

FURTHER CHARACTERIZATION OF
PARTICULATE FRACTIONS FROM LYSSED CELL
ENVELOPES OF *HALOBACTERIUM HALOBIUM*
AND ISOLATION OF GAS VACUOLE MEMBRANES

WALTHER STOECKENIUS and WOLF H. KUNAU

From The Rockefeller University, New York, and the Cardiovascular Research Institute, University of California Medical Center, San Francisco. Dr. Stoeckenius' present address is the Cardiovascular Research Institute, University of California Medical Center, San Francisco, California 94122. Dr. Kunau's present address is the Ruhr-Universität Bochum Institut für Physiologische Chemie, 463 Bochum-Querenburg, Germany

ABSTRACT

Lysates of cell envelopes from *Halobacterium halobium* have been separated into four fractions. A soluble, colorless fraction (I) containing protein, hexosamines, and no lipid is apparently derived from the cell wall. A red fraction (II), containing approximately 40 per cent lipid, 60 per cent protein, and a small amount of hexosamines consists of cell membrane disaggregated into fragments of small size. A third fraction (III) of purple color consists of large membrane sheets and has a very similar composition to II, containing the same classes of lipids but no hexosamines; its buoyant density is 1.18 g/ml. The fourth fraction (IV) has a buoyant density of 1.23 g/ml and contains the "intracytoplasmic membranes." These consist mainly of protein, and no lipid can be extracted with chloroform-methanol. Fractions I and II, which result from disaggregation of cell wall and cell membrane during lysis, contain a high proportion of dicarboxyl amino acids; this is in good agreement with the assumption that disruption of the cell envelope upon removal of salt is due to the high charge density. The intracytoplasmic membranes (IV) represent the gas vacuole membranes in the collapsed state. In a number of mutants that have lost the ability to form gas vacuoles, no vacuole membranes or any structure that could be related to them has been found.

In an earlier paper (28) the morphology of *H. halobium* and the lysis of its cell envelope at low salt concentrations have been described. It was shown that the lysis is incomplete. One major soluble fraction and several morphologically distinct breakdown products were recovered. In the cytoplasm of the intact cell, membrane-like structures were observed and provisionally called intracytoplasmic membranes. These were also present in the envelope fraction but did not form part of the envelope structure. It was recognized that the intracytoplasmic membranes were related to the presence of gas vacuoles in these cells

and that they might represent collapsed gas vacuoles. However, at that time no convincing evidence for this conclusion was available.

We now report separation of the different membrane fragments in the envelope lysate, the isolation of the intracytoplasmic membranes, and their identification as collapsed gas vacuoles.

MATERIALS AND METHODS

The strains of halobacteria, the growth conditions, and fractionation procedures used in this work were those described previously (28). In addition, mutant strains were isolated, and a fractionation procedure

for whole cells was developed that made the time-consuming isolation of envelopes unnecessary. Freeze-etching and sectioning of whole cells and lysates avoiding centrifugation had to be employed to study the gas vacuoles. These techniques are described here.

Mutant Strains

On agar plates *H. halobium* forms opaque whitish-pink colonies. During the course of our earlier work, a translucent colony of deeper red color was observed on a plate inoculated with the NRL strain of *H. halobium*. From this a new strain, *H. halobium* R₁, which formed translucent red colonies only, was isolated. Another strain, Halobacterium sp. strain 5, isolated by Dr. H. Larsen (Trondheim, Norway), frequently formed such translucent red colonies or translucent red sectors in opaque pink colonies. Several of these were isolated and designated H. No. 5 c₁, c₂, etc. After they had been replated they yielded translucent red colonies only. Upon examination with dark field or phase light microscopy or with electron microscopy, cells from agar or liquid cultures of these strains and of R₁ showed no gas vacuoles under conditions where the parent strains appeared highly vacuolated. No spontaneous reversion to gas vacuole formation has been observed in these strains. The difference in the appearance of colonies on agar is obviously caused by the strong light scattering effect of the gas vacuoles.

Addition of 1 mg/ml 2-aminopurine nitrate (Calbiochem, Los Angeles, California) to the agar plates resulted in the appearance of numerous, translucent red colonies in the NRL strain of *H. halobium* and of a number of pink, opaque colonies in the red transparent colony-forming mutants isolated previously. From these opaque colonies, stable strains which grew as opaque colonies only could be isolated. Examination in the light microscope showed that they were vacuolated. In addition, a number of colonies were observed, and stable strains which showed difference in pigmentation were isolated. These have not been investigated in detail so far.

Fractionation of Lysates from Whole Cells and Envelope Preparations

Whole cells or envelope preparations were lysed by dialysis against distilled water for 12 hr at 4° C. To lysates from whole cells DNase (Worthington Biochemical Corp., Freehold, N. J.) was added, and the lysate was stirred for 30 min at room temperature to decrease the viscosity.

For the centrifugation schedule followed in the fractionation of the lysates of whole cells, see Fig. 1. Spinco L2, L2-50, and L2-65 and International B60 centrifuges with angle head rotors for differential centrifugation and swinging bucket rotors for gradient

centrifugation were used in this work. Differential centrifugation of lysates from envelope fractions has been described before (28). Linear sucrose-density gradients from 1.3 to 2.0 M were found to give optimal resolution of the components in the purple fraction. To reach density equilibrium for the intracytoplasmic membranes and "large membrane sheets" found in the purple fraction, 30-hr runs at 400,000 g were found to be necessary. The bulk of the small cell membrane fragments, the red fraction (when present), had not reached density equilibrium even at that time. Separation of these components, however, could be obtained at lower gravity force and in shorter runs. That equilibrium had actually been reached was established in experiments in which identical fractions were layered on top and at the bottom of identical gradients and centrifuged until they had moved to identical positions.

The gradients were analyzed by collecting drops through a hole punched into the bottom of the tube. Two drops per collecting tube were used for small centrifuge tubes (rotors SW-39, SW-65, and SB-405), and five drops were used for large tubes (rotors SW-25.2 and SB-489). They were then brought to 1.0 ml with distilled water, and the absorption at 280 nm and 260 nm was read in a Zeiss PMQ II spectrophotometer.

Chemical Analysis

Phosphorus was determined by the method of Ames and Dubin (2), protein with Lowry's technique (17), and RNA with orcinol (19) after hot trichloroacetic acid was extracted using purified yeast RNA as a standard.

For amino acid determination, the cell fractions were hydrolyzed in 6 N HCl at 110° C for 22 hr and then processed through an automatic amino acid analyzer (27). The accelerated procedure of Spackman (26) was used.¹

For lipid determinations whole cells and envelope fractions were dried at 40° C under N₂ in a Rotavapor (Büchi, Switzerland). The lipids were extracted with chloroform:methanol (2:1) overnight at room temperature and further purified by washing with distilled water, drying under N₂, and reextraction of the dry residue with chloroform.

Thin-layer chromatography was carried out on a 0.25 mm layer of silica gel G (Merck, Darmstadt, Germany) on 20 × 20 cm plates with diisobutyl ketone:acetic acid:water:ethanol = 80:50:10:10 (system 1) or with butanol:acetic acid:water =

¹ We are grateful to Dr. Teh-Yung Liu and Miss Sandra Walsh in Dr. S. Moore's laboratory at The Rockefeller University, who carried out the amino acid analyses, and to Dr. S. Moore for discussion of the results.

TABLE I

	Protein	260 m μ	RNA	Lipids	Hexosamine
	PO ₄ ³⁻	280 m μ			
	mg/ μ moles		μ g/mg protein		
Purple membranes	2.25	0.890	3.3	+	-
Gas vacuole (intracyto- plasmic) membranes	87	0.970	3.2	-	+
Red pellet	2.19	1.070	19.2	+	+
Colorless supernatant	43	0.770	—	-	+

60:20:20 (system 11). The lipids were detected by spraying them with 40% sulfuric acid and charring (23) and by staining with ammonium molybdate-perchloric acid (29). The Zindzadze reagent (7) was used for phospholipids.

