THE CELL ENVELOPES OF TWO EXTREMELY HALOPHILIC BACTERIA

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ABSTRACT

The cell envelope of *Halobacterium halobium* was seen in thin sections of permanganate-fixed cells to consist of one membrane. This membrane appeared mostly as a unit membrane but in a few preparations it resembled a 5-layered compound membrane. The cell envelope of *Halobacterium salinarium* at high resolution was always seen as a 5-layered structure different in appearance from the apparent compound membrane of *H. halobium*. The "envelopes" which were isolated in 12.5 per cent NaCl from each organism were indistinguishable from each other in the electron microscope and comprised, in each case, a single unit membrane with an over-all thickness of about 110 A. Some chemical analyses were made of isolated membranes after freeing them from salt by precipitating and washing with trichloroacetic acid. Such precipitated membranes consisted predominantly of protein, with little carbohydrate and no peptido-aminopolysaccharide (mucopeptide). Sectioned whole cells of *H. halobium* contained intracellular electron-opaque structures of unknown function.

INTRODUCTION

The extremely halophilic bacteria *Halobacterium halobium* and *H. salinarium* grow optimally in the presence of 4 to 5 M NaCl and quite well in saturated aqueous solutions of NaCl. These unusual organisms are prone to morphological changes and ultimately complete lysis when their suspending or growth medium is diluted (1, 9). Osmotic effects are obviously involved when a suspending medium is diluted rapidly, but osmosis is not the only and possibly not the most important factor in the ability of salts to maintain the structural and functional integrity of halophilic bacteria during growth. For example, Baxter and Gibbons (3) and Baxter (2) have demonstrated that the glycerol and lactic dehydrogenases of *H. salinarium* are active only at a relatively high salt concentration and are apparently denatured irreversibly at low ionic strengths. Gibbons and associates have demonstrated a specific role of Na^+ in preserving the normal morphology of halophilic bacteria and have suggested that this effect is distinct from an osmotic one (1, 9).

Cell envelopes isolated from *H. halobium* and *H. salinarium* rapidly dissolve when the salt solution in which they are suspended is diluted. The extent of the dissolution is approximately inversely proportional to salt concentration within the range 0.3 to 2.5 M NaCl (5). An investigation of the dissolution process was undertaken, but, because little information was available about the cell envelopes of extreme halophils, it was first necessary to ascertain general properties of these structures. In particular, it was sought to establish whether or not the "unit membrane" (as defined by Robertson (16)) occurs in and can be isolated from halophilic bacteria. The present article describes a method of fixing halophilic bacteria for ultrathin sectioning, the fine structure of these organisms, and some

chemical properties of their cell envelopes. A preliminary account of some of the findings has been given elsewhere (7).

MATERIALS AND TECHNIQUES

Organisms

Halobacterium halobium and *H. salinarium* were grown at 30°C for 2 to 6 days with aeration in a salts solution (25 per cent *w/v* NaCl plus smaller quantities of other salts, see reference 18) containing Oxoid peptone (1 per cent *w/v*).

Fixation Methods

Slightly different fixation procedures were used for each organism. *H. halobium* was harvested, washed once with the basal salts solution at 4°C, and mixed either with an equal volume of cold $KMnO_4$ (4 per cent *w/v*) buffered with barbiturate to pH 7.3–7.4 (12) (method A) or with an equal volume of similarly buffered cold $KMnO_4$ solution (4 per cent *w/v*) containing NaCl (25 per cent *w/v*) (method B). In method A the fixative was added slowly (during 2 to 3 minutes) with stirring in order to minimize osmotic shock. In method B the fixative was added rapidly. The suspension was immediately centrifuged in the cold (taking about 20 minutes), washed three times for 15 minutes at 2000 *g* with a cold aqueous solution of uranyl nitrate (2 per cent *w/v*) and three times with cold water, and then dehydrated conventionally with ethanol. The preparations were passed once through xylene, embedded in Araldite, sectioned with glass knives using an LKB ultramicrotome, and examined as described previously (6).

