A MORPHOLOGICAL STUDY OF *HALOBACTERIUM HALOBIUM* AND ITS LYSIS IN MEDIA OF LOW SALT CONCENTRATION

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ABSTRACT

The reported absence of a cell wall in halobacteria cannot be confirmed. Improved fixation techniques clearly show a cell wall-like structure on the surface of these cells. A stepwise reduction of the salt concentration causes the release of cell wall material before the cell membrane begins to disintegrate. The cell membrane breaks up into fragments of variable but rather small size, which are clearly different from a 4S component reported by others to be the major breakdown product of the cell membrane. It appears more likely that the 4S component arises from the dissolution of the cell wall. A residue of large membranous sheets remains even after prolonged exposure of halobacteria envelopes to distilled water. The lipids in these sheets do not differ significantly from the lipids in the lysed part of the cell membrane. The sheets, however, contain a purple-colored substance, which is not present in the lysed part. The easily sedimentable residue that remains after lysis of the cells or envelopes in distilled water also contains "intracytoplasmic membranes" with unusual structural characteristics. They can also be identified in sections through intact bacteria or envelope preparations. Their function is at present unknown but seems to be related to the formation of gas vacuoles in these organisms.

INTRODUCTION

Recently speculations that cellular membranes may consist of discrete identical subunits have attracted increasing attention. Attempts have been made to isolate and characterize these hypothetical subunits. Success has been claimed not only in their isolation but also in the reconstitution of membranes from these subunits. It has been known for some time that by morphological criteria membrane-like structures can be reconstituted from isolated membrane lipids and protein (31, 32, 33). However, the crucial test for membrane reconstitution requires the demonstration that typical membrane functions are simultaneously restored. One can only hope to achieve this if methods are used for isolation that do not irreversibly alter the properties of the membrane components. The membranes of halobacteria, which disintegrate readily when the salt concentration of the medium is reduced, would appear to be a favorable object for such studies. It has also been reported that the product of membrane breakdown in this case is a small particle that sediments with a sharp boundary in the analytical ultracentrifuge (6). These findings have prompted the study reported here.

The extremely halophilic bacteria, such as

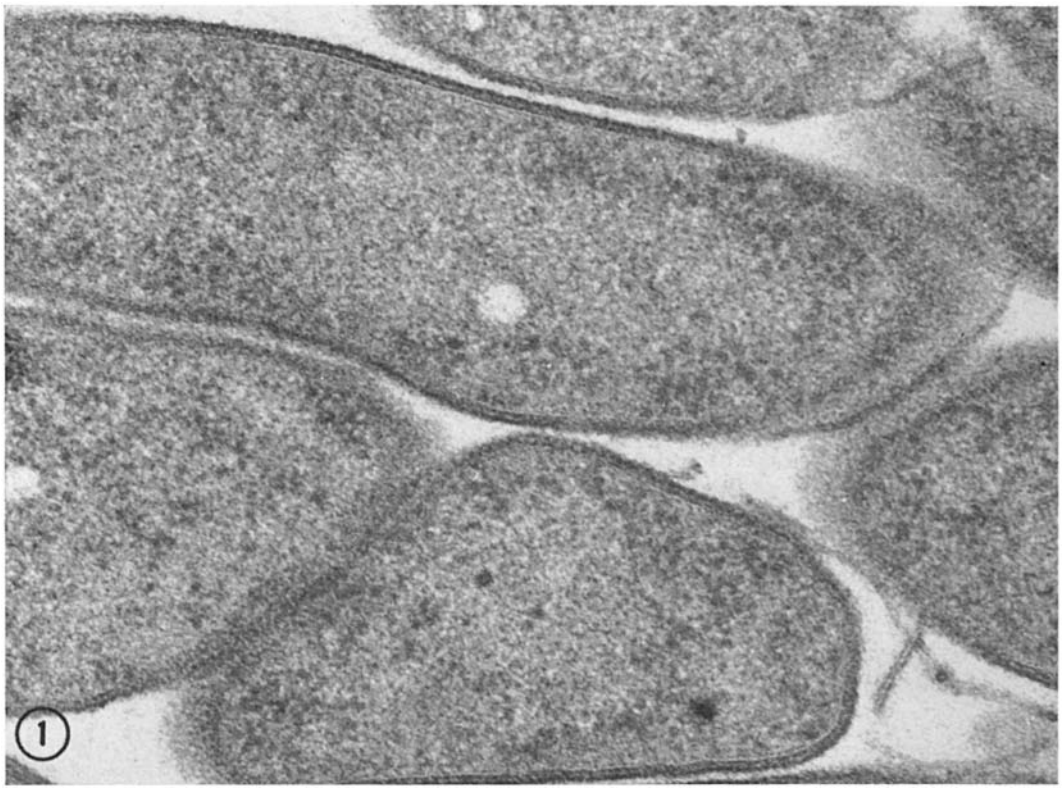


FIGURE 1 Typical cells from a shake culture in log phase. The rather dense nucleoids are surrounded by a cytoplasm containing numerous ribosomes. The central light layer of the cell membrane is clearly visible where the envelope is sectioned at right angles. The dense cell wall, clearly visible on the outer surface of the membrane, shows indications of a periodic structure. × 96,000.

Halobacterium halobium, *H. cutirubrum*, and *H. salinarium*, require high concentrations of salt for growth and preservation of their structure. When the salt concentration is lowered, growth ceases and the long slender rods assume first irregular and finally spherical shapes. Ultimately they will lyse.[1] The concentration of NaCl required for growth and maintenance of shape ranges between 3 and 5 M. Concentrations of 0.1–0.5 M Mg^{++} and 1.3–2.5 × 10^{-3} M K^{+} are also required for optimal growth. No salt has been found that could replace NaCl to any significant extent. Lysis, however, can be prevented in the absence of NaCl by other salts, such as NH_4Cl, KCl, and LiCl, in high concentrations, and also by relatively low concentrations of $MgCl_2$ and $CaCl_2$ (19).

Under the light microscope in phase contrast the intact organisms show no unusual features. In older cultures most cells of *H. halobium* develop central light areas, apparently gas vacuoles, which occupy a considerable part of the total cell volume. The chemical nature of the gas is not known. Under the electron microscope shadowed preparations reveal a surface structure consisting of a regular arrangement of spherical particles about 130 A in diameter (for references and a review of the earlier literature on halophiles, see reference 19).

Ribosomes have been isolated from *H. cutirubrum* and have also been shown to require high concentrations of salt for function and maintenance of structural integrity (3, 4).

Chemical analyses of whole cells and subcellular

[1] The term "lysis" as used here describes a disintegration of whole cells or isolated structural components into particles small enough to reduce the turbidity of a suspension by one or two orders of magnitude and not readily sedimentable at centrifugal forces up to 100,000 *g*.

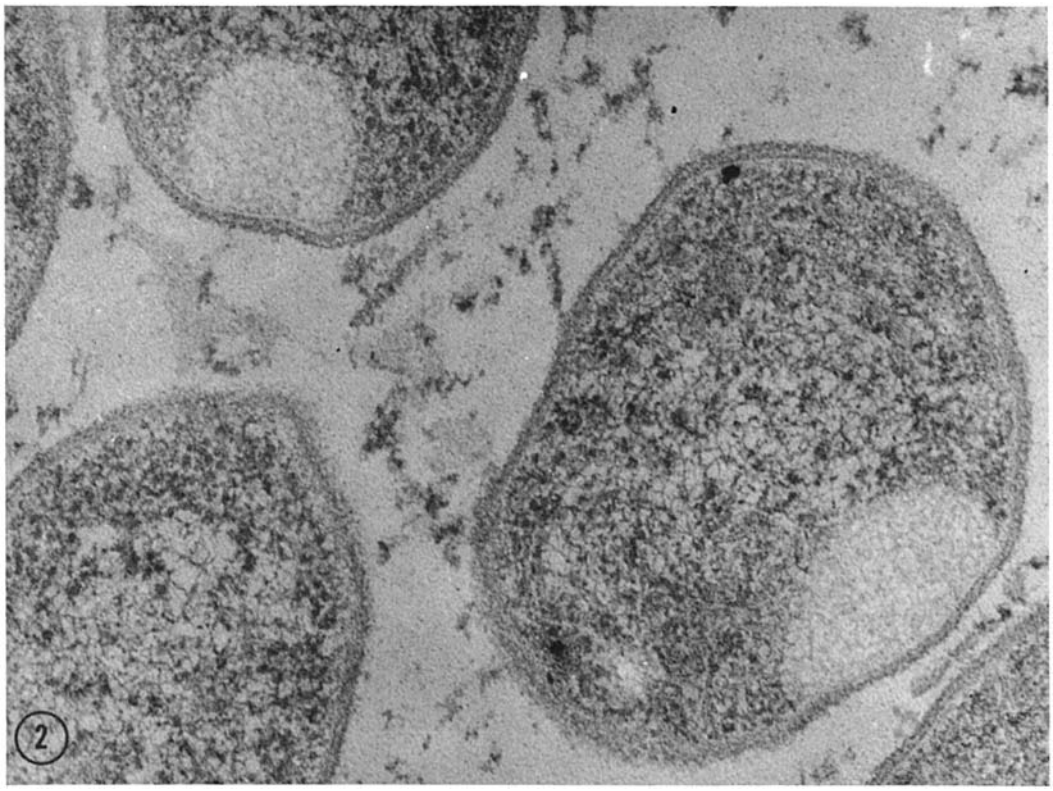


FIGURE 2 Section through cells showing the large round or oval bodies attached to the inner surface of the cell membrane. The chemical nature of these bodies has not been identified. × 96,000.

components have yielded interesting results. No muramic acid or diaminopimelic acid has been found in these organisms. The cell envelope fraction consists mainly of protein, lipid, and a smaller amount of unidentified carbohydrates (28, 6, 9, 17). The main lipid component has been identified by Kates and his associates as the diether analogue of phosphatidylglycerophosphate (27, 15). The fatty alcohols contain no double bonds; only isoprenoid hydrocarbon chains appear to be present. At least one carotenoid is usually found; it has been identified as α-bacterioruberine (20, 21).

Examination of the DNA isolated from halobacteria has shown no unusual features, but two components differing in their buoyant density have been observed in all strains so far investigated (13).[2]

The loss of structural rigidity and lysis upon salt depletion is poorly understood at present. It is generally assumed that a high charge density exists in the surface "membrane," which, upon removal of the counterions, disrupts the structure (2, 6). An unusually high content of dicarboxylic amino acids in the protein of cell envelope fractions has been demonstrated (6, 17, 18). While these findings are consistent with the assumed mechanism of lysis, they do not explain the specific NaCl requirement for growth and maintenance of the rod shape of the cells.

No electron microscope study of the morphological changes occurring during lysis seems to exist.

MATERIALS AND METHODS

Organisms

The strain of *Halobacterium halobium* employed in most of the experiments to be described was obtained from the National Research Council, Ottowa, Canada. In some early experiments a strain of *H. halobium* kindly made available by Dr. A. D. Brown was

[2] W. Stoeckenius. Unpublished results.

used. Both strains yielded identical results but the former was favored because it appeared to grow more rapidly under the conditions used.

Medium and Growth Conditions

The medium employed was that described by Brown (6), consisting of 1% Oxoid peptone in the basal salt solution (BS) used by Sehgal and Gibbons (26).

Organisms were grown at 37°C either as 1 liter shake cultures or in 10 liter carboys using forced aeration and constant stirring (Microferm Apparatus, New Brunswick Scientific, New Brunswick, N.J.). The carboys were inoculated with 300 ml of a 2–3 day shake culture grown at 37°C. Foaming was prevented by the addition of 10 ml of Dow-Corning Antifoam A Compound. Unless otherwise indicated, bacteria were harvested at the end of the log phase of growth (shake cultures, 5–6 days; aerated 10 liter carboy, 3 days).

Preparation of Envelopes

Cells were washed once in BS (containing 25% NaCl) and resuspended in sufficient BS to give a final volume which was about $\frac{1}{50}$ of the original culture. Highly vacuolated cells floated to the surface and were discarded with the supernatant. Mechanical disruption was accomplished by shaking the suspended organisms with equal volumes of 0.11–0.12 mm glass beads in a Bronwill Disintegrator (Bronwill Scientific, Rochester, N.Y.) for a period of 2 min using a jet of liquid CO_2 for cooling the disruption vessel. After removal of the glass beads and further dilution with an equal volume of BS to decrease viscosity, the material was spun for 60 min at 20,000 *g*. The supernatant was discarded and the pellet was resuspended in BS and centrifuged for 15 min at 5000 *g* to remove unbroken cells. The alternate high and low speed centrifugations were repeated four to five times until the pellet obtained from the 20,000 *g* run appeared free of unbroken cells and the supernatant contained little or no pigment. The final pellet suspended in a small volume of basal salt solution constituted our "envelope preparation." This was stored at 4°C and usually used within 2–3 days.

FIGURE 3 Tangential section through the cell wall showing indications of a regular hexagonal structure. × 160,000.

Analytical Procedures

Protein was determined by the method of Lowry et al. (22) with a freshly prepared solution of bovine serum albumin as standard. Lipids were extracted from envelopes and envelope fractions overnight at room temperature with chloroform-methanol, 2:1. Purification of the lipid extract involved washing with distilled water, drying under nitrogen, and reextraction of the dried material with chloroform (29). Total lipids were determined gravimetrically and lipid phosphorus was measured by a modification of the method of Stewart and Hendry (30). RNA content was estimated after hot trichloroacetic acid extraction by an orcinol procedure (23) using purified yeast RNA as standard.

