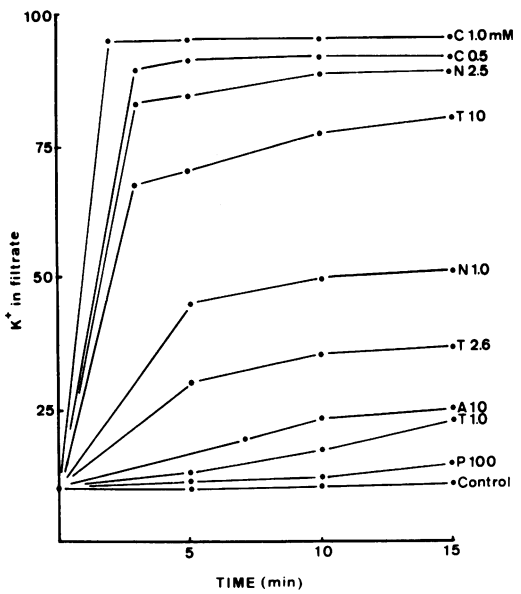

FIG. 5. *K^+ loss from control and anesthetic-treated B. cereus. C, Chlorpromazin; N, nupercain; T, tetracain; P, procain; A, sodium azide. All drug concentrations are in mM. All experiments were carried out at 20°C and pH 7.0. Ordinate gives filtrate K^+ as percentage of total culture K^+.*

clude change in the asymmetric membrane staining pattern (24) to a symmetric geometry; formation of complex mesosome-like structures, not present in the control cells (28); and, with long treatment times or with high concentrations, membrane fracturing and membrane solubilization. This is also the case with *Streptococcus* and *Bacillus* subjected to treatments with dicoumarol (18), moist heat (26, 29), phenethyl alcohol (27), or Nitro Blue Tetrazolium (28). The anesthetic-induced lysis of protoplasts can also be interpreted as a consequence of the disturbance in the membrane molecular organization produced by the anesthetic molecules. Bacterial protoplasts have been found to be lysed by moist heat (14), phenethyl alcohol (27),

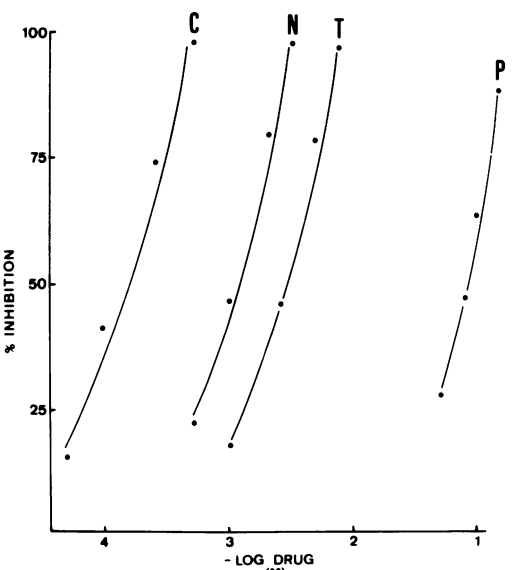

FIG. 6. *Inhibition of tetrazolium-reducing activity of B. cereus membranes as a function of drug concentration. C, Chlorpromazin; N, nupercain; T, tetracain; P, procain.*

TABLE 1. *Inhibition of membrane-bound enzyme activities induced by four local anesthetics*

Drug	Log P[a]	$-\text{Log } I_{50}$[b]	
		B. cereus	*B. subtilis*
Chlorpromazin	5.16	3.88	4.05
Nupercain	4.40	2.98	3.30
Tetracain	3.32	2.58	2.92
Procain	1.87	1.12	1.50

[a] Octanol-water partition coefficients taken from ref. 7.

[b] I_{50}, 50% inhibition concentration (M). Values are taken from Fig. 6. Tetrazolium-reducing activity of *B. cereus* membranes was assayed as described in the text. Succinic dehydrogenase activity of isolated membranes of *B. subtilis* was assayed as described in the text.

and Nitro Blue Tetrazolium (M. T. Silva et al., XI Ann. Meet. Portug. Soc. Electron Microscopy, 1976, abstr. 11).

The above-reported inhibition of membrane-bound enzymatic activities induced by the local anesthetics can be interpreted as a result of the perturbation of the membrane lipid compartment. In several other similar situations, disturbances in the membrane phospholipids have been considered responsible for alterations in the protein-lipid hydrophobic interactions, with a consequent perturbation of membrane-bound enzymatic activities (12, 31).

It is interesting that the concentration of tetracain that produced 50% inhibition of membrane-bound enzymatic activity after 10 min of treatment (Table 1) induced a significant K^+ leakage within 5 to 15 min (Fig. 5) and changed the membrane staining pattern from asymmetric to symmetric. The treatments for 30 min under these conditions stopped growth (Fig. 1) but did not kill the cells (Fig. 1). On the contrary, the higher concentrations of tetracain also studied in this work were strongly cytotoxic and induced irreversible damage to *B. cereus,* as deduced from the counting of the number of survivors (Fig. 1); the treatment under these conditions quickly resulted in extensive cell lysis, as shown by the fall in the absorbancies of the cultures (Fig. 1) as well as by the ultrastructural alterations we described.

The results here presented, as well as previous ones on the same topic (24, 27), show that bacterial cells can be successfully used as tools to study the interaction between biomembranes and membrane-directed molecules of biological or pharmacological interest. It is interesting that the concentrations of chlorpromazin and tetracain that induce a 50% inhibition of the membrane-bound enzyme activities in *B. cereus* and *B. subtilis* were close to those that produce the same degree of inhibition of the Na^+, K^+-adenosine triphosphatase activity of a microsomal membrane fraction from bovine brain cortex (16). However, in our opinion bacterial membranes offer several important advantages over eucaryotic membranes for such a study.

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