Halobacterium salinarium lysed when washed in the basal salts solution, so the fixation procedure was modified by washing and resuspending the organism in one of the following two solutions (at 4°C): solution 1, a saturated aqueous solution of NaCl containing 0.5 M $CaCl_2$; solution 2, an aqueous solution of NaCl (30 per cent *w/v*) containing 0.02 M $CaCl_2$. In both cases the cell suspension was mixed slowly with an equal volume of cold buffered $KMnO_4$ (4 per cent *w/v*) and thereafter treated as described above for *H. halobium*.

In our hands, buffered OsO_4 did not fix the organisms satisfactorily.

Isolation of the Cell Envelopes

The organisms were harvested, resuspended in cold (4°C) half strength basal salts solution, and disrupted mechanically as previously described (4). Cell envelopes were isolated from the disrupted suspensions by a series of alternate fast and slow centrifugations (4) in cold half strength salts solution. When such preparations were required for electron microscopy they were centrifuged, washed once in cold buffered $KMnO_4$ solution (2 per cent *w/v*), once in cold uranyl nitrate (2 per cent *w/v*), and three times in water, sectioned as described above or else deposited on carbon-coated nitrocellulose supporting film, and examined after shadowing with gold-palladium. In some preparations the uranyl nitrate treatment was omitted.

Envelope preparations which were required for chemical analyses were freed from salts by precipitating and washing them three times with cold aqueous trichloroacetic acid (10 per cent), once with ethanol, and three times with diethyl ether.

Chemical Analyses

Amino acids were identified in acid hydrolyzates (6 N HCl for 16 hours at 100°C) of the precipitated envelopes by two-dimensional paper chromatography and reaction with ninhydrin as described elsewhere (4). α,ϵ-Diaminopimelic acid was sought specifically in the one-dimensional solvent system of Rhuland *et al.* (15). Amino sugars were identified by paper chromatography in the same two-dimensional system

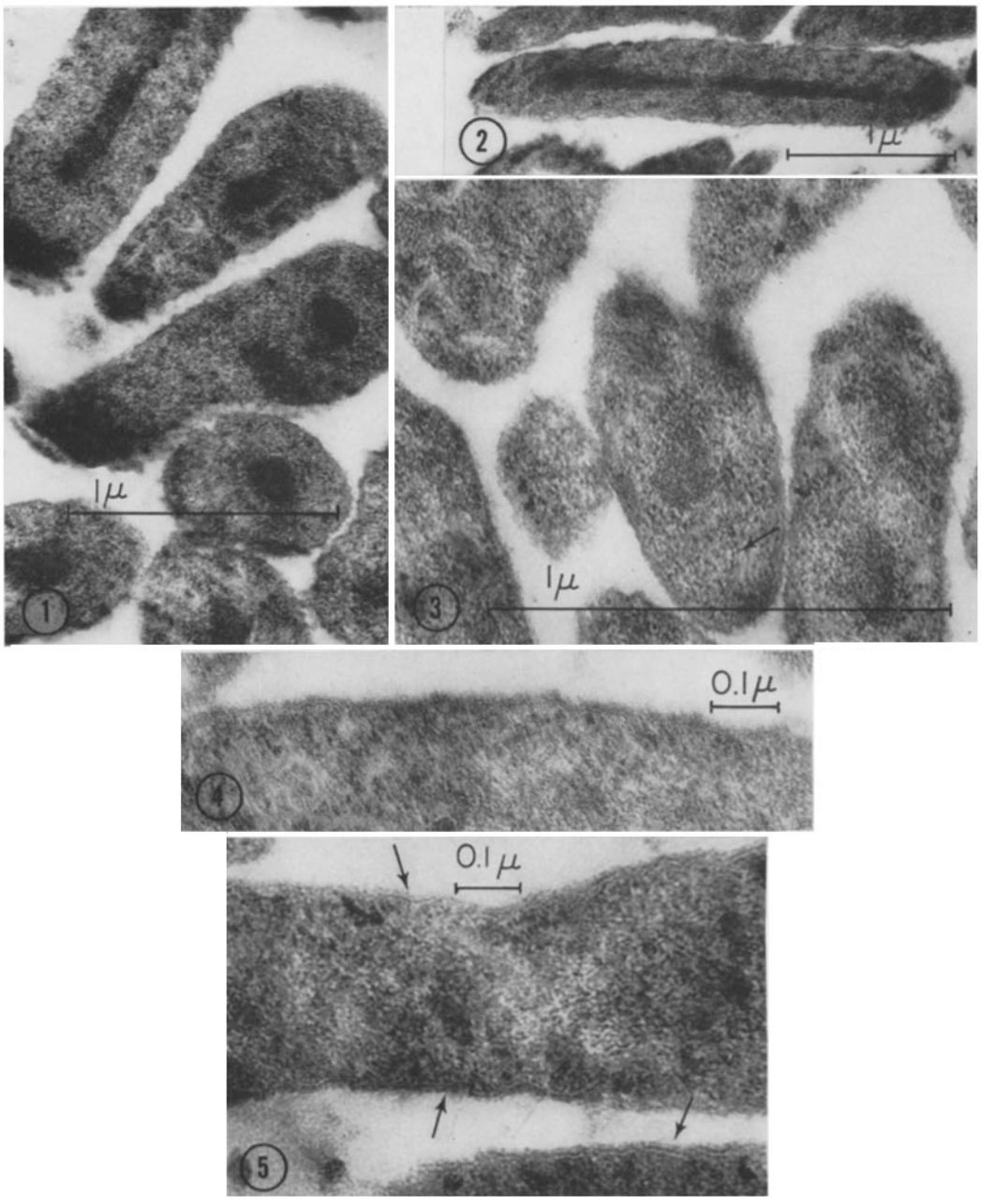
Note added in proof: The magnifications of Figs. 4, 5, 9, 10, and 11 are 70 per cent of those shown on the illustrations.