Preparation for Electron Microscopy

Techniques for negative staining and for the fixation and embedding of halobacteria cells and cell fractions collected by centrifugation have been described (28). For the study of gas vacuoles, centrifugation could not be used because only in the few cells floating on the surface of the tube were the vacuoles preserved. Cells were therefore collected from the surface of agar plates or from liquid cultures on Millipore filters (Millipore Filter Corp., Bedford, Mass.) where suction was applied to increase the filtration rate. Lysis was induced by dropping a warm (approximately 37°C) solution of gelatin in distilled water onto the cells thus collected. After gelling, the surplus of gelatin was trimmed off with a razor blade, and the material was divided into small blocs and processed in the usual way.

For negative staining of lysed cells, 1:20–1:50 dilutions of concentrated cell suspensions with distilled water were found to give suitable concentration of material.

For freeze-etching, suspensions of cells obtained by filtration or from agar plates were quickly frozen in liquid Freon 22 (Du Pont, Wilmington, Del.) cooled with liquid N₂. The apparatus and techniques used were those of Moor and Mühlethaler (20) or of Bullivant and Ames (6) as modified by McAlear and Kreutziger (18).²

² We are indebted to Drs. J. H. McAlear and G. O. Kreutziger at the Electron Microscope Laboratory and Dr. D. Branton at the Department of Botany, University of California, Berkeley, in whose laboratories the freeze-etching experiments were carried out and with whom the results were discussed. Miss Susan Whytock and Mr. R. Waaland rendered expert technical assistance with the freeze-etching. R. Waaland prepared the specimen and took the micrograph reproduced in Fig. 12.

Light Microscopy

A Zeiss photomicroscope was used with dark field or phase optics. Because it is difficult to immobilize the cells without formation of salt crystals, Zeiss electronic flash equipment was used for photomicrography.³

Exposure to Hydrostatic Pressure

Cell suspensions from liquid cultures, or cells collected on Millipore filters or from agar surfaces were placed in a pressure chamber which was equipped with a manometer and two needle valves and was connected to a pressurized CO₂ or N₂ tank. By opening the inlet valve slowly, the pressure in the chamber was allowed to rise to several hundred pounds per square inch. The inlet valve was then closed, and the pressure was slowly released through the outlet valve. When the cell suspensions were removed from the chamber, a marked drop in turbidity and a change of color to a deeper red were noted.

RESULTS

Fractionation of Envelope Lysates

Dialysis of envelope preparations against distilled water yields a clear red lysate. When this is centrifuged at 50,000 *g* for 30 min, a purple pellet is obtained and the color of the supernatant has changed to orange-red. There is still considerable cross-contamination between these two fractions, and it may be necessary to repeat the centrifugation several times before this is reduced to insignificant amounts. The intense and different colors of the two fractions serve as convenient markers in the purification. The morphological components of these fractions have been described (28); the purple pellet contains large membrane sheets and intracytoplasmic membranes. The orange-red

³ The light micrographs were taken with Dr. W. M. Laetsch's equipment at the Department of Botany, University of California, Berkeley.

TABLE II

	Purple membranes		Red pellet		Gas vacuole (intracytoplasmic) membranes		Colorless supernatant	
	moles per 100 moles	μ moles mg protein	moles per 100 moles	μ moles mg protein	moles per 100 moles	μ moles mg protein	moles per 100 moles	μ moles mg protein
Lysine	5.0	0.446	2.1	0.158	4.7	0.456	2.6	0.296
Histidine	0.6	0.053	1.1	0.084	2.5	0.243	1.0	0.118
Arginine	5.9	0.525	4.0	0.308	5.5	0.531	3.5	0.402
Aspartic acid	5.7	0.509	12.9	0.984	11.6	1.119	16.9	1.940
Threonine	6.8	0.602	8.1	0.619	6.5	0.627	9.6	1.100
Serine	5.0	0.446	6.8	0.520	8.1	0.780	6.8	0.785
Glutamic acid	6.5	0.577	11.1	0.845	8.6	0.825	12.3	1.410
Proline	4.6	0.405	4.3	0.328	4.2	0.405	3.6	0.409
Glycine	9.6	0.850	9.5	0.725	9.4	0.906	8.4	0.970
Alanine	11.8	1.050	10.7	0.814	14.7	1.443	8.7	1.000
Valine	7.3	0.645	8.2	0.627	8.8	0.852	9.0	1.040
Methionine	4.1	0.362	1.8	0.136	—	—	—	—
Isoleucine	5.6	0.500	4.6	0.350	9.1	0.882	4.7	0.540
Leucine	13.2	1.170	8.0	0.609	0.9	0.084	6.4	0.730
Tyrosine	3.7	0.326	2.9	0.223	2.3	0.225	2.0	0.227
Phenylalanine	4.6	0.405	3.3	0.251	2.5	0.237	2.4	0.277
Glucosamine	—	—	0.4	0.032	—	—	1.1	0.130
Galactosamine (or mannosamine)	—	—	0.3	0.020	0.3	0.030	1.0	0.120
Ratio $\frac{\text{Lys} + \text{His} + \text{Arg}}{\text{Asp} + \text{Glu}}$	0.94		0.30		0.63		0.24	

Single amino acid analysis of cell fractions by ion exchange chromatography. No corrections have been applied for destruction of sensitive amino acids during acid hydrolysis. The amino sugar values were corrected approximately by assuming 50 per cent destruction. The column used does not separate galactosamine and mannosamine, but preliminary results indicate that the amino sugar is galactosamine (Haines, T. H., Data unpublished.).

supernatant contains small cell membrane fragments and a colorless soluble component.

The orange-red supernatant can be further fractionated by centrifuging it at 105,000 *g* for 20–30 hr or for shorter times if higher gravity forces are used. An orange-red pellet and a colorless supernatant are obtained. This supernatant contains mainly protein, some hexosamines, but no lipid (see Table I). The amino acid composition is given in Table II. When the colorless supernatant is adjusted to 0.1 M NaCl by the addition of salt, analytical ultracentrifugation shows one component with a sedimentation coefficient of $S_{w20.0} = 4.0$ which is apparently identical with the material first described by Brown (5).

The orange-red pellet shows a small contamination with the colorless 4S component, which may be reduced by resuspension in distilled water and by repeating the sedimentation. The pellet contains a small amount of hexosamine, approxi-

mately 60% proteins, 40% lipid, and 2% RNA (see Table I). The amino acid composition of this fraction is given in Table II, and the thin-layer chromatogram of its lipids is shown in Fig. 2. The lipid composition appears identical with that of the total lipid extract from *H. cutirubrum* described by Kates and his collaborators (12).⁴

Subfractionation of the Purple Fraction

The work described earlier (28) has already demonstrated that the readily sedimentable "purple fraction" from envelope lysates contained two morphologically different components, the large membrane sheets and the intracytoplasmic membranes. It soon became obvious that a considerable amount of this material, especially of the intracytoplasmic membranes, was lost during

⁴ Dr. M. Kates kindly sent us a sample of *H. cutirubrum* phospholipids for comparison.

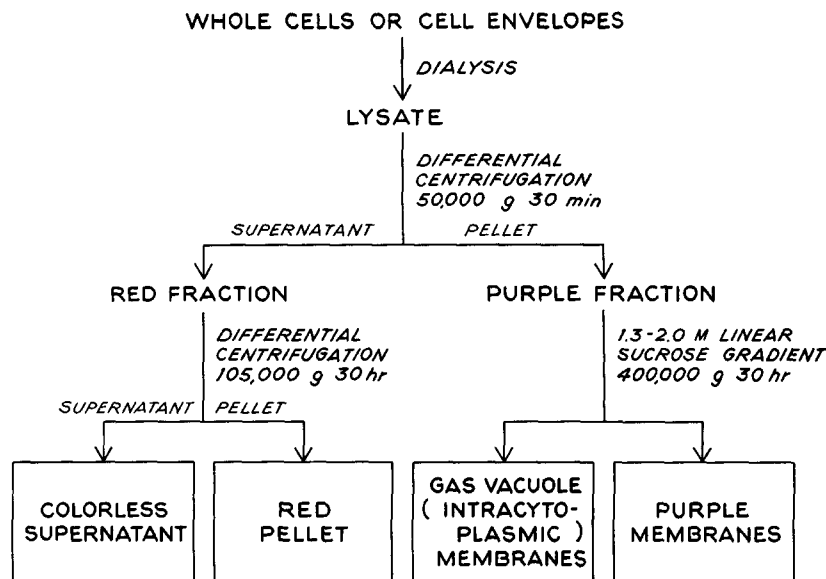


FIGURE 1 Centrifugation scheme for fractionation of whole cells or cell envelope preparations. With whole cells as the starting material only the purple pellet is used for further study, because the red fraction is too heavily contaminated with cytoplasmic components. With envelope preparations as the starting material both fractions can be used, but the amount of material in the purple pellet is considerably reduced.