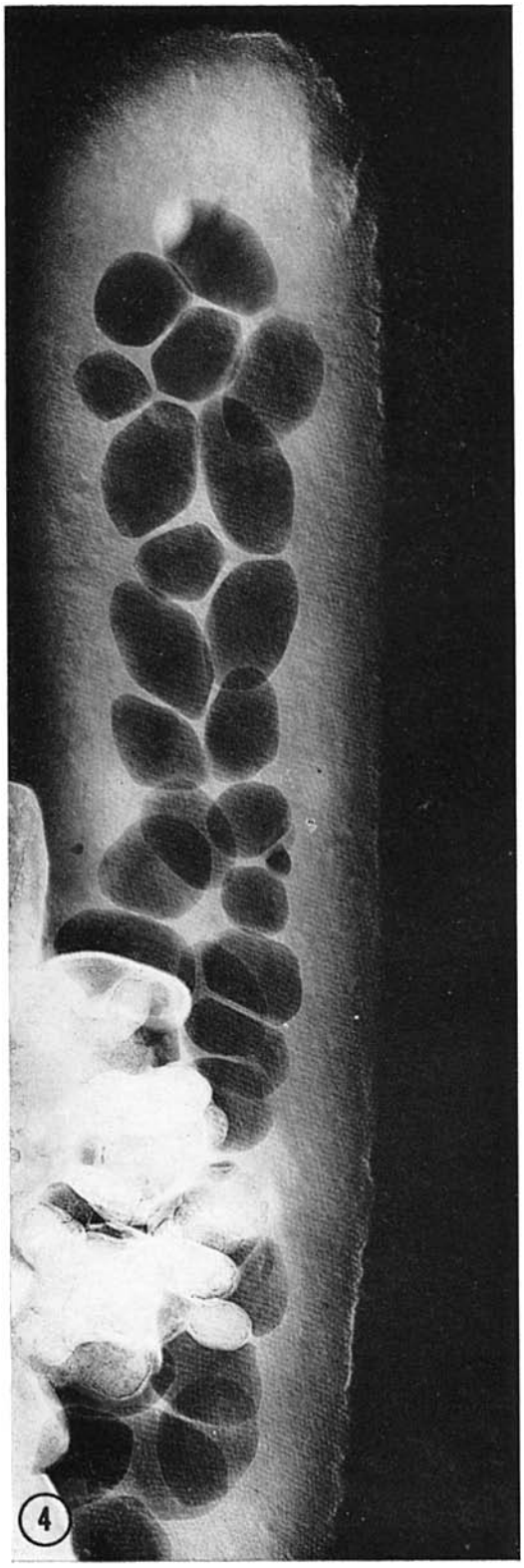
Thin layer chromatography was carried out on Silica Gel H (Merck, Darmstadt, Germany) using a solvent system of chloroform-methanol-water, 65:25:4.

Preparation of Samples for Electron Microscopy

SHADOWING TECHNIQUES: To examine surface structure of cells and envelope preparations, the material was diluted to barely visible turbidity in the appropriate salt solution, applied directly to hydrophilic, carbon-coated Formvar grids, and shadowed with uranium (10 mg at 10 cm and 12°).

For examination of envelope lysate fractions the material was sprayed onto freshly cleaved mica, shadowed with platinum-carbon (at an angle at 6°), and coated with a film of evaporated carbon. The preparation was reinforced with a thin backing of collodion, floated off onto a water surface, and mounted on grids.

NEGATIVE STAINING: Suitable dilutions of the lysate or lysate fractions were applied to Formvar-carbon-coated grids and the grid edge was touched with a filter paper. Before the remaining film of fluid had dried, one or more drops of the negative stain were successively applied to the grid and removed in the



same manner. The grids were then dried in a desiccator and coated with a second thin layer of carbon. 1% uranyl acetate or uranyl formate or 2% phosphotungstic acid neutralized with KOH were used as negative stains.

FIXATION AND EMBEDDING: Pellets of washed cells, envelope preparations, and envelope fractions, approximately 2 mm in diameter, were obtained. Many different fixation procedures were tried and the following was found to give the best results. Pellets were covered with a 4% solution of formaldehyde containing 25% NaCl and 1% $CaCl_2$ at pH 7.0 and allowed to remain at 4°C overnight. All subsequent fixation steps were also carried out in the cold. The pellets were then washed twice for 30 min intervals with 25% NaCl containing 1% $CaCl_2$ and exposed to freshly prepared 2% $KMnO_4$ solution containing 25% NaCl and 1% $CaCl_2$ (pH 7.0) for a period of 30–60 min. In some of the experiments involving partial or total lysis of cells or envelopes, the salt concentration of the fixative and washing solutions was reduced to the same amount as in the solution used for lysing the cells or envelope preparations. The specimens were then washed in salt solution and treated with 2% aqueous solution of uranyl nitrate for 30 min. After several washings with water the fixed material was dehydrated with acetone and portions were embedded in Epon for sectioning. In most cases the material was oriented during embedding so that a cross-section through the center of the pellet could be obtained. Thin sections were cut with a diamond knife and mounted on Formvar films that were lightly coated with carbon. Sections were double-stained with magnesium uranyl acetate and lead citrate (11).

Effects of Salt Concentration

Freshly prepared cells or envelopes were suspended in BS to give in a Bausch & Lomb Spectronic 20 an OD of 0.2–0.4 at 700 mμ when diluted 1:100. To examine the changes induced at different salt concentrations, aliquots were mixed with 100 volumes of BS diluted to the appropriate concentration with respect to NaCl. The test suspensions were maintained at room temperature for 30 min. Extent of lysis was measured as decrease in OD at 700 mμ. The material was then centrifuged for 15 min at 15,000 *g* (cells) or 30 min at 20,000 *g* (envelopes). The pellets were retained for electron microscopy and the supernatant fluids were examined for released material.

FIGURE 4 Shadowed whole cell from a culture at the end of the log phase. There is extensive vacuolization of the central part of the cell. The regular surface pattern, which does not appear hexagonal in this case, is clearly visible over most of the surface. The mass of material on the lower left is salt crystals partially evaporated and distorted under the electron beam. × 56,000.

RESULTS

Intact Cells

A thin section through cells of *H. halobium* from a 3 day shake culture (early log phase) is shown in Fig. 1. The rod-shaped cells have a rather dense cytoplasm with nucleoids of almost the same density. Numerous, ill-defined, denser particles in the cytoplasm probably represent ribosomes. A few considerably denser and slightly bigger particles visible in some of the cells have not been identified. Clear round areas of variable size in the cytoplasm could represent sections through gas vacuoles.

Where the cell envelope is sectioned approximately radially, several layers can be clearly discerned. An innermost clear band about 30 A wide is the most constant feature observed. That it actually represents the central light layer of a typical unit membrane will become obvious from inspection of sections through isolated envelopes to be described later. On its surface bordering the cytoplasm a thin dense band can sometimes be seen, but is often not visible at all, presumably because its density equals that of the cytoplasm. A dense band on the outer surface of the light band is much more regularly and clearly observed, but sometimes it merges imperceptibly with the 130–150 A wide layer of material that forms the outer surface of the cell. In Fig. 1 the outer part of this 130–150 A layer is much denser than the inner, but Fig. 2 shows a micrograph where this stratification is not so obvious, and sometimes, especially in older cultures, the outer 130–150 A layer appears frayed and less dense (Fig. 6). This variability in appearance may be attributable, at least in part, to accidents of fixation and staining, because the appearance of the cells sometimes differs in different regions of the same pellet.

In some places (see Fig. 1) the outermost dense layer of the envelope shows indications of a regular beaded appearance with a periodicity of approximately 150 A. Indications of a regular structure can occasionally also be seen in tangential section (Fig. 3). This appearance is probably related to the well-known surface structure (12, 16, 24) seen in shadowed preparations of whole bacteria (Figs. 4 and 5). It has been described as a hexagonal arrangement of spherical particles with a spacing of 120–150 A (16). In our shadowed preparations we usually did not find a clearly hexagonal lattice, but this may be an artifact due to distortion during drying. Bundles of polar flagella are also frequently seen in shadowed preparations.

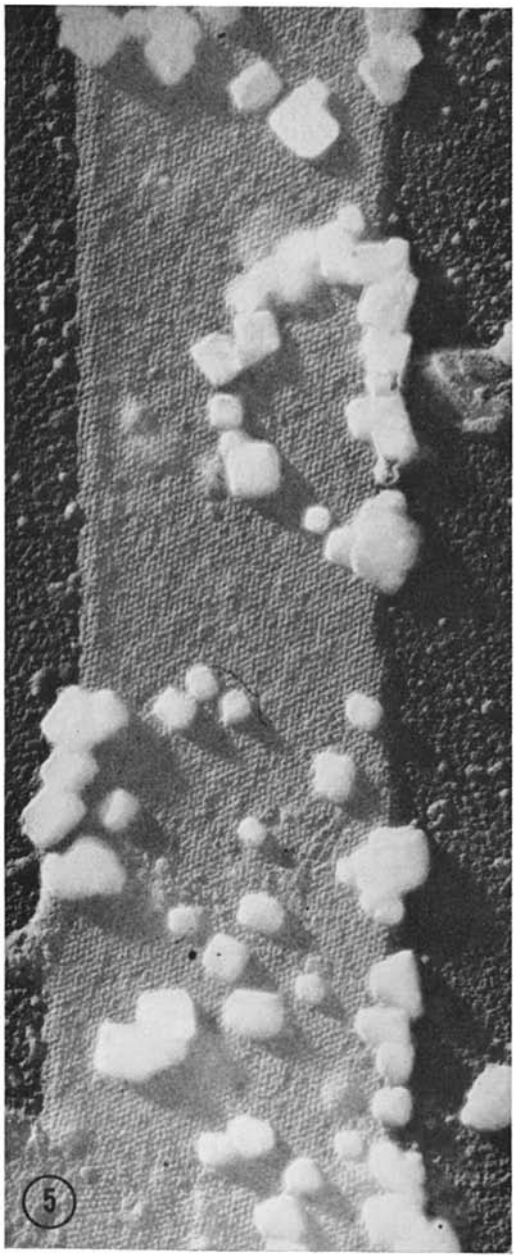


FIGURE 5 Empty envelope of a cell, to show the surface structure more clearly. Numerous salt crystals are also present. Again the pattern is clearly not hexagonal over most of the area. × 56,000.

The envelope of these cells therefore seems to consist of the following components: (*a*) an inner

unit membrane, the cell membrane proper, whose central light layer is always clearly visible; the outer dense layer is usually visible while the inner dense layer is rarely discernible; and (*b*) a cell wall with a regular surface structure; it may also show a layered appearance in sections, with an outer denser and an inner lighter layer.

It is, of course, possible that the outer dense layer of the cell membrane or at least part of it actually belongs to the cell wall. This problem cannot be resolved before considerably more is known about the chemical composition of these structures, and a separation has been achieved.

Older cells from a 6 day shake culture (end of log phase) show essentially the same envelope structure and general morphology. Their outline is somewhat more irregular, the cytoplasm usually appears less dense, the fewer ribosomes stand out with higher contrast, and the DNA strands of the nucleoids are better defined. In some cells a round or oval, rather homogeneous body is found (Fig. 2). Usually it appears to be in contact with the inner surface of the cell membrane on one side and may measure more than 0.5 μ on its longest dimension. The contents are granular; the over-all density is variable. Other cytoplasmic components appear to be excluded from this body.

Another morphological feature of these cells is easily overlooked; it is, however, of considerable importance for an understanding of the results obtained in the lysis and fractionation studies to be reported below. In the cytoplasm structures are observed that look like sections through short pieces of unit membrane up to 0.3 μ long and about 80 A wide (Fig. 6). Though they are often found close to the cell membrane, no continuity with it has ever been clearly established. If these structures actually should be membranes, as their appearance in sections suggests, they show very unusual features. They apparently do not separate two compartments, but appear as plates with a free edge and they sometimes show short side branches. We shall provisionally call them "intracytoplasmic membranes." Since they are hardly visible in the dense cytoplasm of younger cells it is difficult to establish at what time in the growth cycle they appear. They are present in small numbers on the third day in a shake culture. Their number definitely increases with the age of the cells. On the sixth day as many as three or four may be seen in cross-section of a single cell, and more in longitudinal sections (Fig. 7).

Envelope Preparations

A thin section through a pellet from an envelope preparation is shown in Fig. 8. The envelopes form closed, roughly spherical vesicles with a diameter approaching the diameter of whole cells. Some cylindrical vesicles are also present. The wall of these vesicles has the same layered structure seen in the envelope of intact cells. Because the vesicles contain little material, the inner dense band of the

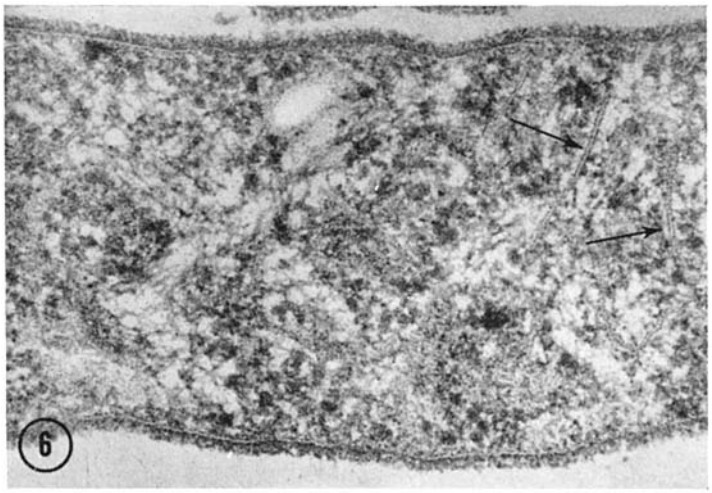


FIGURE 6 Intracytoplasmic membranes (arrows) in section through a cell from a culture at the end of the log phase. × 96,000.