FIGURES 1 TO 5 Sections of *Halobacterium halobium.*

FIGURES 1 TO 3 Organisms grown for 6 days and fixed by method A. The electron-opaque intracellular structures are apparent. In Fig. 1 the dark zones at the left-hand ends of the three long cells are sectioning artifacts. In Fig. 3 there appears to be a "membrane" (arrow) running between an intracellular structure and the cell periphery. Fig. 1, × 38,000; Fig. 2, × 24,000; and Fig. 3, × 66,000.

FIGURE 4 A section of *Halobacterium halobium* fixed by method A, showing a single unit membrane at the periphery. × 70,000.

FIGURE 5 *Halobacterium halobium* fixed by method B. In places the cell envelope appears to be a compound (5-layered) membrane (arrows). × 70,000.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as the amino acids and by reaction with ninhydrin or *p*-dimethylaminobenzaldehyde. In some cases the amino sugar–ninhydrin complex was eluted with water, chromatographed in butan-1-ol + acetic acid + water (6:1:2, *v:v:v*) and examined for pentoses (see ref. 20). Sugars were identified tentatively by paper chromatography in the butanol + acetic acid solvent system and by reaction with aniline phthalate. Glucose was identified specifically by reaction with glucose oxidase (Glucostat, Worthington Biochemical Corporation). The presence of heptose(s) was established by the H_2SO_4-cysteine reaction of Dische (8). Protein was estimated by the method of Lowry *et al.* (11). Amino sugars were estimated in acid hydrolyzates (2 N HCl for 2 hours at 100°C) by the method of Rondle and Morgan (17); "reducing substances," total hexoses, and glucose were estimated in the same hydrolyzates by the methods of Park and Johnson (13) and of Dische (8) and by the use of glucose oxidase (6), respectively.