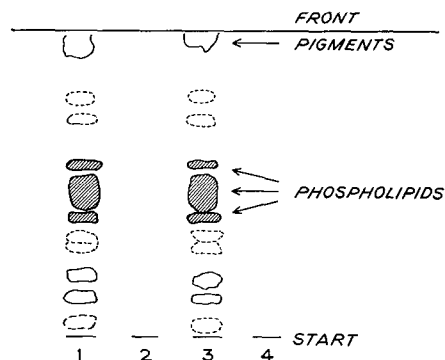


FIGURE 2 Thin-layer chromatogram with solvent System I (see Materials and Methods) of chloroform-methanol extracts from: 1, purple membranes; 2, gas vacuole (intracytoplasmic) membranes; 3, red pellet; 4, colorless supernatant.

preparation of the envelopes. This was the reason for developing the isolation procedure for an essentially identical purple fraction directly from lysates of whole cells (Fig. 1).

When the purple fraction, prepared from whole cells or envelopes, is centrifuged in a continuous sucrose density gradient, two bands can be resolved (Fig. 3). The lighter band has a deep

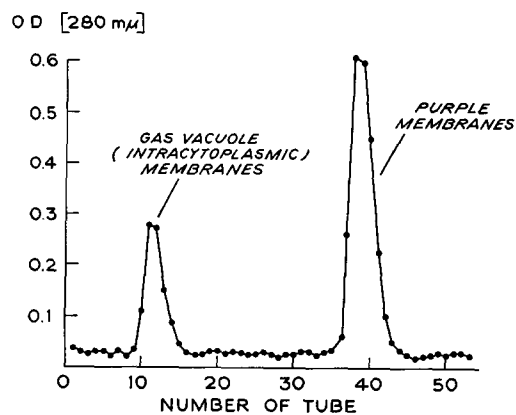


FIGURE 3 Density gradient fractionation of the purple fraction. The heavier band appears slightly yellow and contains the gas vacuole (intracytoplasmic) membranes. The lighter band has a deep purple color and contains the purple membranes. Electron micrographs of material from the two bands are shown in Figs. 4 and 5.

purple color and a buoyant density of 1.18 g/ml. The material in the heavier band, when present in high concentration, shows a very faint yellowish color and in lower concentration is detectable through its absorption at 280 mμ. It equilibrates at

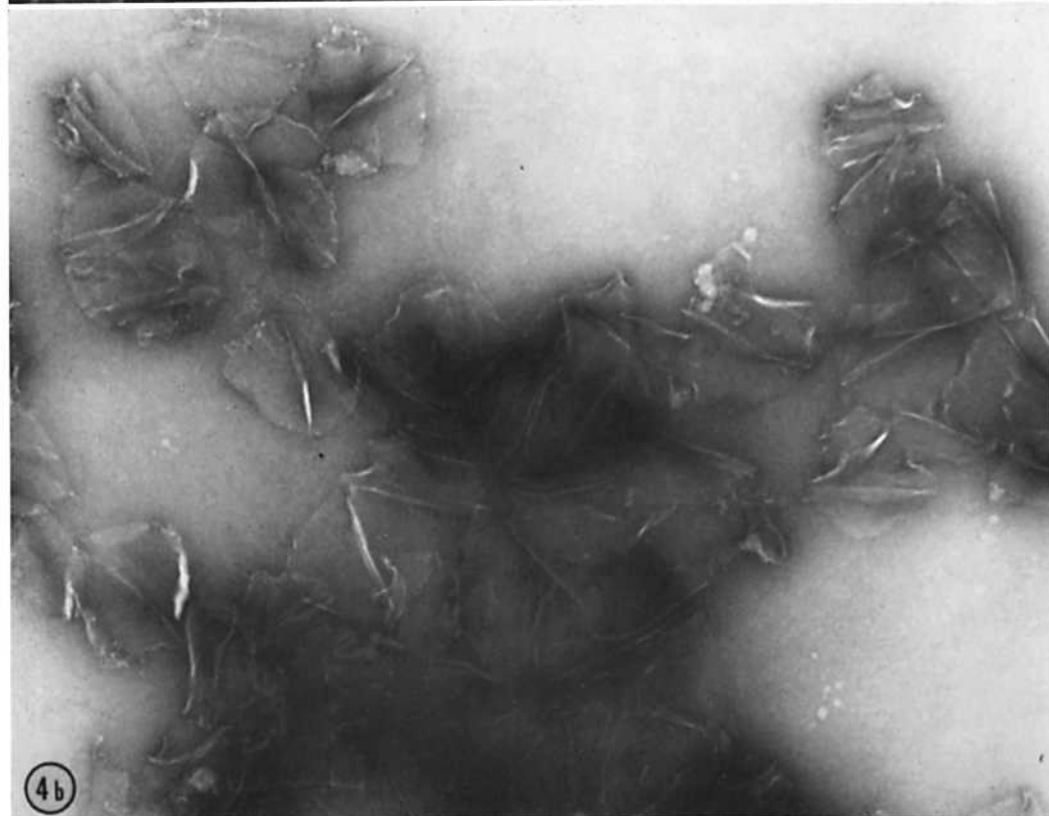
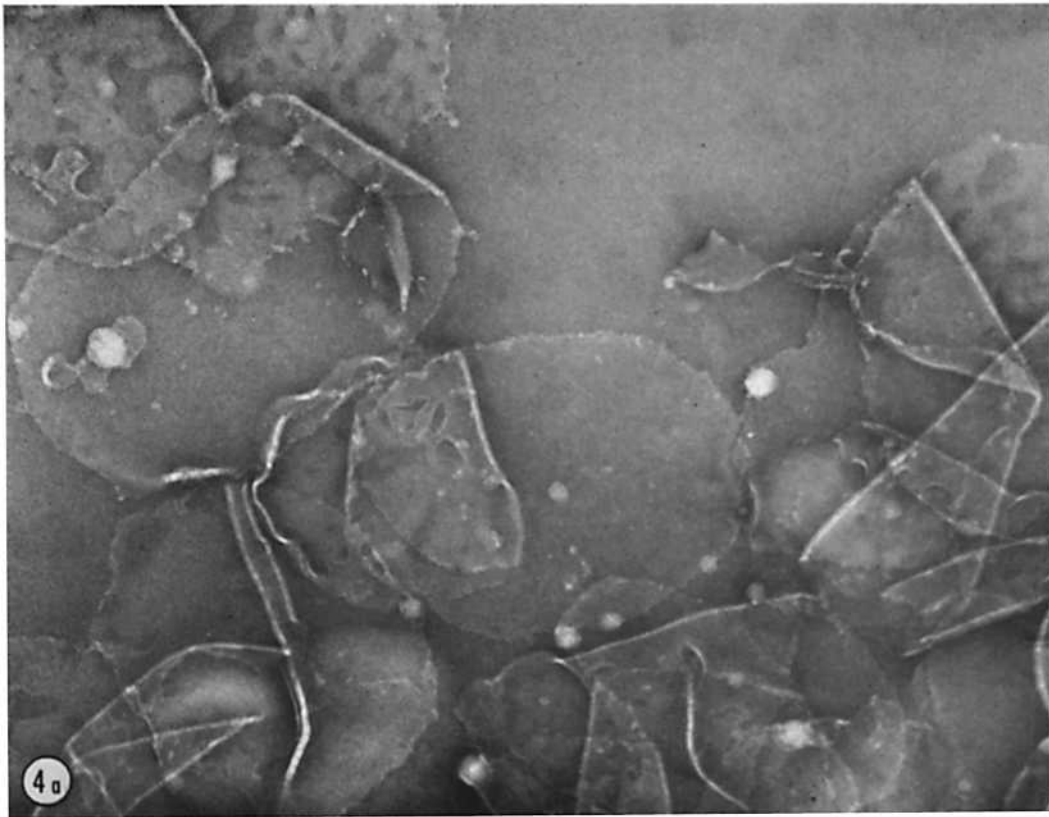


FIGURE 4 *a* Negatively stained material from the lighter band of the density gradient shown in Fig. 3, containing the purple membranes. $\times 82,000$.