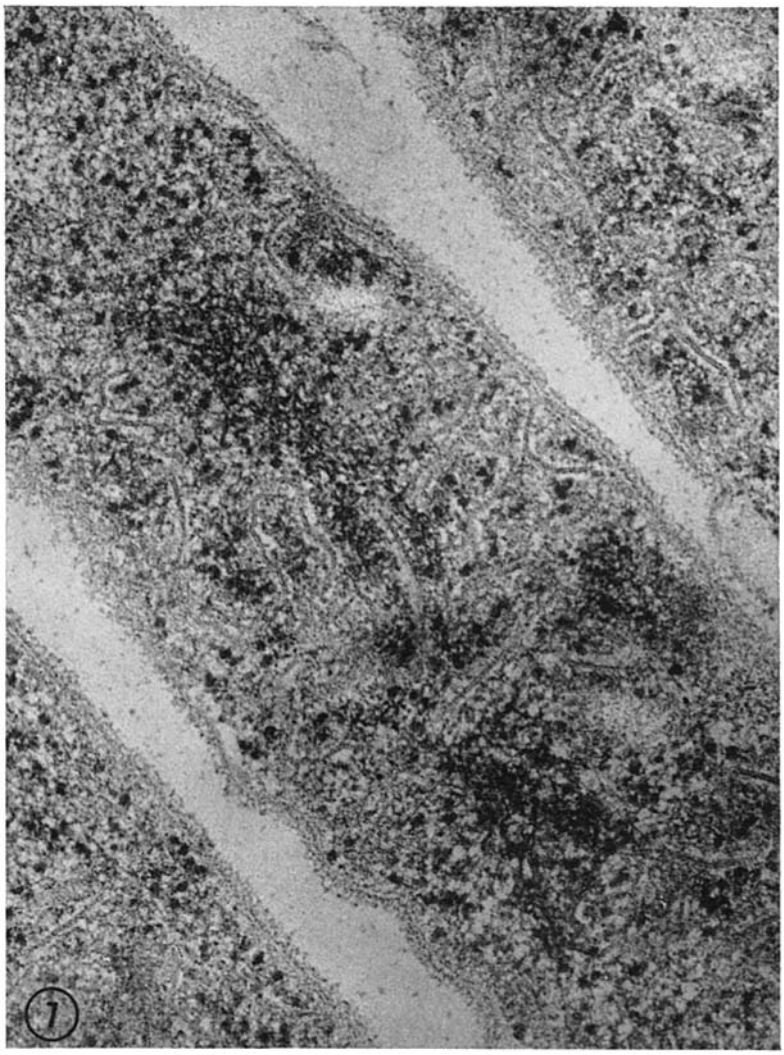
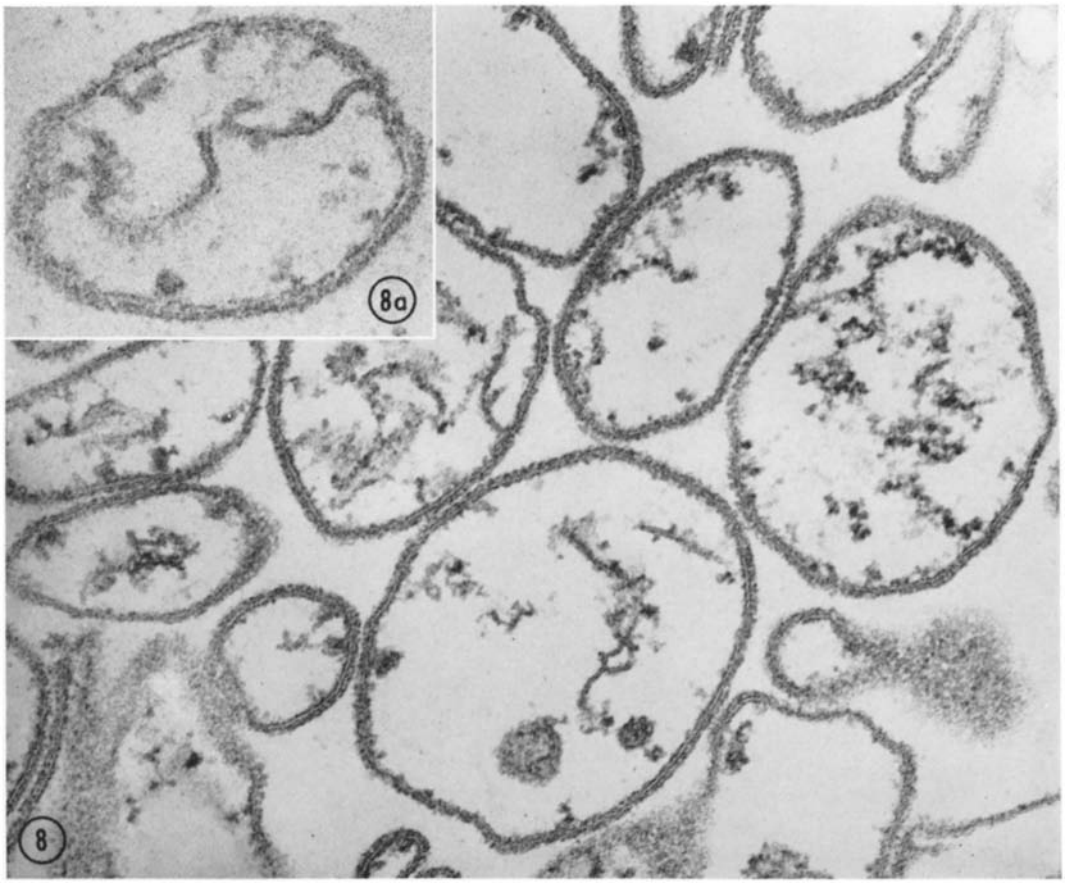


FIGURE 7 Section through cells from a 6 day shake culture, showing numerous intracytoplasmic membranes. × 96,000.

cell membrane can now be clearly observed. Its rather fuzzy and irregular border suggests that some cytoplasmic material adheres to it. The intracytoplasmic membranes are now also very clearly seen. Often they appear to be attached to the cell membrane at one end (Fig. 8 *a*), but no continuity between the three layers of these two structures has ever been observed. In addition to intracytoplasmic membranes the envelope vesicles contain a variable, but usually small, number of dense particles, presumably ribosomes. The big granular bodies seen in whole cells are also occasionally encountered in the vesicles. The younger the culture from which the envelopes are prepared, the fewer the structural elements contained in the vesicles.

The surface pattern seen in shadowed preparations of whole cells is also found on the isolated envelopes. Occasionally encountered, disrupted envelope vesicles show that this pattern exists only on the outside of the envelope; the inner surface appears smooth (Fig. 9). Spherical particles (most of them 200–250 A in diameter), which are also found in shadowed preparations of whole cells,



FIGURES 8 and 8 *a* Section through an envelope preparation. The cell membrane and cell wall are clearly discernible. The content of the vesicles seems to consist mainly of some ribosomes and intracytoplasmic membranes. An area was chosen where the number of intravesicular components is considerably higher than average. Fig. 8 *a* (inset) shows one vesicle at higher magnification. × 74,000 and 148,000 (inset).

and some free intracytoplasmic membranes are other morphological components visible in these envelope preparations.

Effects of Low Salt Concentrations on Intact Cells and Envelope Preparations

A reduction of the salt concentration in the suspending medium caused large changes in the turbidity of the suspension accompanied by morphological changes and the release of material from the cells. These changes were followed qualitatively by electron microscopy and quantitatively by measuring the change in optical density and the release of nonsedimentable material. The changes in optical density were found to be quite reproducible, but the percentage of material that was no longer sedimentable varied considerably from one experiment to another. The concentration of NaCl at which a given percentage of material was found in the supernatant differed as much as 0.5 M in different experiments. RNA showed the most erratic behavior. The cause of this variability has not been established. However, the general pattern of release was found to be the same in all experiments. This is true for envelope preparations as well as for whole cells.

WHOLE CELLS: Changes in turbidity and the amount of material released from cells of *H. halobium* as a function of decreasing NaCl concentration are shown in Fig. 10. Between 2.5 M and 1.0 M NaCl a sharp increase in turbidity occurs, followed by a steep decrease below 1.0 M. At 0.5

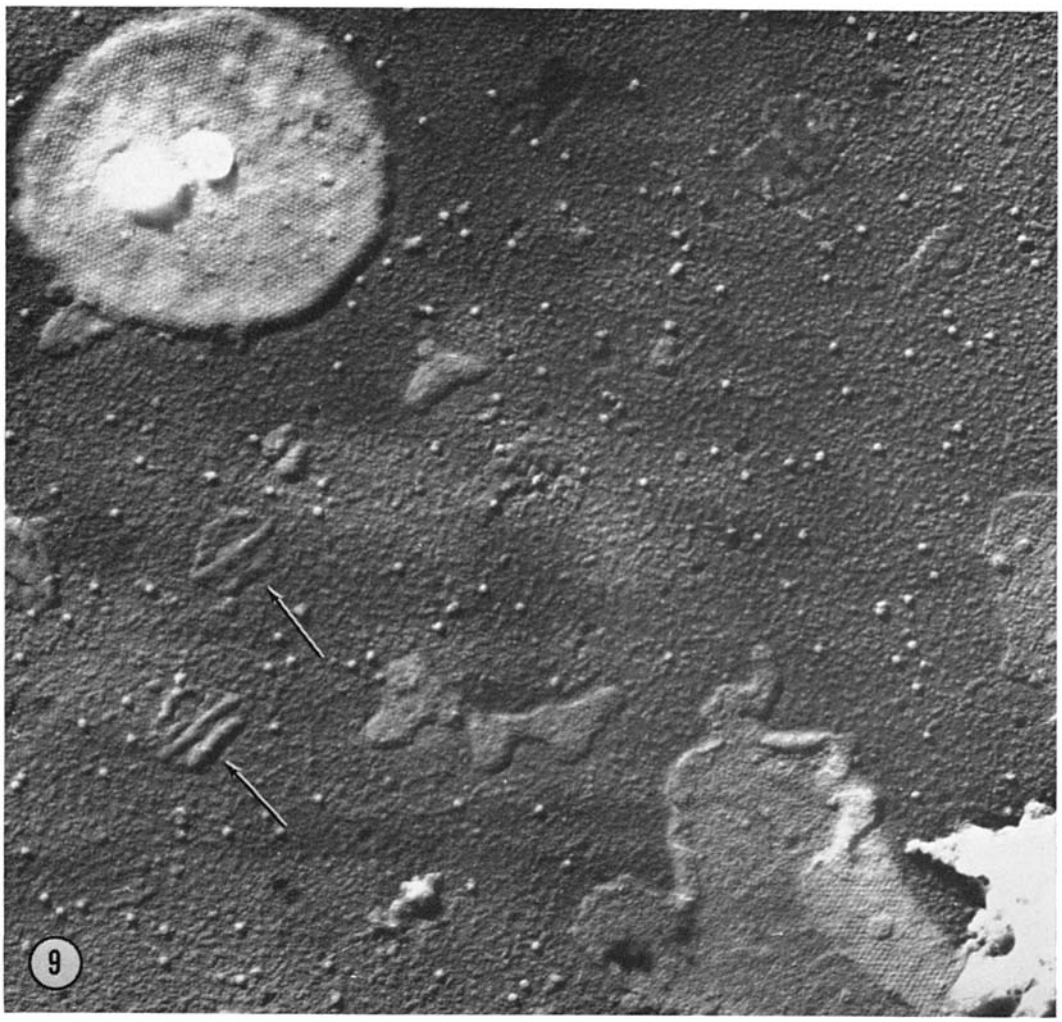


FIGURE 9 Envelope preparation shadowed with uranium. In the lower right-hand corner are ruptured vesicles showing the smooth inner surface and the periodic pattern on the outer surface, where the envelope is apparently folded back on itself. In the upper left-hand corner is an intact vesicle. In the background are numerous 250 A particles and two intracytoplasmic membranes (arrows). × 59,000.

M only about 10% of the original turbidity remains. Little material is found in the supernatant between 4.0 and 2.0 M. Protein release begins to increase between 1.5 and 1.0 M and reaches about 75% of the final value at 0.5 M.

In electron micrographs of sectioned material, the shape of the cells becomes irregular below 2.5 M NaCl concentration, but little change in fine structure could be detected until a concentration of 1.0 M was reached. At this stage the cells are spherical and material apparently is lost from the cell wall, while the cell membrane is well preserved. Ruptured and nearly-empty–appearing cells are also encountered which contain intracytoplasmic membranes as the major remnant of cytoplasmic structures. At 0.5 M NaCl no intact cells remain. In addition to some amorphous debris, only cell membranes and intracytoplasmic membranes are seen. The limiting cell membranes are broken up into fragments, which no longer form closed structures. They can be distinguished from the intracytoplasmic membranes by their greater length, apparently lower rigidity, and a slightly fuzzy appearance of both surfaces. At 0.04 M NaCl the picture is essentially the same except that less amorphous material is present and fewer unlysed

large membrane fragments are seen. The pellet now contains a much higher proportion of intracytoplasmic membranes.

These isolated intracytoplasmic membranes show other unusual structural features that are not easily observed in sections of whole cells. They often have short side branches. At higher magnification and in favorable sections it can be seen that the central light layer and the outer dense layers are actually continuous at the branching point, and the dense layers often appear as rows of dense dots with a spacing of about 50 A. In oblique sections parallel, straight, denser lines with the same spacing are occasionally observed on their surface, especially when the formaldehyde in the fixation is replaced by glutaraldehyde (Figs. 11 and 11 *a*).