RESULTS

Fine Structure of the Bacteria

Figures 1 to 3 show thin sections of *Halobacterium halobium* taken from 6-day-old cultures. The appearance of the organism differs in several respects from that of $KMnO_4$-fixed bacteria from other sources. Firstly, there are few intracellular electron-transparent zones, with which nuclear material is often associated; secondly, the organism contains electron-opaque structures whose fine structure is different from that of the cytoplasm; thirdly, the electron opacity of the envelope is similar to that of the cytoplasm, and the envelope is accordingly less readily discernible than in many other types of organism. The function of the intracellular electron-opaque structures is unknown. In one instance there was evidence of a membrane (?) running between such a structure and the cell periphery (Fig. 3). In another isolated instance (not illustrated) the intracellular body was seen to be laminated like a concentric series of membranes. In organisms from 2-day cultures the bodies were less evident, but the effect of age on their appearance was not examined systematically. In agreement with the Gram reaction of these organisms (Gram-negative), no cell wall of the Gram-positive type is present, but the envelope differs also from the corresponding structures of other types of Gram-negative bacteria. Whereas Gram-negative bacteria from other habitats are bounded by two separable structures which in section resemble unit membranes (6), only one

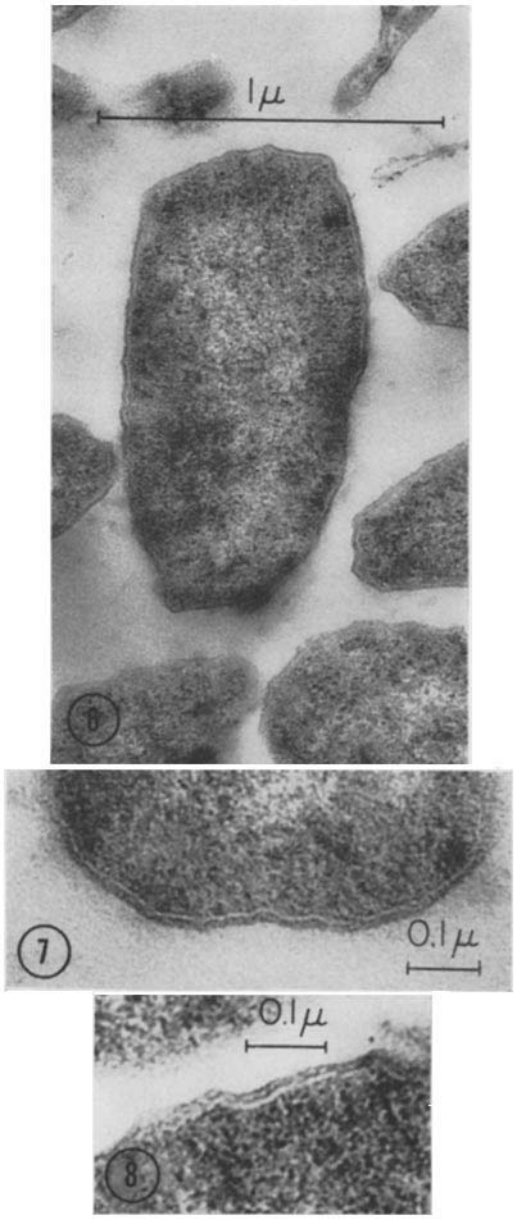

FIGURE 6 A section of *Halobacterium salinarium* in which the cell envelope resembles a thin cell wall with a closely underlying cytoplasmic membrane. Fixed while suspended in solution 1. Similar results were obtained with solution 2. × 46,000.

FIGURES 7 AND 8 As in Fig. 6. The "cell wall" is resolved into a layered structure 100 to 120 A in over-all thickness. The electron-transparent layer of the underlying "cytoplasmic membrane" is 50 A thick. Fig. 7, × 100,000; Fig. 8, × 100,000.