FIGURE 4 *b* Negatively stained material from the heavier band of the density gradient shown in Fig. 3, containing the gas vacuole (intracytoplasmic) membranes. $\times 84,500$.

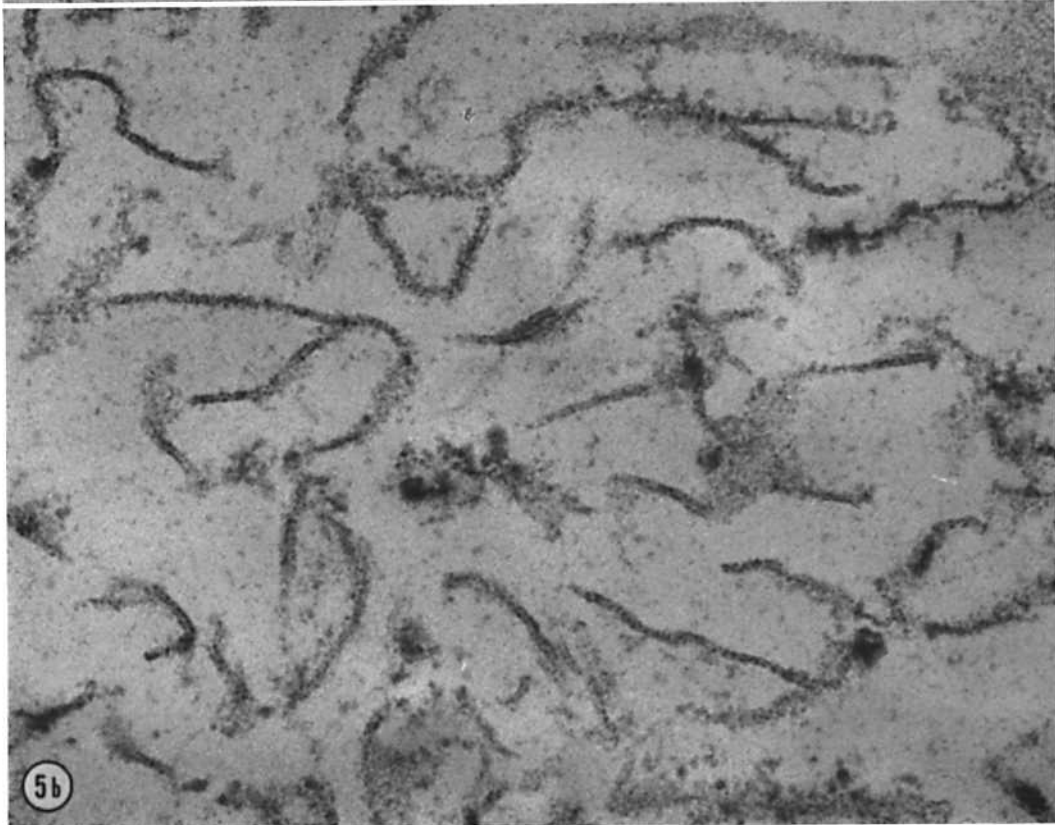
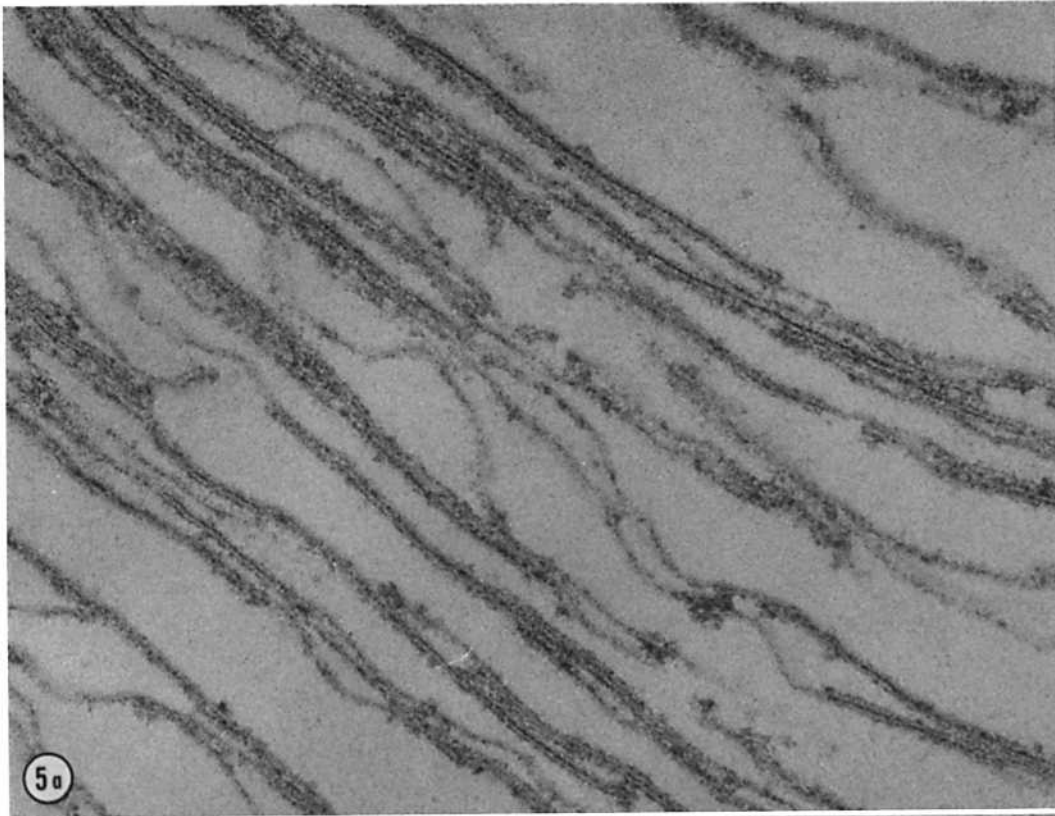


FIGURE 5 *a* Section through pellet obtained from the lighter band of the density gradient shown in Fig. 3, containing the packed purple membranes. $\times 158,000$.

FIGURE 5 *b* Section through pellet obtained from the heavier band of the density gradient shown in Fig. 3, containing the gas vacuole (intracytoplasmic) membranes. $\times 157,500$.

a density of 1.23 g/ml. When the purple fraction is contaminated with the orange-red fraction or when whole lysates of envelope fractions are layered on top of the gradient, the orange-red material is spread out through the upper part of the gradient, and the bulk of it does not reach density equilibrium even after 35 hr at 400,000 *g*. Often there is, however, a sharp lower boundary slightly above and clearly resolved from the purple band. It has been shown before that the orange-red material consists of cell membrane fragments of varying sizes. The largest cell membrane fragments are found in the fractions collected from this boundary. They are probably very close to, if not at, density equilibrium, indicating that they have a density slightly lower than that of the material of the purple band.

Electron microscopy of the purple band shows only large membrane sheets which confirms our earlier conclusions that the purple pigment is associated with this component (Figs. 4 *a* and 5 *a*). The colorless band apparently consists only of intracytoplasmic membranes with their characteristic shape and surface pattern (Figs. 4 *b* and 5 *b*). The purple band, henceforth called purple membranes, contains considerably less RNA and slightly less lipid than the orange-red fraction (see Table I); this is consistent with its slightly higher buoyant density. No hexosamine was found in this fraction. The amino acid compositions of the purple membranes and the intracytoplasmic membrane fractions are given in Table II. Protein is the only major constituent detected in the intracytoplasmic membranes. A small amount of hexosamine is also present. It must, however, be pointed out that the total amount of protein calculated from the amino acid analysis accounts for only 70% of the dry weight of this fraction. This must be considered as a preliminary result since only one amino acid analysis was carried out.