The process of lysis has also been followed in shadowed preparations. Even at 4.3 M NaCl numerous broken or distorted cells and envelope fragments are found. Intact cells as well as fragments show the regular surface pattern already described. In addition to amorphous debris, intracytoplasmic membranes, flagella, and numerous round 200–250 A particles are regularly seen in the background. At lower salt concentrations the amount of distorted cells and envelope fragments increases. The surface pattern of the envelope becomes indistinct at 2.0 M NaCl. The 250 A particles are still present but not easily recognizable in the large amount of amorphous background material. At the lowest concentration studied (0.04 M) the amount of background material is decreased; large membrane fragments, intracytoplasmic membranes, and round particles are now more clearly visible. The major breakdown products which can be recognized in electron micrographs will be described in more detail when we discuss the lysis of isolated envelopes, because there is less interference from amorphous background material.

ENVELOPE PREPARATIONS: Fig. 12 shows the effect of different salt concentrations on the turbidity and release of protein and RNA from envelopes. Only a slight increase in turbidity is observed at concentrations between 4.3 and 2.0 M NaCl and these values have been omitted. The turbidity begins to decrease sharply at 1.4 M NaCl and then drops almost linearly to 8% of the original turbidity at 0.04 M. Assays of the 600,000 *g* min supernatant show that the release of protein follows an inverse course and begins to increase

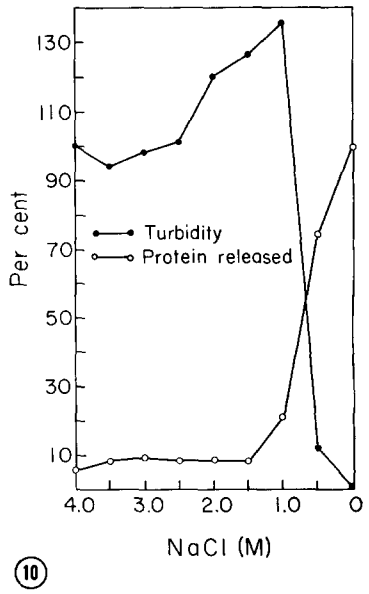
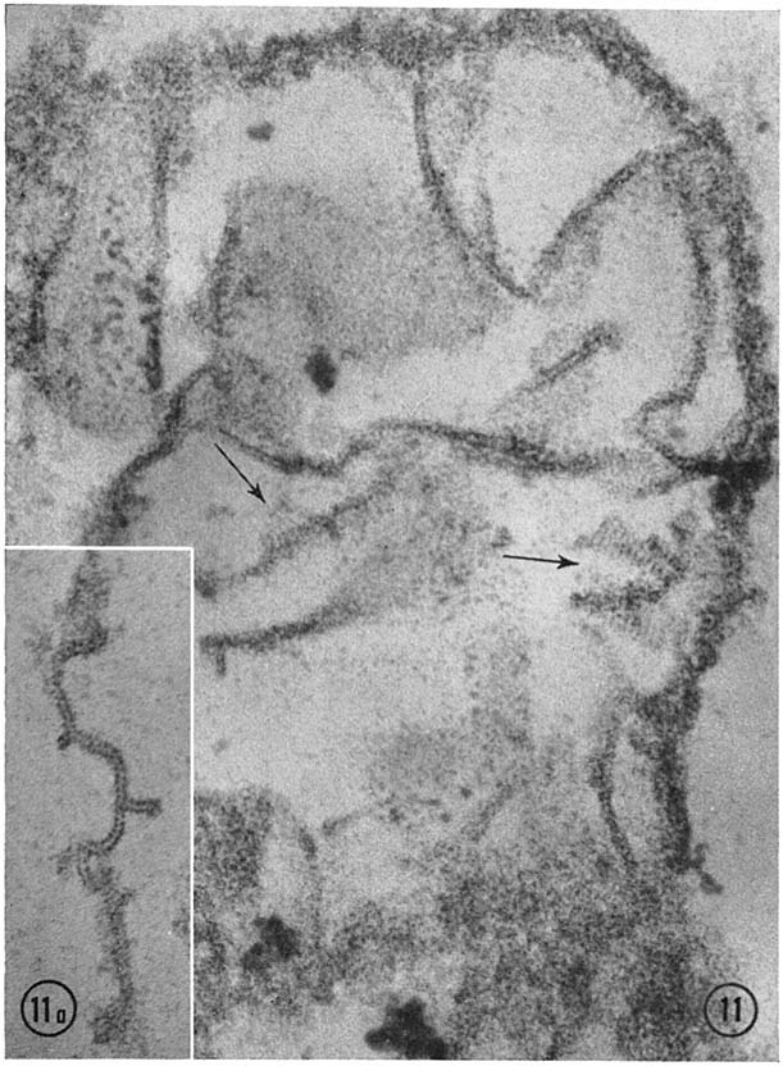


FIGURE 10 Changes in the turbidity and the release of protein of whole cells at different salt concentrations expressed as per cent of the initial turbidity and per cent of the protein released at 0.04 M NaCl.

rapidly at 1.4 M NaCl. The release of RNA shows a different pattern. The amount in the supernatant increases to 30% between 1.6 and 0.8 M NaCl and then remains approximately constant until a concentration of 0.2 M is reached. An abrupt increase follows between 0.2 M and 0.04 M. It is possible that this reflects a difference in the salt response between envelopes and contaminating ribosomes. After centrifugation the supernatants of the envelope preparation appear essentially clear and colorless down to salt concentrations of 1.4 M, and the pellets appear red. However, at 1.2 M NaCl the supernatant shows a faint orange-red color which increases in intensity at lower salt concentrations. In this same NaCl concentration range the color of the pellets, which are rapidly diminishing in size, becomes a deeper red and slowly changes to purple. Usually a small, nearly colorless portion is found at the bottom of the pellet. Fig. 13 shows this release of pigment, measured as absorbance at 480 mμ in the supernatant. When the orange-red material is first released it partially sediments even in the low centrifugal field used here, so that the upper part of the supernatant still appears colorless. This sedimentation diminishes upon further reduction of the salt concentration until the supernatant is uniformly colored.



FIGURES 11 and 11 *a* Nearly tangential sections through intracytoplasmic membranes show a regular striation on their surface (arrows). Cross-section through isolated intracytoplasmic membrane shows the branching and a globular-appearing structure in the dense layers (Fig. 11 *a*). This material was prefixed with glutaraldehyde instead of formaldehyde. × 192,000.

Sections through pellets obtained from envelope preparations exposed to decreasing salt concentrations show no major morphological changes down to 2.0 M NaCl solutions. At 1.6–1.4 M NaCl many of the vesicles appear collapsed or distorted, which may indicate a loss in the rigidity of the envelope. Some ruptured vesicles are also observed. In general the layered structure of the envelope is still intact. At 1.2 M, longer stretches of the vesicle envelopes have lost material from their outer layer, the cell wall, and in these places the vesicles are now bounded only by a typical unit membrane, the cell membrane proper (Fig. 14), which carries some ill-defined, fuzzy material on its outer surface. This loss of wall material has further progressed at 1.0 M NaCl, when virtually no intact wall is left on the vesicles. Only the thicker and slightly fuzzy outer border of the cell membrane suggests that remnants of wall material still adhere to it (Fig. 15). Cross-sections through the pellet

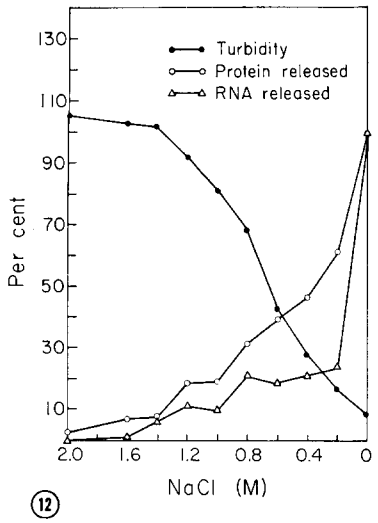


FIGURE 12 The decrease in turbidity and the release of protein and RNA as a function of salt concentration observed in envelope preparations. The values are given as per cent of initial turbidity in 4.3 M NaCl and as per cent of release in the lowest salt concentration (0.04 M).

now also show a greater number of open vesicles or large sheets of membrane and amorphous debris accumulated at one end of the pellet. These sheets of membrane still partly retain the unit membrane structure. In other parts, however, they appear only as a single dense line about 50–60 A wide, as if the central light layer had been removed and the two dense bands collapsed onto each other. No big pieces of free intact cell wall can be identified in the preparations exposed to 1.0 M salt solution. The cell wall probably either is detached in very small pieces or disintegrates rapidly after peeling off. There is, however, a possibility that fragments of the wall have only been reduced in thickness and cannot be distinguished from those membrane sheets, which show only one dense band.

With the appearance of broken vesicles, free intracytoplasmic membranes also appear in increasing number. They can be distinguished from the large membrane sheets by their limited length, higher contrast, clearer unit membrane structure and the characteristic branching. They usually also appear straighter, as if they were more rigid.

At 0.8 M NaCl the relative amount of large membrane sheets is much increased; there are few, if any, closed vesicles left (Fig. 16). From 0.6 to 0.2 M NaCl little change in the appearance of the sedimentable material is observed. The large membrane sheets prevail and the unit membrane structure in these is less and less frequently seen. The intracytoplasmic membranes show no changes in their morphology.

At the lowest salt concentrations used in these experiments, 0.04 M NaCl, or even after prolonged dialysis against distilled water, a considerable amount of the large membrane sheets persists (Fig. 17). The relative concentration of the intracytoplasmic membranes, however, appears to increase (Fig. 18).

Shadowed preparations of the envelope fraction show no gross changes in morphology when the salt concentration is decreased from 4.3 to 2.0 M. At 1.6 M the surface pattern becomes less distinct and disappears completely between 1.4 and 1.2 M. The round, 200–250 A particles also seem to be-

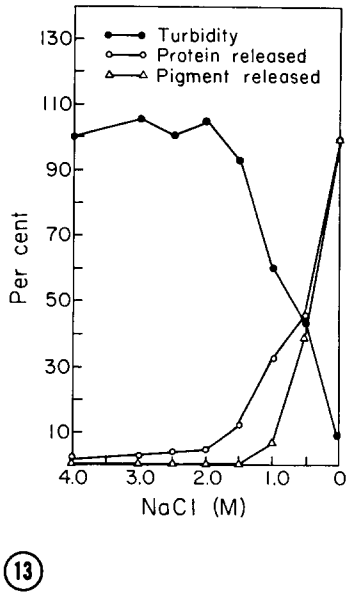


FIGURE 13 The release of orange-red pigment and protein from an envelope preparation as a function of salt concentration. The values are given as per cent of the release observed at 0.04 M NaCl.

come less distinct and frequent, but this is difficult to assess because of the large number of salt crystals and some amorphous material present in the background of these preparations. Below concen-

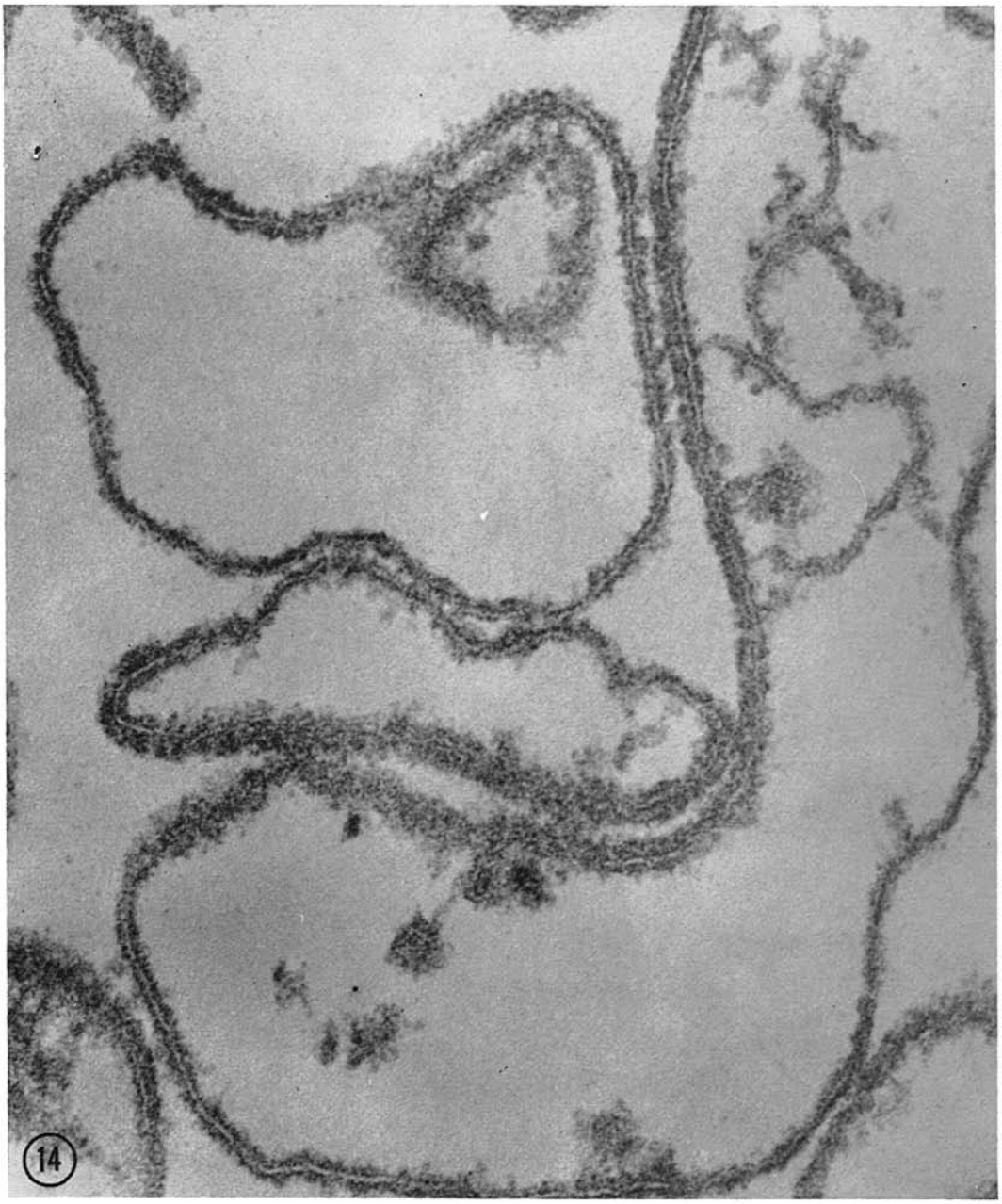


FIGURE 14 Section through pellet from an envelope preparation exposed to 1.2 M NaCl. The vesicles have lost wall material from most of their surface, but in some places the wall appears to be still intact. × 176,000.