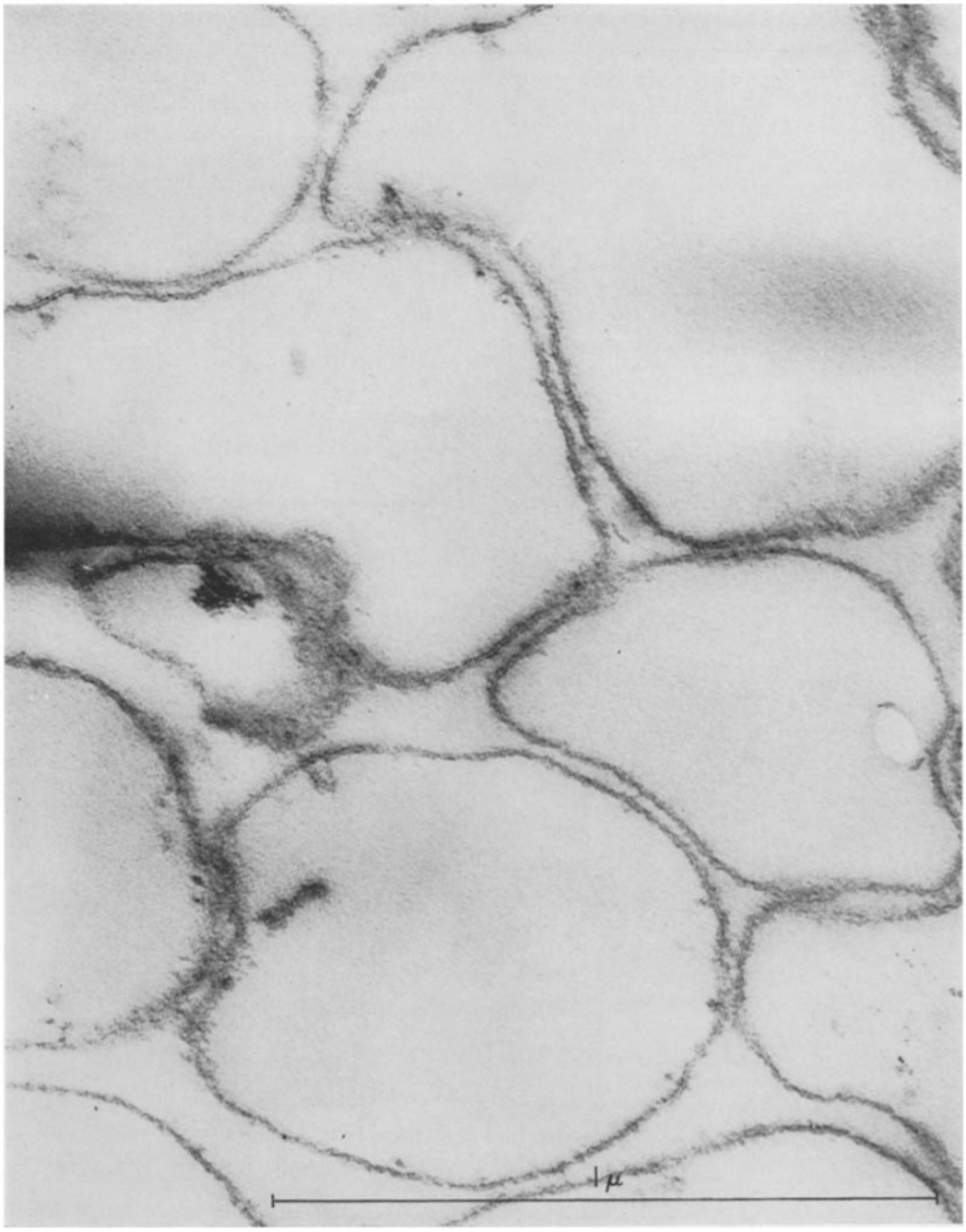

FIGURE 9 A section of "envelopes" isolated from *Halobacterium salinarium*. They are unit membranes about 110 A in over-all thickness. Similarly treated "envelopes" of *H. halobium* were identical. × 70,000.

structure has been detected in sections of *H. halobium*. Fig. 4 shows this membrane at higher magnification, under which conditions it resembles a unit membrane from other sources. The over-all thickness of the membrane lay within the range 90 to 120 A with approximately equal contributions by the two electron-opaque layers and one electron-transparent layer. In some preparations the

membrane was not located at the extreme outside of the cell but was recessed to a distance of about 100 A. The external material was often stained lightly and was difficult to identify. Occasionally, as in Fig. 5, however, it had the layered appearance of a membrane and formed, together with the more obvious membrane, a structure resembling a compound membrane of the type described by Sjöstrand (19). The "elements" (Sjöstrand's term) of the compound membranes were never observed to separate from each other.