The composition of the purple fraction has also been studied in two mutants derived from the NRL strain. One is the red transparent colony-forming strain R_1 , the other the pink, opaque colony-forming strain R_{101} derived from R_1 (see Material and Methods). This means we compared a strain which, through a spontaneous mutation, had lost the ability to form gas vacuoles (R_1) with a mutant strain derived from it, which under the action of a mutagen had regained the ability to form gas vacuoles (R_{101}). The density-gradient separation of the purple fraction from both strains,

prepared from whole cells under identical conditions, is given in Fig. 6. The purple membranes (large membrane sheets) appear in identical positions and comparable amounts in the gradient. The intracytoplasmic membranes are completely absent in R_1 but are present in R_{101} . They appear in the same position in the gradient and in roughly the same amount as in the parent NRL strain.

Identification of the Intracytoplasmic Membranes

LIGHT MICROSCOPY: When cells from a vacuole forming culture of *H. halobium* in the second half of the logarithmic growth phase are observed in the light microscope under phase contrast conditions, nearly all cells are seen to contain apparently highly refractile light bodies (Fig. 7 *a*). At the end of the log-phase, they fill the central portion of the cells and can no longer be distinguished as separate bodies. Only small regions at the poles appear free of them. After the cells have been exposed to hydrostatic pressure, these bodies are no longer present and the cells often appear narrower or sometimes wider in the middle than at the poles (Fig. 7 *b*). Under growth conditions the "refractile bodies" will gradually reappear after a few hours.

If a cell suspension that has not been exposed

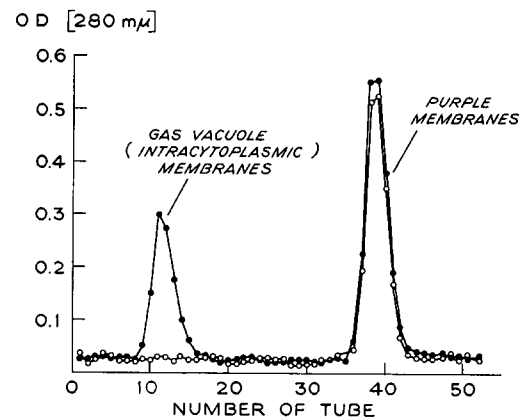
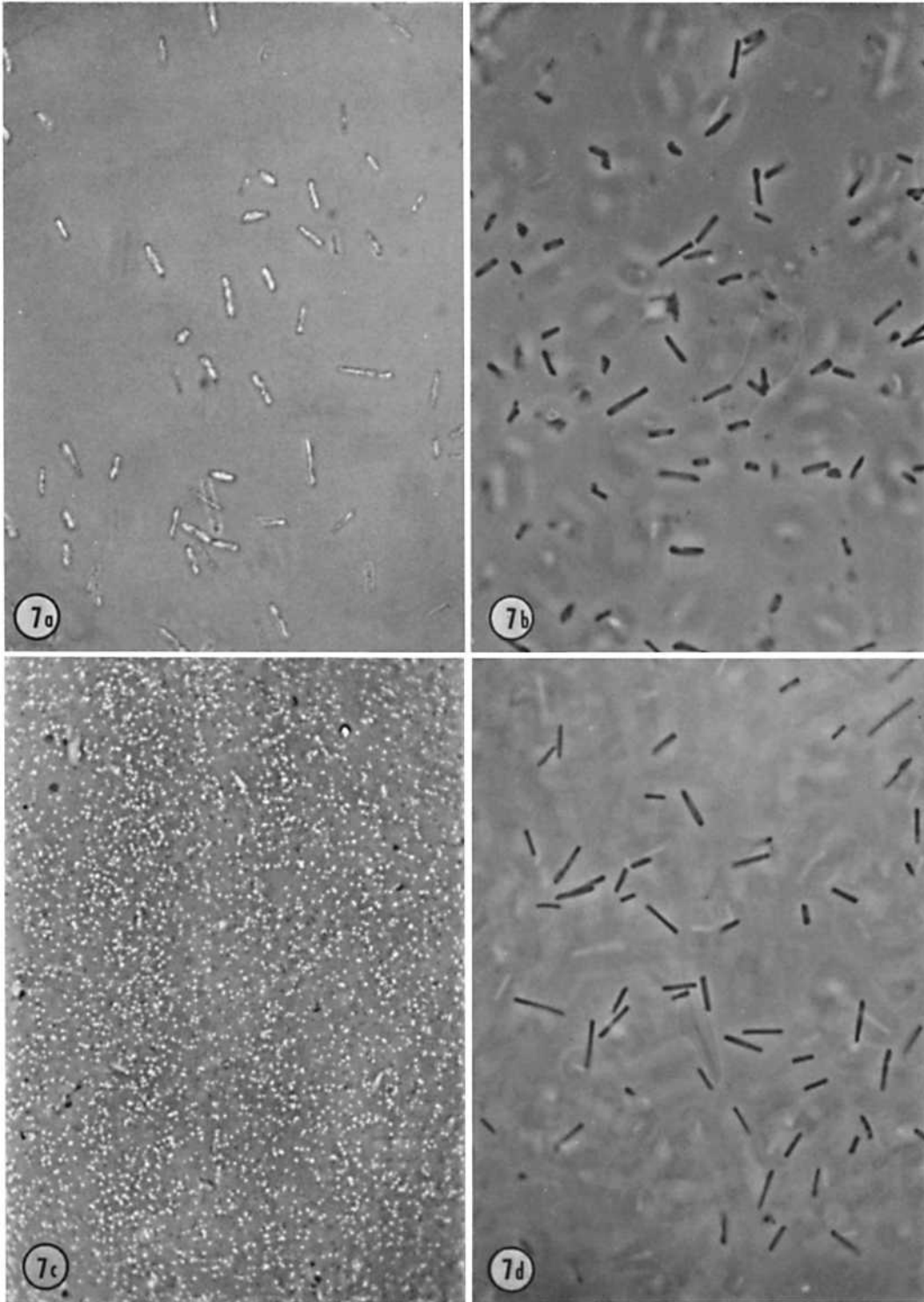


FIGURE 6 Density gradient of the purple fractions from two mutant strains. *H. halobium* R_1 which does not form gas vacuoles completely lacks the heavier band, whereas the double mutant *H. halobium* R_{101} which has regained the ability to form gas vacuoles shows the same pattern as the wild type, i.e., both the gas vacuole (intracytoplasmic) membrane band and the purple membrane band are present (compare with Fig. 3).



FIGS. 7 *a-d* Phase-contrast light micrographs of *H. halobium* cells from stationary phase cultures in basal salt solution. $\times 1400$.

FIGURE 7 *a* Wild-type cells filled with gas vacuoles.

FIGURE 7 *b* Same cell suspension as in Fig. 7 *a* after exposure of cells to hydrostatic pressure. The vacuoles have collapsed and are no longer visible.

FIGURE 7 *c* Same cell suspension as in Fig. 7 *a* after dilution with distilled water. The cells have lysed, and the gas vacuoles have been set free.

FIGURE 7 *d* Mutant strain that has lost the ability to form gas vacuoles.

to pressure is left standing undisturbed, the cells will slowly float to the surface. If the same suspension is centrifuged even at moderate speed, most of the cells will collect at the bottom of the tube in a red pellet; only a minor portion will form a thin, pink layer on the surface of the liquid. This surface layer can be redispersed and re-centrifuged at the same speed, whereupon most of the cells will sediment to form a red pellet, and the rest will again form a thin pink layer on the surface. In the microscope the cells from the red pellet do not show vacuoles. The cells from the pink surface layer appear highly vacuolated.

These observations essentially confirm the results reported by Petter (22), Houwink (9), and others (see references 15, 18, and 24 for further references) and are consistent with their conclusion that the highly refractile bodies are gas-filled vacuoles.