trations of 1.0 M NaCl more and more irregularly shaped, slightly flattened particles, a few 100 A in diameter, appear in the preparations. They probably represent pieces of disintegrating cell membrane, because they appear sometimes in rows continuous with bigger sheets of membrane and

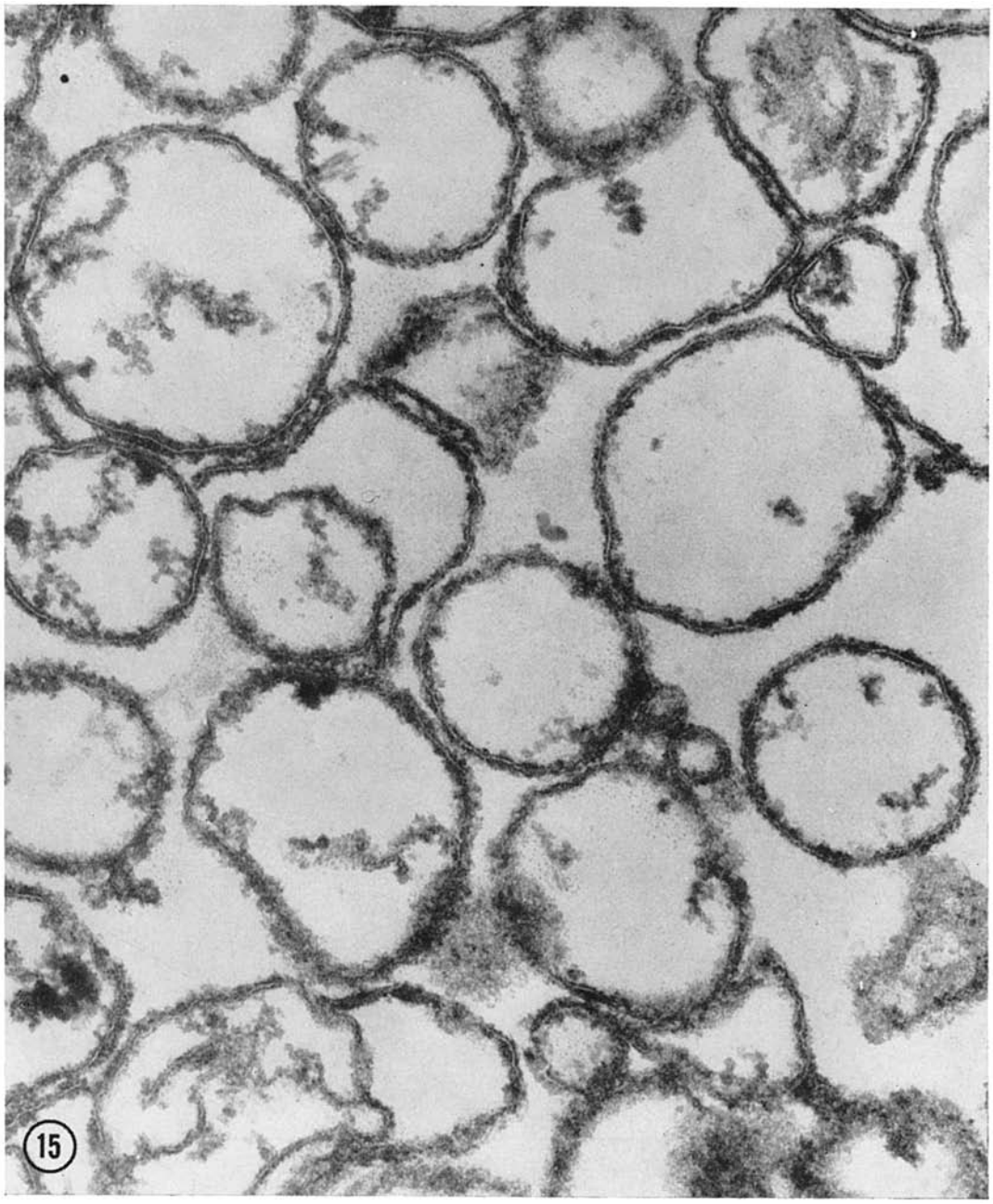


FIGURE 15 Pellet from an envelope preparation after exposure to 1.0 M NaCl. No intact wall remains on the vesicles. × 89,000.

have approximately the same thickness (Fig. 19). Even after prolonged dialysis against distilled water large sheets of membrane can be found. In addition there are small, irregular pieces, which are apparently fragments of the sheets, and a large number of roundish, smaller particles close

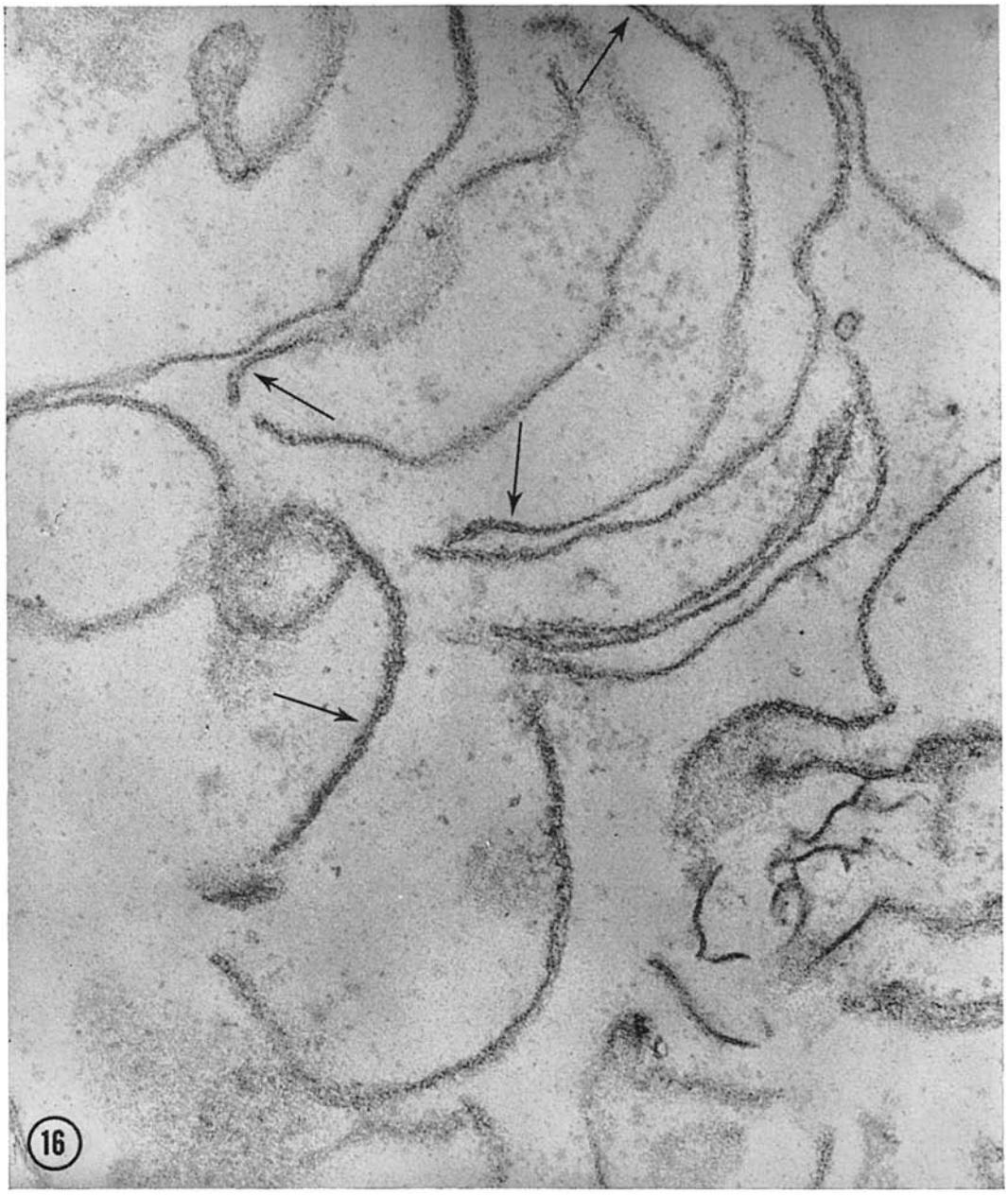


FIGURE 16 Pellet from an envelope preparation after exposure to 0.8 M NaCl. Intact vesicles are no longer found. The unit membrane appearance of the cell membrane is visible in only a few places (arrows). In the lower right-hand quadrant is a group of intracytoplasmic membranes. They can be recognized by their limited length and higher contrast. × 89,000.

to the limit of resolution obtainable in these shadowed preparations. The intracytoplasmic membranes can be recognized by their characteristic size and shape. They are oval and often somewhat angular in outline, with a width of 0.15–0.2 μ and a length of 0.2–0.3 μ. Ridges running across

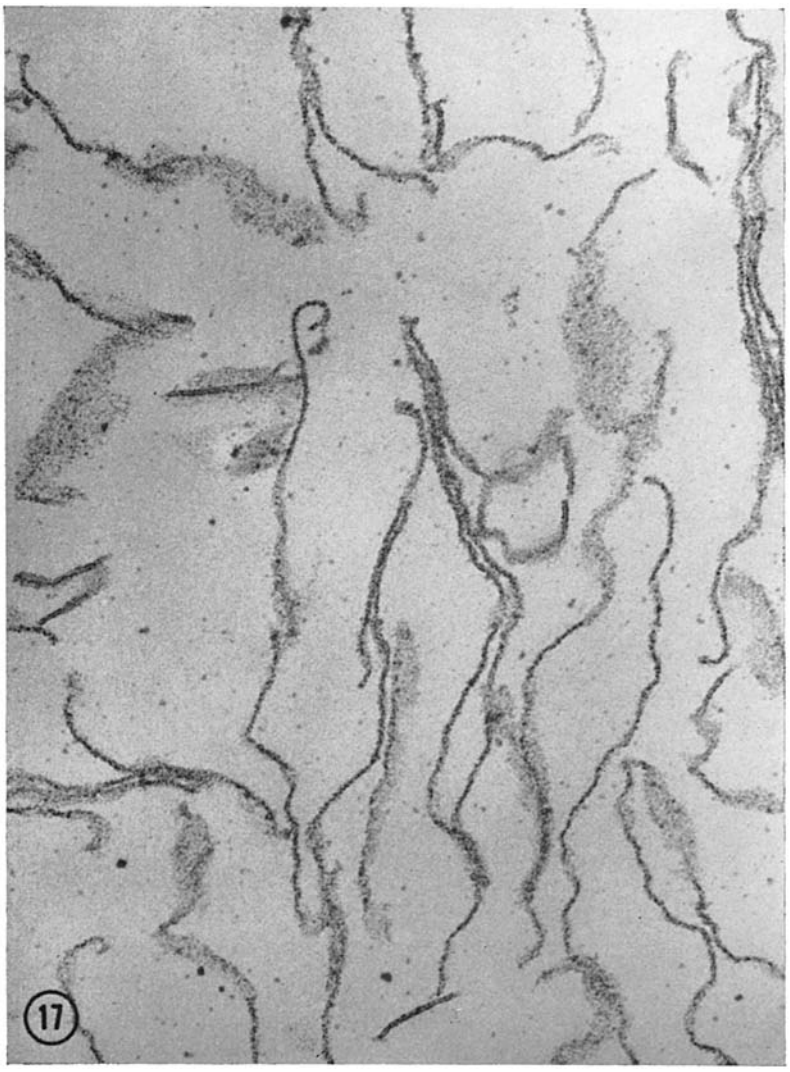


FIGURE 17 Pellet from an envelope preparation after dialysis against distilled water. It consists mainly of large membrane sheets. × 96,000.

the entire width of these membranes may represent the short side branches seen in sections. The appearance does not change with changes in salt concentration.