Halobacterium salinarium differed from *H. halobium* in several respects. There were no intracellular electron-opaque structures such as were seen in *H. halobium*, and electron-transparent zones were present which resembled those of other bacteria. At low magnification the cell envelope seemed to comprise a cell wall with an underlying cytoplasmic membrane (Fig. 6). At higher magnification, however, the "cell wall" could be resolved into a 3-layered membranelike structure 100 to 120 A in over-all thickness (Figs. 7 and 8). The electron-transparent layer underlying the outer membrane measured about 50 A across. Essentially the same results were obtained with both fixation methods. The outer membrane was never observed to separate from the underlying transparent layer, and formed with it a 5-layered compound structure which was visibly different from the corresponding structure in *H. halobium*. This difference persisted when *H. halobium* was fixed by the same procedures as those used for *H. salinarium*.

The Isolated Envelopes

In thin section, isolated "envelopes" appeared as simple unit membranes about 110 A in over-all thickness rather than the compound structures

FIGURE 10 An "envelope" isolated from *Halobacterium salinarium*, fixed with $KMnO_4$ washed with uranyl nitrate and shadowed with gold-palladium. The "envelope" is obviously a single structure with no evidence of a separate internal or cytoplasmic membrane. Similarly treated "envelopes" of *H. halobium* were identical. × 42,000.

described above (Fig. 9). Under these conditions membranes from the two organisms were indistinguishable from each other. Membranes which had been treated with uranyl nitrate and shadowed had a granular appearance which is attributed to the constituent subunits on the membrane surfaces (Fig. 10). There was no visible difference between shadowed membranes isolated from the two organisms. Membranes which had not been treated with uranyl nitrate lacked the granules (Fig. 11) and in some cases there was evidence of partial disaggregation, suggesting that treatment with $KMnO_4$ was not in itself sufficient to stabilize the

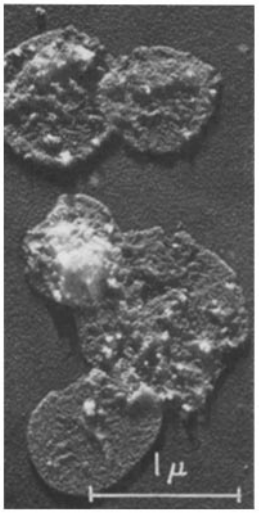

FIGURE 11 "Envelopes" isolated from *Halobacterium halobium*, fixed with $KMnO_4$ without subsequent treatment with uranyl nitrate. They have suffered partial disintegration. × 14,000.

membranes. The "envelope" isolated from each organism was always a single structure. No evidence was ever seen of two concentric membranes such as are isolated by comparable methods from NCMB 845 (4, 6).

The precipitated membranes of each organism released, on acid hydrolysis, the normal constituent amino acids of proteins (with the possible exception of cysteine/cystine), hexosamine, glucose, and a hexose which, in the butanol + acetic acid solvent system, could not be distinguished from galactose. The H_2SO_4-cysteine reaction was positive for heptose with membranes from *H. halobium* but not with membranes from *H. salinarium*. The amino sugars of *H. halobium* membranes consisted predominantly of glucosamine with probably a smaller amount of galactosamine. The amino sugar(s) from *H. salinarium* membranes was not examined further. No muramic acid nor α,ϵ-diaminopimelic acid was detected in hydrolyzates of the precipitated membranes (2 mg) of either organism, nor in trichloroacetic acid-precipitated whole cells (42 to 53 mg) from 2-, 4-, and 6-day-old cultures of *H. halobium*. Table I lists the results of some quantitative analyses of precipitated membranes of the two organisms. No analyses were made for lipids or related materials because of the ether washing to which the precipitates were subjected during preparation.