When vacuolated cells are lysed under the light microscope, the cells suddenly disappear, and numerous small bodies in rapid Brownian motion appear free in solution (Fig. 7 *c*). Their size appears to be at the limit of resolution of the light microscope. That these bodies are actually the gas vacuoles set free by lysis of the cells is confirmed by electron microscopy of the lysate to be discussed later, but this can also be inferred from light optical observations. When such a lysate is left standing in a test tube, the turbid solution can be seen to slowly clear from the bottom toward the top. This is evidently caused by the slow rise of the vacuoles to the top and may be used for concentrating the vacuoles. However, the vacuoles are not completely stable when isolated, and the turbidity of the solution decreases slowly. If the cells have been exposed to hydrostatic pressure before lysis, no such bodies are liberated, and the lysate appears empty in the light microscope. Compression of the lysate containing the bodies results in an immediate clearing of the suspension and disappearance of the bodies from the light microscope image.

Osmotic pressure can also cause collapse of the vacuoles. Lysis of the cell envelope is apparently so rapid when cells are exposed to dilute salt solutions that little rise in intracellular pressure occurs. However, when cells are fixed with formaldehyde and then brought into low salt concentrations, they swell considerably and reversibly but do not lyse (1). This rise in intracellular

pressure causes collapse of the vacuoles which can be observed in the light microscope.

Liquid cultures from the mutant strains that grow in translucent red colonies show no floating cells and no vacuoles when seen under the microscope (Fig. 7 *d*), and no small bodies are liberated upon lysis.

ELECTRON MICROSCOPY: The appearance of vacuolated cells in shadowed preparations has been described (28). In sections the vacuoles appear as empty areas in the cytoplasm, usually round or slightly angular, surrounded by a continuous dense line approximately 30 Å wide. (Fig. 8). This line is often difficult to see when the vacuoles border on the dense cytoplasm. However, they are preferentially located in the nucleoid region where this limiting membrane usually stands out clearly against the light background. The denser cytoplasm is mainly confined to a narrow band between the plasma membrane and the nucleoid region with two more extended areas of cytoplasm at the poles of the cell. Very few intracytoplasmic membranes can be found in these vacuolated cells. Some of these occasionally show a wider central light space.

If cells that have been exposed to pressure are sectioned, the appearance is the same as that previously described for centrifuged cells (28). No vacuoles are present, and intracytoplasmic membranes are observed. However, these are difficult to detect in the dense cytoplasm or, in the nucleoid region, are difficult to distinguish from parallel DNA strands. One therefore hesitates to assume that the few clearly visible intracytoplasmic membranes can account for the numerous and very prominent gas vacuoles seen in the cells before exposure to pressure, and because of the difficulty in identifying the intracytoplasmic membranes unambiguously, no quantitative relationship can be established.

Lysates, especially after negative staining, yield more conclusive results. In lysates of vacuolated cells, numerous characteristic bodies are found (Fig. 9 *a*). They are typically spindle-shaped, of a size comparable to that of the gas vacuoles, and are not penetrated by the stain so that they appear light against the dark background. Indications of a regular surface structure consisting of parallel lines perpendicular to the long axis of the spindle can often be observed (Fig. 9 *b*). If a sample of the same cell suspension is exposed to hydrostatic pressure before or immediately after lysis, no such

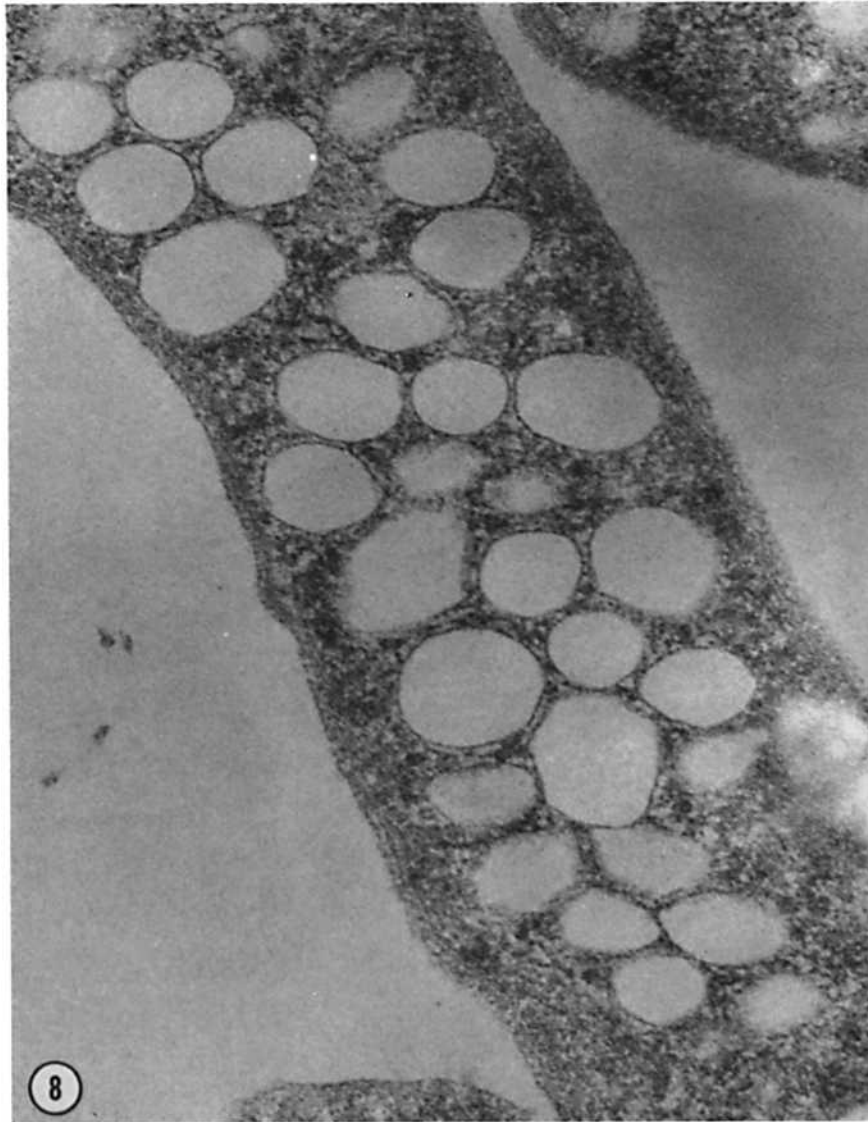


FIGURE 8 Section through a highly vacuolated cell of *H. halobium*. The single dense line surrounding the vacuoles is clearly visible. $\times 108,000$.

bodies are seen. Instead, numerous typical intracytoplasmic membranes appear in the negatively stained preparations (Figs. 10 *a* and 10 *b*). They are not as conspicuous as the light spindle-shaped bodies. However, a close inspection shows that they are present in large numbers comparable to the number of spindle-shaped light bodies in uncompressed preparations.

If these lysates are fixed and sectioned, essentially the same results are obtained. The prepara-

tions from vacuolated cells show numerous free vacuoles (Fig. 11 *a*), whereas otherwise identical preparations from compressed cells show no vacuoles but typical intracytoplasmic membranes, i.e., two parallel dense lines separated by a light band (Fig. 11 *b*). The beaded appearance of the two dense lines in the intracytoplasmic membranes is usually quite pronounced, whereas in the vacuoles it is often not as distinct. This corresponds to the observations in the negatively stained material

where the surface structure is also more clearly visible when the vacuoles are collapsed.

It should be noted that, in lysates of vacuolated cells, a few intracytoplasmic membranes are usually present. It seems possible that some of the gas vacuoles exist in the collapsed state in vivo. Alternatively a transient rise in osmotic pressure may occur before the cells lyse, and this may be sufficient to cause collapse of some of the vacuoles.

Further results confirming the conclusion that intracytoplasmic membranes represent collapsed gas vacuoles are obtained with the freeze-etching technique. The vacuoles in intact cells are clearly visible, and their typical shape and regular surface structure are easily recognized (Fig. 12). Usually the inner surface of the vacuoles appears to be exposed in these preparations. Its fine structure does not differ significantly from that seen in negatively stained preparations of lysates. In specimens from compressed cells, no vacuoles are seen after freeze-etching. Instead, flat or slightly curved membrane sheets are present in the cytoplasm showing the typical shape and surface pattern also seen in negatively stained lysates which contain collapsed vacuoles (Fig. 13). They are easily identified as intracytoplasmic membranes.