The large unlysed membrane sheets and intracytoplasmic membranes, which are resistant to distilled water, can also be observed in negatively stained preparations (Fig. 20). The intracytoplasmic membranes can again be identified by their size and shape. In addition to this, however, they display a very characteristic regular surface pattern of parallel lines with a somewhat variable spacing of approximately 40 A. The lines always run approximately parallel to the short axis of the particle and often appear slightly curved, with the convex side pointing to the center of the plate (Fig. 20 *a*). This striation may correspond to the dotted or striated appearance of the outer dense layers occasionally seen in sections. The slight discrepancy in spacing, between sectioned and negatively stained material (~50 A versus ~40 A), may be a preparation artifact or due to errors introduced by the small number of micrographs of sectioned material in which the surface structure

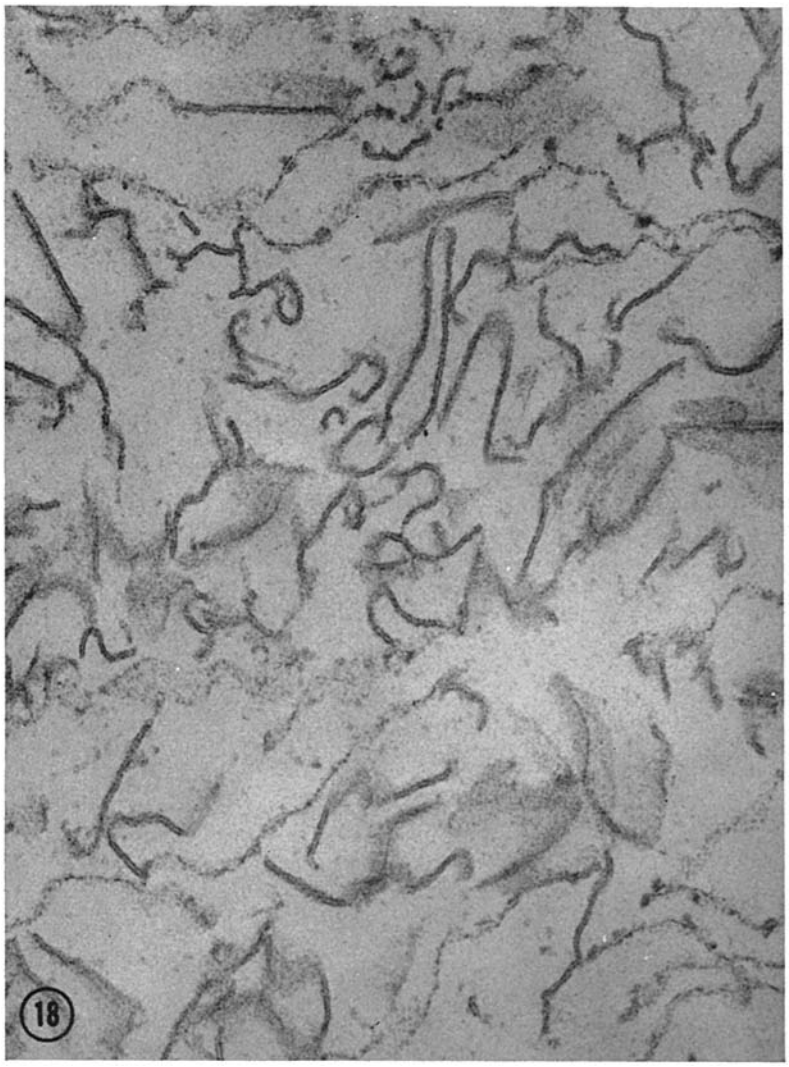


FIGURE 18 Bottom part of pellet from a preparation identical with that shown in Fig. 17. The intracytoplasmic membranes are concentrated here. × 96,000.

was visible. The large unlysed membrane sheets have a very different morphology. They are usually considerably bigger in area and more irregular in outline, but appear thinner. They display a finely granular surface structure, with faint indications of a possibly regular structure, which has never been clearly resolved (Fig. 21). Some bigger particles or "bumps" can usually be seen, scattered irregularly over the surface of the membrane. They are thought most likely to be small fragments produced during lysis. Small fragments are also found adhering to the edge of these membrane sheets, very similar to the structures seen in shadowed preparations. Negative staining is the best technique to distinguish between unlysed large membrane sheets and intracytoplasmic membranes because it reveals the difference in surface structure.

Fractionation of the Envelope Lysate

Dialysis of the envelope preparation against distilled water yields a clear red lysate with a residual turbidity of about 3%. Centrifugation at 20,000 *g* for 60 min separates a clear orange-red

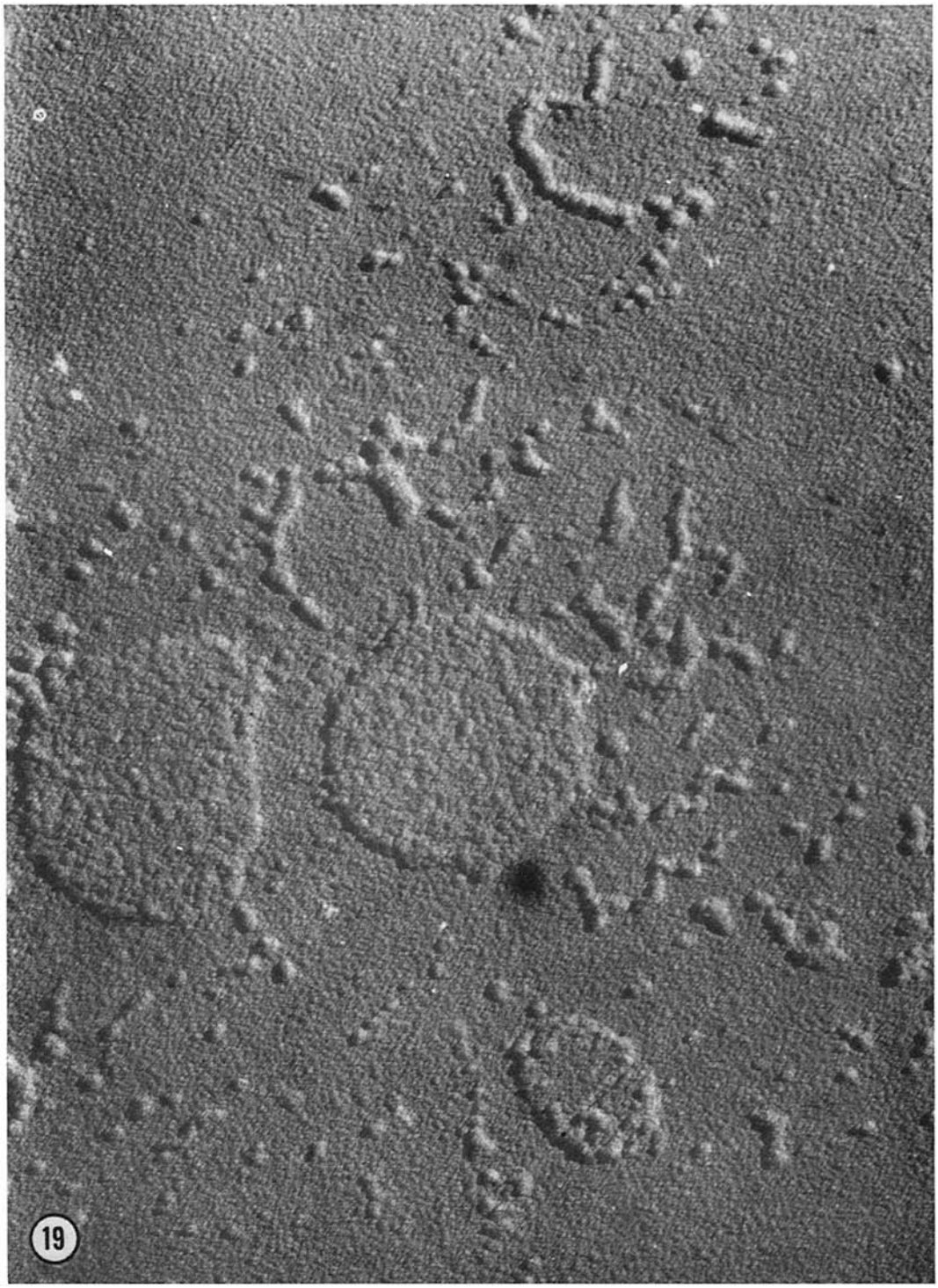
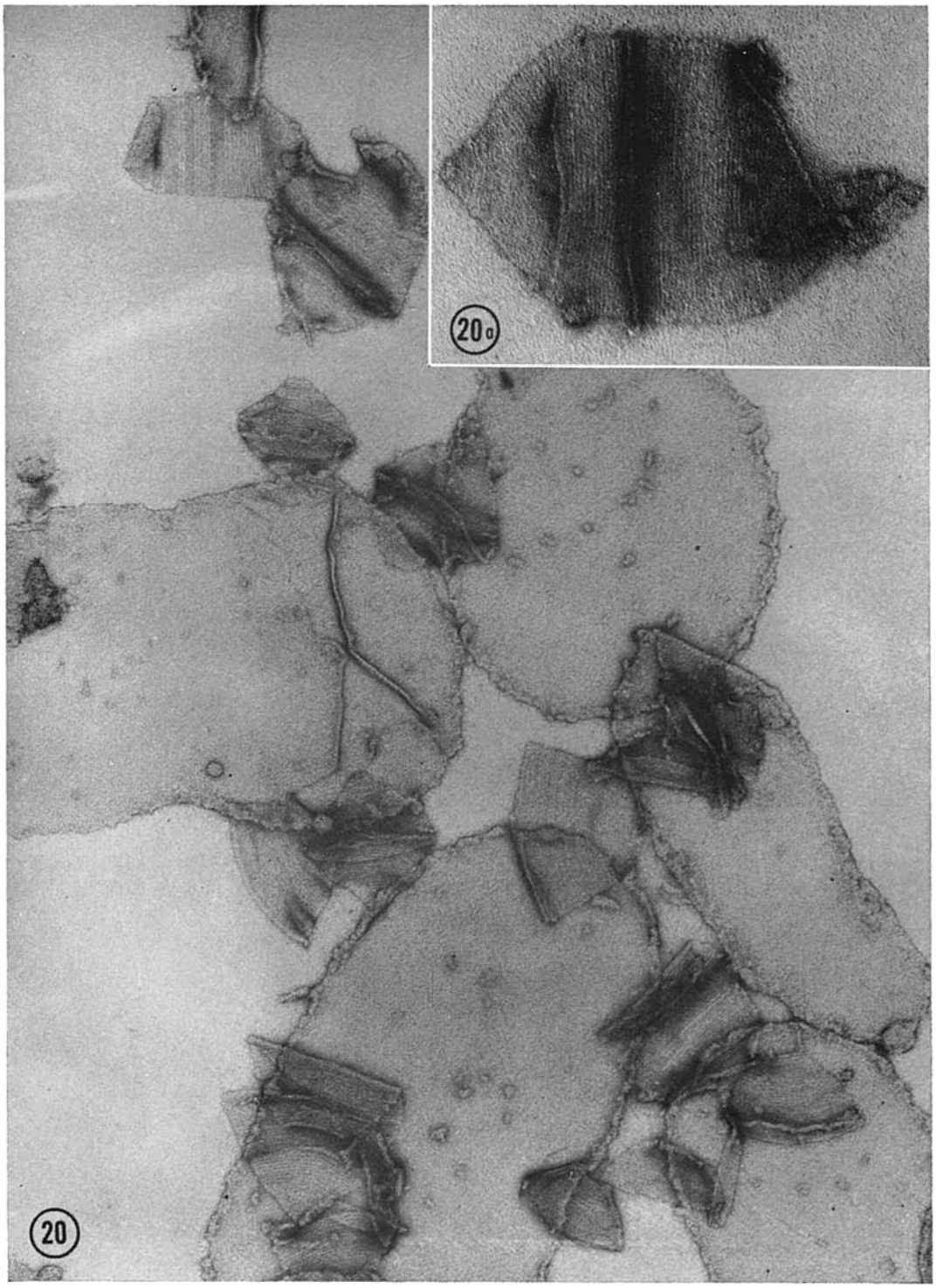


FIGURE 19 Shadowed specimen showing disintegrating membrane sheets from an envelope preparation exposed to 0.5 M NaCl. × 60,000.



FIGURES 20 and 20 *a* Negatively stained preparation from the purple residue remaining sedimentable after exposure of envelope preparations to distilled water. The intracytoplasmic membranes are easily recognized by their characteristic size, shape, and surface pattern (inset). The large membrane sheets show some smaller fragments attached to their surface. × 114,000 and 224,000 (inset).

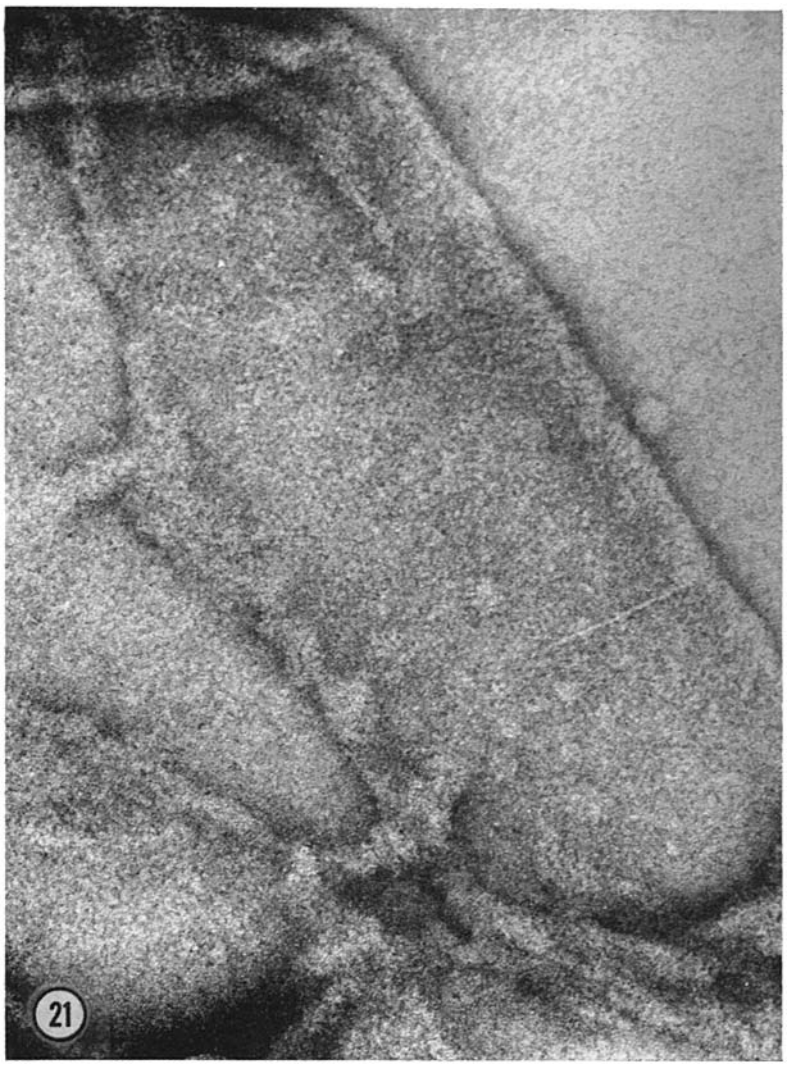


FIGURE 21 Higher magnification of a large membrane sheet to show the finely granular surface structure with indications of a regular pattern. × 384,000.

supernatant from a small purple pellet which still contains some orange-red material. Resuspension of the pellet in distilled water and centrifugation repeated two or three times removes all traces of orange-red color from it.[3] The yield of sedimentable material in the envelope lysate varied from trace amounts in early log phase to approximately 14% in late log phase and slightly higher values in stationary phase cells (see Table I).