TABLE I

Chemical Analyses of Acid-Precipitated Membranes

	H. halobium	*H. salinarium*
	%	%
Amino sugar (as glucosamine)	1.2	1.3
"Reducing substances" (as glucose)	6.2	5.2
Total hexose (as glucose)	4.3	4.9
Glucose	0.65	0.60
Protein	65	75

In addition to these substances, small amounts of heptose were detected in membranes of *H. halobium* but not in those of *H. salinarium*.

DISCUSSION

Microbial cell envelopes usually consist of two distinct structures. Gram-positive bacteria, yeasts, actinomycetes, and related organisms are bounded by a cytoplasmic membrane and a rigid cell wall. Where sufficient resolution has been obtained, both the peripheral structures of Gram-negative bacteria have been shown to have the layered appearance and approximate dimensions of unit membranes (see, for example, refs. 6 and 22). There have been occasional reports of layering in cell walls of Gram-positive bacteria (14, 21), but the dimensions and chemical composition of these walls are such that confusion with membranes is unlikely.

The cell envelopes of the two halophils are different from those of either Gram-positive or Gram-negative bacteria, but the extent of the difference is uncertain. It is probably safe to conclude that the envelope of *H. halobium* is a single structure. It is more difficult to decide whether this structure is a unit membrane or a compound membrane. If the structure is, in fact, a compound membrane,

then one of its elements is difficult to preserve and/or visualize in the electron microscope. A factor which complicates the interpretation of the present results is the possibility of artifacts' arising during fixation in the high salt concentrations required by these organisms. We are not aware of electron micrographs of any other thin-sectioned extremely halophilic organisms with which comparisons can be made. The possibility of artifact is perhaps more obvious in the case of *H. salinarium*, in which the morphologically internal 50 A electron-transparent layer might be interpreted as a shrinkage space. The fact that this layer occurred after fixation under two sets of ionic conditions and could not be obtained in *H. halobium* reduces but does not eliminate this possibility. Whichever interpretation is preferred, however, the envelope of this organism also comprises one rather than two structures

There is less doubt about the isolated "envelopes." The chemical composition of the isolated structures is, in each case, consistent with that of a lipoprotein membrane (see also ref. 5). When adequate resolution was obtained in sectioned isolated "envelopes," they were seen always as simple unit membranes although of slightly greater dimensions than the ~75 A measured in other material by Robertson (16). It is not known whether this apparently greater thickness is significant. We conclude, therefore, that a structure essentially similar to a unit membrane occurs in the periphery of and can be isolated from these organisms. If the envelopes of the whole organisms should be shown unequivocally to be compound membranes, then it would follow that some components are lost during the isolation procedure. The observation which is relevant to the principal aims of this investigation, however, is that the structure which is isolated in 12.5 per cent (*w/v*) NaCl and which dissolves when the salt solution is diluted is a unit membrane.

The granular appearance of isolated membranes which had been treated with uranyl nitrate could have arisen either by aggregation of membrane subunits or by preservation of an existing structure. Membranes which had not been treated with uranyl nitrate were not granulated and showed evidence of disintegration. Houwink (10) noted a macromolecular pattern on the surface of *H. halobium*, but that pattern was more uniform than the granulation encountered in the present work.

Failure to detect either muramic acid or diaminopimelic acid in hydrolyzed membrane or whole cell precipitates indicates that these organisms do not contain measurable amounts of peptido - aminopolysaccharide (mucopeptide). Substances of this type, which constitute the major fraction of the cell walls of Gram-positive bacteria, occur in and are thought to stiffen the envelopes of Gram-negative bacteria. The cell envelopes of halophils, unlike those of other bacteria, do not normally have to withstand high pressures of osmotic origin. The Halobacteria are rigid under favorable conditions, however, and the means by which rigidity is acquired is thought to involve interaction between negative charges on the membrane and environmental cations (5).

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