None of the strains that grow only as translucent red colonies show vacuoles or intracytoplasmic membranes in the electron microscope. This has been confirmed not only for sectioned whole cells where the intracytoplasmic membranes are difficult to see at best but also for envelope preparations and negatively stained lysates where they are easily recognized. Mutant strains derived from these "vacuoleless" mutants that have regained the ability to form pink opaque colonies again show the vacuoles and, after exposure to hydrostatic pressure, the intracytoplasmic membranes.

DISCUSSION

The results described here leave little doubt that the intracytoplasmic membranes represent collapsed gas vacuoles.

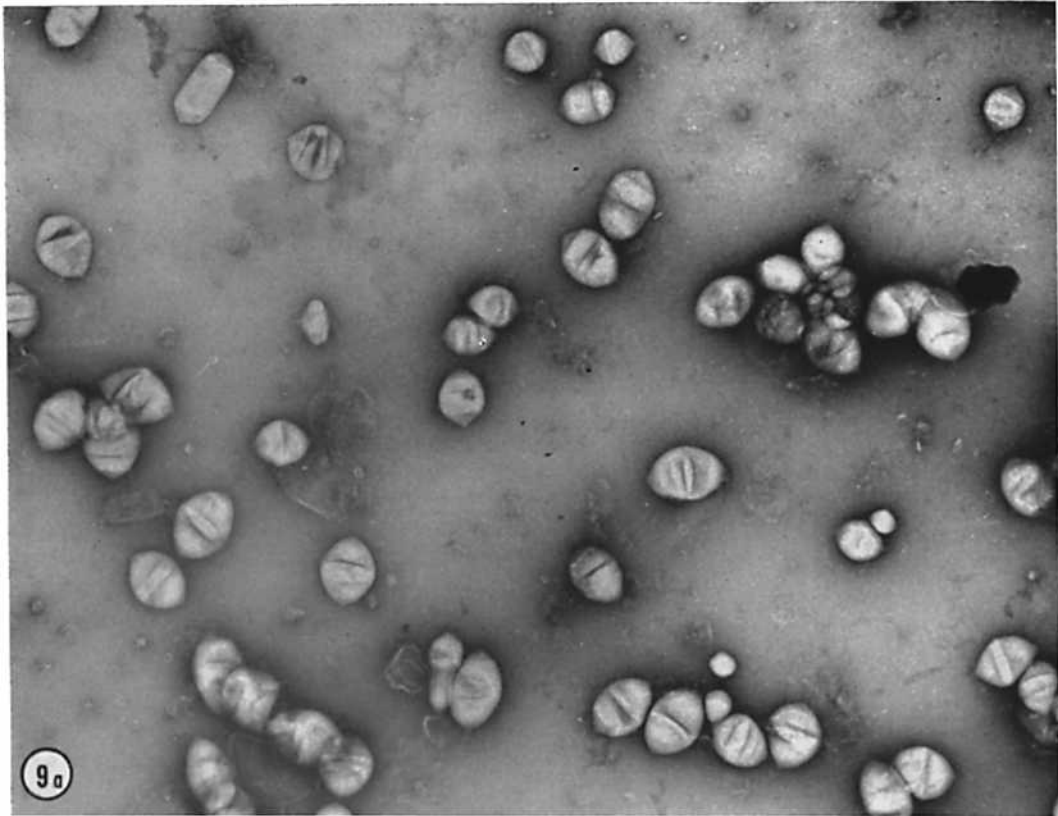
The following observations support this conclusion. Intracytoplasmic membranes have only been observed in strains that form gas vacuoles. Both these structures, gas vacuoles and intracytoplasmic membranes, remain intact when the cells are exposed to distilled water. The size and shape of the vacuoles, the appearance of their limiting membranes in sections, and their characteristic surface structure correspond closely to the morphological characteristics of the intracytoplasmic membranes. Application of hydrostatic pressure to vacuolated cells or to their lysates results in disappearance of the vacuoles, and intracytoplasmic membranes appear in the preparation in comparable numbers.

Once this conclusion has been accepted, the unusual morphological features of the intracytoplasmic membranes are more easily understood. It is now clear why they do not appear to form closed structures as typical cellular membranes do. The short side branches seen in sections or the ridges seen in shadowed and negatively stained preparations (28) can now be explained as folds in the membrane of the collapsed vacuole. The constant distance between the two dense lines as well as the "open ends" seen in sections of the collapsed state require additional assumptions. It seems most likely that the total thickness of the membrane is greater than the dense line seen in sections and that unstained material is present on the inner, and possibly also on the outer, surface of the membrane. Freeze-etching shows that the striated pattern apparently is present on the inner surface of the vacuole, and observations on the collapsed isolated vacuoles

FIGURES 9 *a* and *b* Lysate from vacuolated cells of *H. halobium* negatively stained.

FIGURE 9 *a* The spindle-shaped gas vacuoles apparently not penetrated by the stain are the most prominent structures in the picture. In the upper left-hand corner a vacuole with the not so frequently observed shape of a short cylinder with conical ends. Some purple membranes (large membrane sheets) can also be distinguished. $\times 29,200$.

FIGURE 9 *b* Same as Fig. 9 *a* but at higher magnification. The characteristic striated surface pattern of the gas vacuoles is now clearly visible. $\times 300,000$.



show that it is also present on the outer surface of the membrane. This evidence, together with the beaded appearance of the membrane in cross-sections, suggests the following structure. A gas vacuole is surrounded by a membrane that can be described as a stack of hoops laterally attached to each other. A decrease in the diameter of the hoops toward both ends of the structure results in the typical spindle shape of the vacuole. Only the outer (or central) portion of each hoop takes up enough heavy metal to be visible in sectioned material. This model is borne out by the observation that the membranes appear to break preferentially along the lines seen on the surface (Fig. 14). When the vacuole collapses, the hoops may actually break at the edge of the flattened vacuole or at least bend sharply with a concomitant change in structure that decreases the contrast in the electron micrographs. It is also possible that the decrease in contrast, when the edge is sectioned at an acute angle, may be sufficient to account for the appearance of open ends. Some modification of the hoop structure must, of course, exist to account for closure at the tips of the spindle-shaped vacuoles and the occasionally observed more rounded ends. At present we have no micrographs that indicate clearly what the structure at these points might be.

The surface structure of the vacuole membrane as seen in the electron micrographs would also be consistent with a model in which one or several continuous strands are wound into a conical helix of low pitch. So far we cannot decide between the two models, but electron micrographs of disintegrating vacuole membranes appear to be more compatible with the stacked-hoops model than with the conical helix (Fig. 14).

The thickness of the vacuole membrane (approximately 40 Å) is of molecular dimension, and it appears to be an asymmetric structure. This is

not only deduced from the differences in staining of the outer and inner portion of the membrane but also from their behavior upon isolation. The membrane must be relatively impermeable to water because the vacuoles are stable for many hours after lysis. When the vacuoles have been collapsed, even upon isolation the two apposed inner surfaces of the membrane show little tendency to separate, but there is no strong lateral aggregation of individual collapsed vacuoles; this indicates again a difference between the inner and outer surface. If the inner surface were hydrophobic, these observations could be understood most easily, and it would also help to explain the impermeability to water and the accumulation of gas.