Both fractions, the orange supernatant and the purple pellet, contain mainly protein and lipids. The lipid content of the pellet, determined as lipid

[3] Lysis is accompanied by a marked increase in viscosity, which is unaffected by deoxyribonuclease or trypsin. It necessitates a rather long centrifugation at high speed to sediment the purple material unless the viscosity is reduced by dilution. The viscosity is associated with the supernatant, and subsequent centrifugation, for further purification of the pellet material, can be carried out at lower speed. In most cases only one high-speed centrifugation was used to separate the pellet material from the supernatant.

TABLE I

Distribution of Chemical Constituents in Envelope Lysates

Measurement	Preparation A*		Preparation B‡	
	Total lysate	Proportion in pellet	Total lysate	Proportion in pellet
	mg/ml	*%*	*mg/ml*	*%*
Dry weight	13.24	14	14.85	20
"Protein"§	14.50	10	13.80	15
Total nitrogen	1.60	14	1.73	23
Total lipid	1.37	23	1.84	23
Lipid phosphorus	0.056	25	0.057	21
RNA	0.80	2		
DNA	0.22	0		

* Envelope preparation of cells taken during the late log phase of growth.

‡ Envelope preparation of cells taken during the stationary phase of growth.

§ Determined by the procedure of Lowry et al. (22) using purified bovine serum albumin as standard. While the absolute values for protein obtained are too high, this technique may still be used to determine relative amounts of protein in the different fractions (18).

phosphorus per milligram of nitrogen, is slightly higher than that of the supernatant. A comparison of the thin layer chromatograms shows that the lipid composition of the two fractions is practically identical (see Fig. 22). The orange-red color in the supernatant has been attributed on the basis of the absorption spectrum (Fig. 23) to the presence of α-bacterioruberine, the major carotenoid of *H. halobium* (20, 21). It may also be present in the pellet as a contamination. The pellet contains principally a purple-colored substance that has not been identified so far. It is not extractable with diethyl ether from a suspension of the pellet material in water. After precipitation of the suspended material with protamine, however, it is discolored by shaking with ether and a yellow color appears in the ether phase. Extraction of the pellet material with chloroform–methanol (2:1) also discolors the precipitate, and a yellow-orange color appears in the chloroform layer. When the chloroform is evaporated and the residue taken up in methanol the color changes to yellow. In this solvent a broad absorption peak is found at 360–380 mμ (Fig. 24). In thin layer chromatograms on silica gel the yellow-colored substance moves closely behind the solvent front, similar to the carotenoid α-bacterioruberine.

The morphological components of the purple pellet have been described. In shadowed electron microscope preparations of the orange supernatant no large membrane sheets or intracytoplasmic membranes are found. It contains mainly the small cell membrane fragments and the still smaller, roundish particles also observed in lysates between 0.8 and 0.04 M salt concentrations, and a large amount of amorphous background material. The orange-colored material can be sedimented by prolonged centrifugation at 105,000 *g* (6 hr). It leaves a nearly colorless clear supernatant with a small, loosely packed, orange-red pellet and a fluid, orange-red layer in contact with it. Sections through the pellet of this high speed run show that it consists of very short membrane fragments, mostly 500–1000 A in length, with a unit membrane structure visible only rarely (Fig. 25). In shadowed preparations, mainly the small irregular membrane fragments and round particles are found that have been described in the whole lysate (Fig. 26).

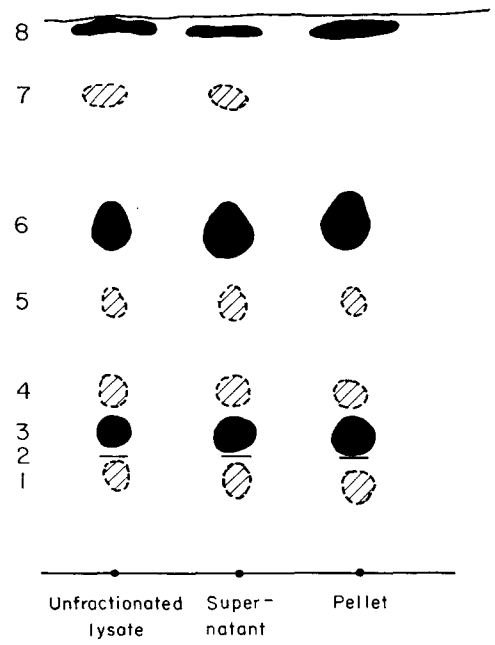


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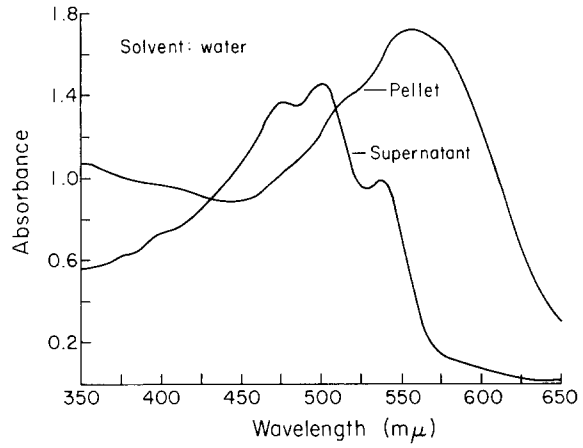
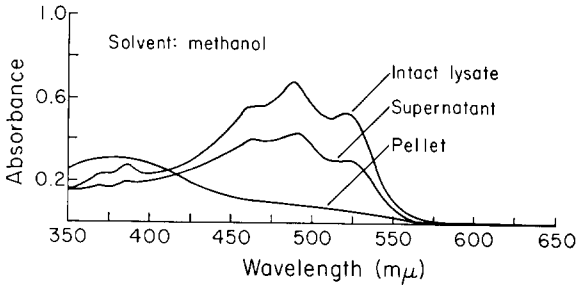
FIGURE 22 Thin layer chromatogram of total lipid extracts from different fractions obtained from the envelope lysate. For comparison, extracts were adjusted to approximately equal concentrations of lipid phosphorus.



(23)

FIGURE 23 Absorption spectra of the envelope lysate fractions.



(24)

FIGURE 24 Absorption spectra of lipid extracts from the envelope lysate fractions.

Preliminary experiments using sucrose density gradients have resulted in a further fractionation of the lysate. A separation into orange-red and purple fractions can be obtained in a discontinuous gradient of 1 and 2 M sucrose. The orange-red material remains essentially at the surface of the 1 M sucrose while the purple material is concentrated at the 1–2 M interface. Using a 1–1.75 M linear sucrose gradient, an upper, orange-red band and two or three rather diffuse, lower, purple bands were obtained. In negatively stained preparations of the upper band only small aggregated membrane fragments of very irregular shape were found. The lower purple bands contained, in addition to typical intracytoplasmic membranes, the large round or oval membrane sheets with a granular surface structure. So far all fractions of purple color have been found to contain intracytoplasmic membranes together with the large membrane sheets.

DISCUSSION

Several investigators have reported that halobacteria cannot be fixed with OsO_4 (6, 16) and we can confirm these observations. Since Kates et al (15) have shown that there are virtually no double bonds present in the hydrocarbon chains of lipids from *H. cutirubrum* and other extreme halophiles this result is not surprising. It has been known for some time that the double bonds are the primary sites for the reaction between most lipids and OsO_4 and that they are necessary for fixation (34). The chemistry of $KMnO_4$ fixation is not understood so well and the mechanism by which it fixes halobacteria is unknown.

The fixation procedure introduced in this study is a further development of the technique used by Brown and Shorey (9). The modified procedure gives improved preservation and/or stainability of a structure on the surface of the cells, which apparently corresponds to the cell wall of other

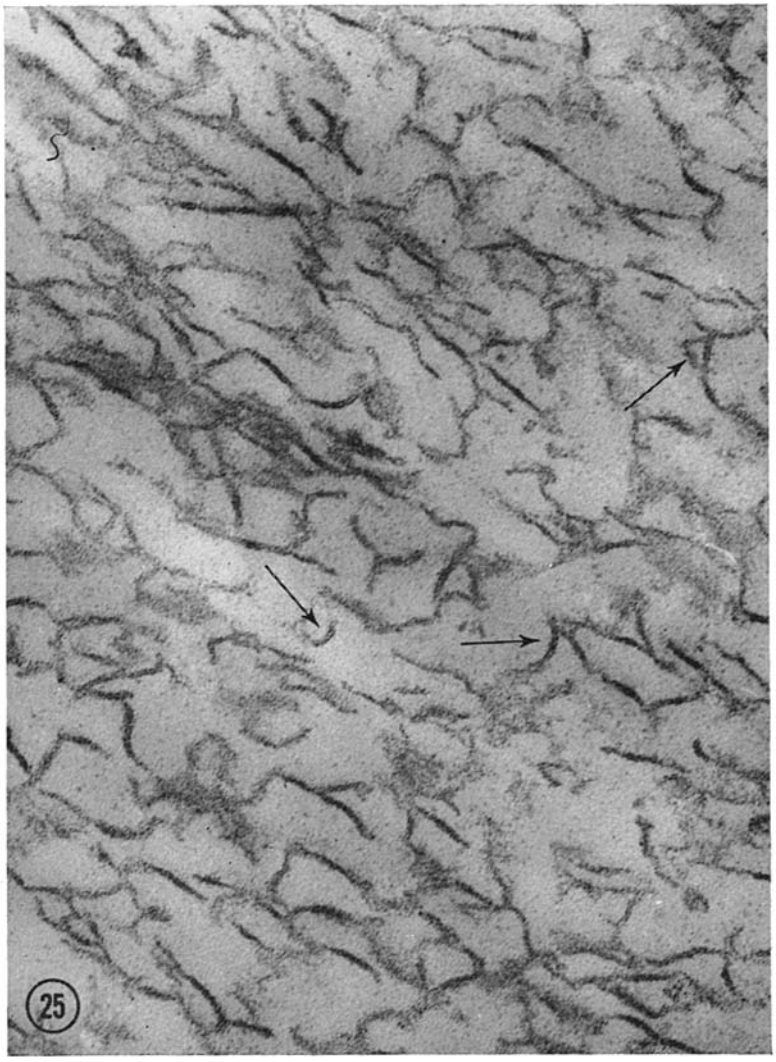


FIGURE 25 Section through the orange-red material that can be sedimented at high speed from the lysate. It consists of membrane fragments, which in places still show a unit membrane structure (arrows). This electron micrograph shows the fastest sedimenting part of the fraction. × 96,000.

bacteria. Brown (6, 7) and Brown and Shorey (9), mainly on the basis of electron micrographs, assumed that halobacteria, especially *H. halobium*, do not possess a cell wall but are bounded only by a cell membrane, or, at least, that the isolated cell envelopes consist of vesicles with only a unit membrane as the limiting structure. The low proportion of lipid in the envelope preparation is difficult to reconcile with this conclusion. Moreover, our own envelope preparations, as well as one obtained following Brown and Shorey's technique, consistently show a cell wall if they are prefixed with formaldehyde in the presence of 1% $CaCl_2$. Other reasons for doubting the validity of Brown's conclusions have been discussed by Kushner et al. (17). The regular arrangement of spherical particles on the surface of halobacteria, first reported by Houwink (12), must therefore be ascribed to the cell wall.

Another structural component observed in whole bacteria and the envelope preparations, which to our knowledge has not been reported before, is the intracytoplasmic membranes. Their preservation cannot be attributed to the special fixation used here. Intracytoplasmic membranes

FIGURE 26 The slower sedimenting part of the material shown in Fig. 25 after shadowing with platinum-carbon. The fragments here are considerably smaller and rather variable in size. × 58,000.

are also present in preparations fixed according to Brown and Shorey (9). However, they are few in number before the cultures reach the end of the log phase, difficult to detect in sections of whole bacteria, and not very conspicuous in shadowed preparations. They are probably more numerous than sections through the envelope preparations seem to indicate because they are found in higher concentrations in lysates of whole cells than in lysates of envelope fractions. Some of them are probably set free with the rest of the cell content, when the cells are broken prior to the separation of the envelopes, and we have found them in the supernatant obtained in the high-speed centrifugations used to separate the envelopes from the soluble part of cell homogenates.

While these intracytoplasmic membranes look like typical unit membranes in cross-section, their branching, their surface pattern, and their plate-like structure distinguish them from all other membranes so far described in bacteria or other cells, with the possible exception of certain blue-green algae (5, 14). The fraction of the lysate in which they are concentrated contains membrane lipids.

However, its lipid content cannot be used as an argument for the membrane nature of these structures until fractions of higher purity are obtained. We should emphasize again that the provisional term intracytoplasmic membranes is not meant to imply that these structures necessarily have a composition, fine structure, and function comparable to those of other cellular membranes. Their function at present is still unknown. They seem to be related to the formation of gas vacuoles because they appear at about the same time and increase in number with the gas vacuoles. Furthermore, a mutant strain of *H. halobium* isolated by us, which has lost the ability to form gas vacuoles, also lacks the intracytoplasmic membranes. The same holds true for another strain isolated from Larsen's *H. halobium* strain 5, where the parent strain has gas vacuoles and intracytoplasmic membranes, and the variant strain lacks both.[4] No reversions to gas vacuole production have been detected in these strains so far and therefore the correlation with the presence of intracytoplasmic membranes has not been carried further. A similar structure has been described in a short note on the morphology of a blue-green alga (5) which also produces gas vacuoles. The interpretation suggested by the authors, that these structures represent collapsed gas vacuoles, cannot be considered definitely proved by the evidence presented, and so far we have no arguments to offer for such a role of intracytoplasmic membranes in halobacteria. In another blue-green alga, Jost (14) has demonstrated, by means of freeze-etching and negative staining, a structure that shows the same type of surface pattern as the intracytoplasmic membranes of *H. halobium*. Although the shape of these *hohlspindeln* is that of a cylinder with conical ends, they may very well have some relationship to the intracytoplasmic membranes presently described.

The first visible change in halobacteria, when the salt concentration is reduced, is an apparent loss of rigidity and the appearance of irregularly shaped cells, which on further reduction of the salt concentration become spherical (1, 2, 24). We have not observed any changes in fine structure of the envelope correlated with these events. The first observed change is the loss of the regular surface pattern, which occurs at approximately 1.6 M NaCl concentration. The next visible step in the lysis is a loss of cell wall material. It begins at 1.4 M NaCl and appears to be complete at 1.0 M NaCl. At the same time some of the vesicles open to form sheet-like structures, but the majority are still closed at 1.0 M NaCl. Below 1.0 M NaCl more of the vesicles open and the cell membranes begin to disintegrate rapidly, so that less and less material remains sedimentable at $1.2 \times 10^6 g$ min. Qualitatively this sequence of events is reflected also in the observations on the release of protein and lipid into the supernatant, using the lipid-soluble red carotenoid as an indicator of lipid release (Fig. 13). Quantitatively, one might expect to find a higher proportion of the total protein in the supernatant at 1.0 M NaCl than the 20–30% actually observed if most of the wall material were released at this salt concentration. However, we do not know how much of the total protein of the envelope preparation is soluble protein that is contained inside the vesicles and is only released when the vesicles break up. Moreover, part of the wall material may still remain attached to the membrane, or be detached and indistinguishable from the membrane sheets. This might explain the apparent discrepancy.

Perhaps the most interesting result of this study was the observed heterogeneity of the envelope lysate and the morphology of its components. Brown (6) has reported that he found one major component in the lysate that sedimented with $s_{20,w} \cong 4.0$ in the analytical ultracentrifuge. He assumed this to be a macromolecular constituent of the cell membrane. He also obtained a small pellet after centrifugation of the lysate at 14,500–17,000 g for 45 min, and an orange component that sedimented ahead of the 4.0S material. Apparently these were not further investigated.

Since our studies indicate that Brown's envelope preparation contained the cell wall in addition to the cell membrane, a reevaluation of his conclusions becomes necessary. Some preliminary data obtained with the analytical ultracentrifuge[5] confirm the presence of the 4.0S component described by Brown. The orange-red material sediments

[4] This strain shows a high frequency of loss of gas vacuole formation. Gas-vacuoleless colonies appear clear, whereas the other colonies appear opaque. Many sectored colonies can be observed on plates. We are grateful to Dr. Helge Larsen (Trondheim, Norway) for drawing our attention to this strain and for making it available.

[5] We are grateful to Mr. R. C. Williams, Jr., and Dr. G. M. Edelman for carrying out the ultracentrifuge experiments.

considerably faster, with a very diffuse boundary. Repeated sedimentation of the orange-red material in the preparative centrifuge before the analytical run reduces the 4.0S component to trace amounts without any apparent loss of orange material. As described in Results section, the orange-red component consists of very small, irregular membrane fragments. It seems more likely, therefore, that the main constituents of the cell membrane are contained in this fraction. The 4.0S component may represent a breakdown product of the cell wall, but further work is required to decide this point.

At least two components have been found in the purple pellet that can be separated from the lysate at low speed. One, on the basis of its morphology, is readily identified as the intracytoplasmic membranes seen in sections of intact bacteria and unlysed envelope fractions and has been discussed above. The origin of the large membrane sheets is much less obvious. Electron microscopy of envelopes at different salt concentrations seems to indicate that these sheets are unlysed parts of the cell membrane. However, this would imply a heterogeneity of the cell membrane, which we find difficult to accept. Another possibility is to assume a heterogeneity of the bacterial population, for instance a structural change in the cell membrane of older cells. This, to us, seems to be a more acceptable explanation, but efforts to fractionate the unlysed cell envelope, in the hope of separating the hypothetical older envelopes from the younger, have so far not yielded any results. The increase in the mass of nonlysable material with age of the culture which would fit this explanation must be attributed at least partly to the intracytoplasmic membranes. A third possibility is that the unlysed membrane sheets constitute a distinct layer of the cell envelope that has not been identified in sections. We feel that so far the electron micrographs of lysing cells do not allow us to exclude this interpretation. At present, however, we have no convincing arguments for or against any of these possible explanations. Further work, especially separation and chemical analysis of the two components found in the pellet, is needed.

The red color of the intact bacteria and of the envelope preparation is apparently due to at least two different colored substances, which can be separated with the fractions of the lysate. The orange-red carotenoid, α-bacterioruberine, is apparently bound to the cell membrane, as are the carotenoids in other bacteria. The purple-colored substance has not been identified but is evidently bound to the membranous material readily sedimentable from the lysate. Of the two identified structural components of this material, the large membrane sheets are the more likely site, because lysates of the *H. halobium* strains which have neither gas vacuoles nor intracytoplasmic membranes (see above) still yield a purple pellet which contains membrane sheets.

It has been shown that lysis of halobacteria upon removal of salt does not depend on enzymic or osmotic processes. It is generally assumed that a high charge density on the structural components of the organism when not shielded by counterions leads to a disaggregation (1, 2, 6, 8, 17, 18, 24, 25). Our data are fully compatible with these conclusions. They show in addition that a cell wall exists in halobacteria and that cell wall and membrane begin to lyse at different salt concentrations and apparently independently of each other. The process of lysis is incomplete. Even after prolonged dialysis against distilled water some large membrane sheets and the intracytoplasmic membranes are still intact and readily sedimentable. The lysed part of the cell membrane appears not as a well-defined subunit, but as a mixture of membrane fragments of small but variable size. The average size of these particles diminishes with diminishing salt concentrations. However, even in distilled water membrane structure is still recognizable in the particles and they are still sedimentable at high speeds in the preparative ultracentrifuge. They are clearly not subunits and there is no indication that they are composed of identical subunits.

This study shows that a thorough characterization by chemical and physical techniques is necessary before the existence and isolation of membrane subunits can be claimed. A similar case, where a supposedly well-defined membrane subunit turned out to be an artifact, has been reported recently (10, 35).

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REFERENCES

1. ABRAM, D., and N. E. GIBBONS. 1960. Turbidity of suspensions and morphology of red halophilic bacteria as influenced by sodium chloride concentration. *Can. J. Microbiol.* **6**:535.
2. ABRAM, D., and N. E. GIBBONS. 1961. The effect of chlorides of monovalent cations, urea, detergents, and heat on morphology and the turbidity of suspensions of red halophilic bacteria. *Can. J. Microbiol.* **7**:741.
3. BAYLEY, S. T. 1966. Composition of ribosomes of an extremely halophilic bacterium. *J. Mol. Biol.* **15**:420.
4. BAYLEY, S. T., and D. J. KUSHNER. 1964. The ribosomes of the extremely halophilic bacterium, *Halobacterium cutirubrum*. *J. Mol. Biol.* **9**: 654.
5. BOWEN, C. C., and T. E. JENSEN. 1965. Blue-green algae: Fine structure of the gas vacuoles. *Science.* **147**:1460.
6. BROWN, A. D. 1963. The peripheral structures of gram-negative bacteria. IV. The cation-sensitive dissolution of the cell membrane of the halophilic bacterium, *Halobacterium halobium*. *Biochim. Biophys. Acta.* **75**:425.
7. BROWN, A. D. 1964. Aspects of bacterial response to the ionic environment. *Bacteriol. Rev.* **28**:296.
8. BROWN, A. D. 1965. Hydrogen ion titration of intact and dissolved lipoprotein membranes. *J. Mol. Biol.* **12**:491.
9. BROWN, A. D., and C. D. SHOREY. 1963. The cell envelopes of two extremely halophilic bacteria. *J. Cell Biol.* **18**:681.
10. ENGELMAN, D. M., T. M. TERRY, and H. J. MOROWITZ. 1967. Characterization of the plasma membrane of *Mycoplasma laidlawii*. I. Sodium dodecyl-sulfate solubilization. *Biochim. Biophys. Acta.* In press.
11. FRASCA, J. M., and V. R. PARKS. 1965. A routine technique for double-staining ultra-thin sections using uranyl and lead salts. *J. Cell. Biol.* **25**:157.
12. HOUWINK, A. L. 1956. Flagella, gas vacuoles and cell-wall structure in *Halobacterium halobium*; an electron microscope study. *J. Gen. Microbiol.* **15**:146.
13. JOSHI, J. G., W. R. GUILD, and P. HANDLER. 1963. The presence of two species of DNA in halobacteria. *J. Mol. Biol.* **6**:34.
14. JOST, M. 1965. Die Ultrastruktur von *Oscillatoria rubescens* D. C. *Arch. Mikrobiol.* **50**:211.
15. KATES, M., L. S. YENGOYAN, and P. S. SASTRY. 1965. A diether analog of phosphatidyl glycerophosphate in *Halobacterium cutirubrum*. *Biochim. Biophys. Acta.* **98**:252.
16. KUSHNER, D. J., and S. T. BAYLEY. 1963. The effect of pH on surface structure and morphology of the extreme halophile *Halobacterium cutirubrum*. *Can. J. Microbiol.* **9**:53.
17. KUSHNER, D. J., S. T. BAYLEY, J. BORING, M. KATES, and N. E. GIBBONS. 1964. Morphological and chemical properties of cell envelopes of the extreme halophile *Halobacterium cutirubrum*. *Can. J. Microbiol.* **10**:483.
18. KUSHNER, D. J., and H. ONISHI. 1966. Contribution of protein and lipid components to the salt response of envelopes of an extremely halophilic bacterium. *J. Bacteriol.* **91**:653.
19. LARSEN, H. 1962. Halophilism. *In* The Bacteria. I. C. Gunsalus and R. Y. Stanier, editors. Academic Press, Inc., New York. **4**:297.
20. LEDERER, E. 1938. Sur les caroténoides des Cryptogames. *Bull. Soc. Chim. Biol.* **20**:611.
21. LIAAEN JENSEN, S. 1960. Bacterial carotenoids. VI. A note on the constitution of bacteriorubereine α. *Acta Chem. Scand.* **14**:950.
22. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265.
23. MEJBAUM, W. 1939. Über die Bestimming kleiner Pentosemengen, insbesondere in Derivaten der Adenylsäure. *Z. Physiol. Chem.* **258**:117.
24. MOHR, V., and H. LARSEN. 1963. On the structural transformations and lysis of *Halobacterium salinarium* in hypotonic and isotonic solutions. *J. Gen. Microbiol.* **31**:267.
25. ONISHI, H., and D. J. KUSHNER. 1966. Mechanism of dissolution of envelopes of the extreme halophile *Halobacterium cutirubrum*. *J. Bacteriol.* **91**:646.
26. SEHGAL, S. N., and N. E. GIBBONS. 1960. Effect of some metal ions on the growth of *Halobacterium cutirubrum*. *Can. J. Microbiol.* **6**:165.
27. SEHGAL, S. N., M. KATES, and N. E. GIBBONS. 1962. Lipids of *Halobacterium cutirubrum*. *Can. J. Biochem.* **40**:69.
28. SMITHIES, W. R., N. E. GIBBONS, and S. T. BAYLEY. 1955. The chemical composition of the cell and cell wall of some halophilic bacteria. *Can. J. Microbiol.* **1**:605.
29. SPERRY, W. M., and F. C. BRAND. 1955. Determination of total lipids in blood serum. *J. Biol. Chem.* **213**:69.
30. STEWART, C. P., and E. B. HENDRY. 1935. The phospholipins of blood. *Biochem. J.* **29**:1683.
31. STOECKENIUS, W. 1959. An electron microscope study of myelin figures. *J. Biophys. Biochem. Cytol.* **5**:491.
32. STOECKENIUS, W. 1962. The molecular structure

of lipid-water systems and cell membrane models studied with the electron microscope. *Symp. Intern. Soc. Cell Biol.* 1:349.

33. STOECKENIUS, W. 1966. Structural organization of the mitochondrion. *Ciba Found. Symp., Principles Biomol. Organ.* 418.
34. STOECKENIUS, W., and S. C. MAHR. 1965. Studies on the reaction of osmium tetroxide with lipids and related compounds. *Lab. Invest.* **14**:1196.
35. TERRY, T. M., D. M. ENGELMAN, and H. J. MOROWITZ. 1967. Characterization of the plasma membrane of *Mycoplasma laidlawii*. II. Modes of aggregation of solubilized membrane components. *Biochim. Biophys. Acta.* In press.