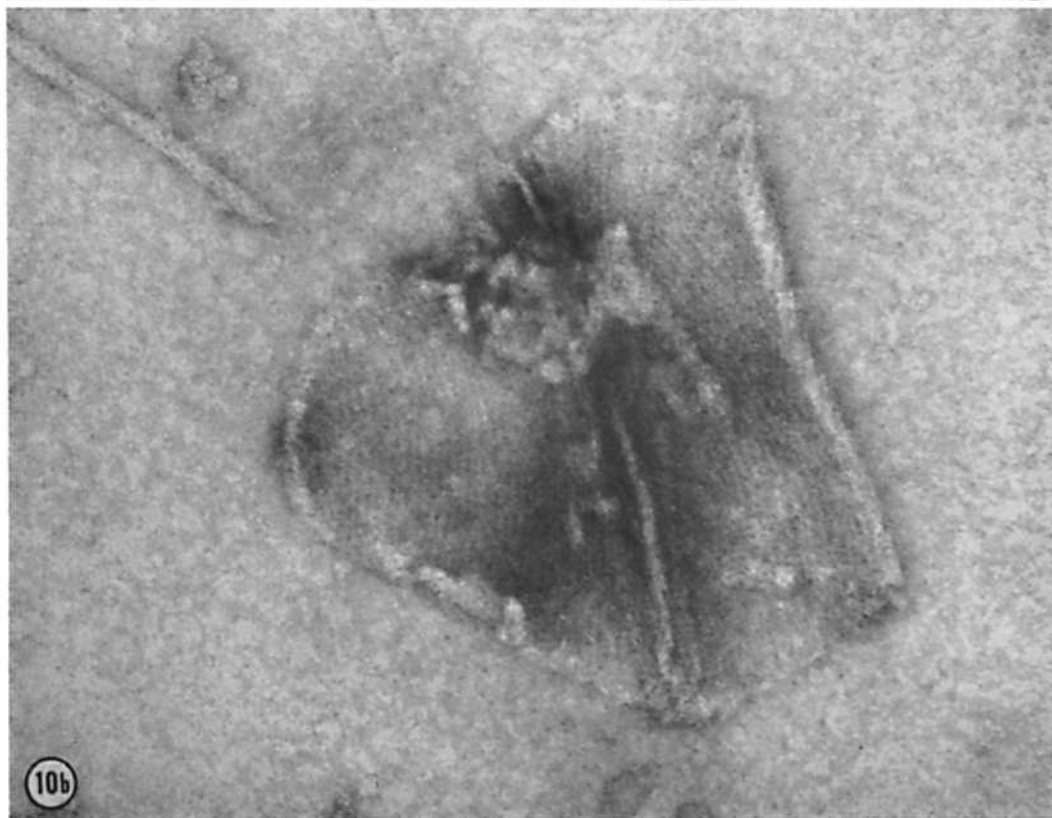
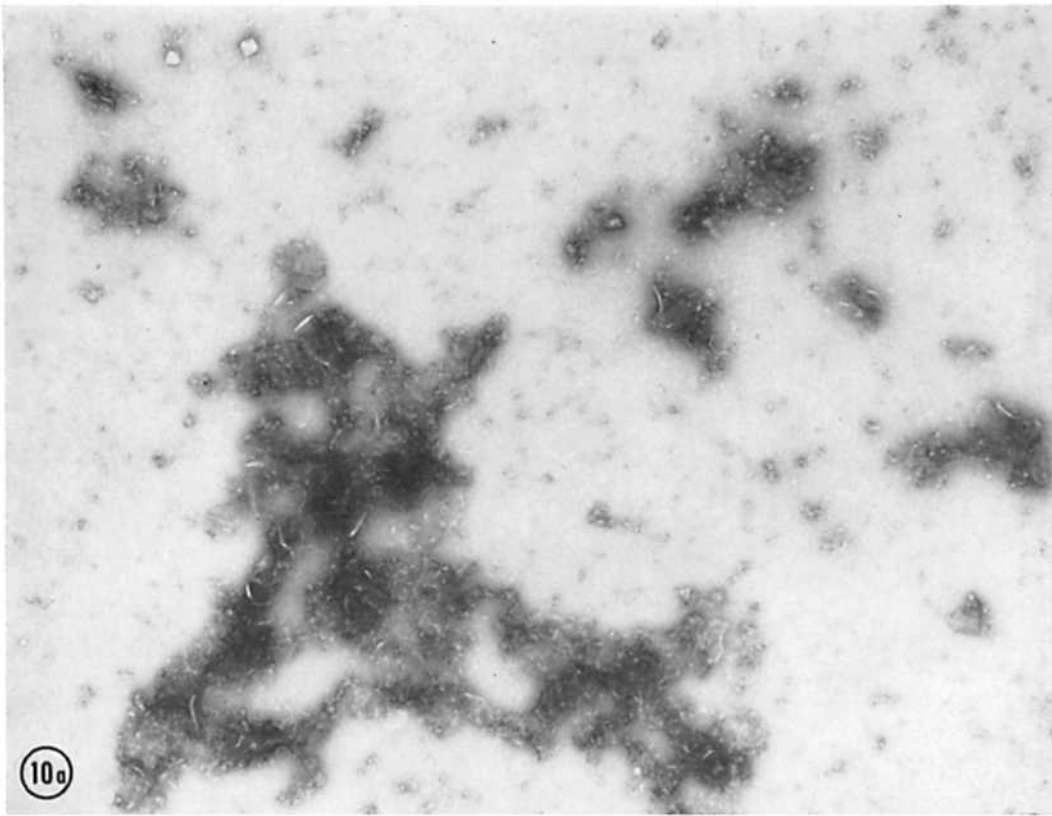
If one accepts the assumption of a hydrophobic inner membrane surface, it is surprising to find that no lipid can be extracted with chloroform-methanol from the isolated vacuole membranes. This, of course, does not exclude the existence of some lipid-like material, i.e., compounds containing long hydrocarbon chains, perhaps lipids covalently bound to membrane substance. However, the relatively high buoyant density of 1.23 g/ml in sucrose makes it unlikely that this membrane has a structure and composition similar to that of other cellular membranes, for instance, that it represents "half of a unit membrane" as was suggested by Bowen and Jensen (4).

Amino acid analysis indicates that probably 70% of the dry weight of the vacuole membrane is protein. Compared to the over-all amino acid composition of the proteins from halobacteria and halobacteria envelopes (3, 14), the proportion of dicarboxyl amino acids to basic amino acids in the vacuole membrane is low. This is in good agreement with the resistance of these membranes to distilled water and the conclusions of Brown (5, 25) and others (13, 15, 16, 21) that

FIGURES 10 *a* and *b* Lysate from vacuolated cells of *H. halobium* exposed to hydrostatic pressure before negative staining.

FIGURE 10 *a* At low magnification it can be seen that the typical spindle-shaped gas vacuoles have disappeared (compare to Fig. 9 *a*). In the collapsed state their membranes are not as conspicuous. The most prominent features seen at this magnification are the folds or curled up edges of the membranes. $\times 29,500$.

FIGURE 10 *b* Same as Fig. 10 *a* but at higher magnification. The striated surface pattern is now clearly visible on the collapsed vacuole membrane. $\times 300,000$.



lysis in halobacteria is caused by the large number of acidic groups in the membrane protein which results in a high negative charge on the structures when counterions are removed. The same argument holds for the purple membranes which are also resistant to distilled water and contain an even lower proportion of dicarboxyl amino acids to basic amino acids. The two other components of the envelope, the colorless fraction and the red membranes which disaggregate in distilled water, consequently show a higher proportion of acidic to basic amino acids than that found for whole envelope preparations (3, 14). Noteworthy is the rather high proportion of amino acids with hydrophobic side chains, especially in the purple membranes.

Analysis of the lipids in the purple membranes and the red membranes confirms our earlier observation that the lipid composition of these two fractions is the same within the limits of resolution of our technique. Their relationship in the intact envelope is still undetermined. Hexosamines were found in highest amounts in the colorless supernatant in good agreement with our earlier conclusion that this fraction is derived from the cell wall. In contrast to other bacteria, no lipids appear to be associated with the cell wall material.

While the properties of the cell envelope of halobacteria are unique, the occurrence of gas vacuoles is not. Similar gas vacuoles have been observed in blue-green algae, and their collapsed state has first been described and correctly interpreted by Bowen and Jensen (4). (For a review of the early literature see Fogg, reference 8, and for references to the more recent work see Smith and Peat, reference 24.) The over-all shape of the gas vacuoles in blue-green algae is different from that found in halobacteria. They usually appear as long cylinders and occur in bundles. However, their diameter, surface structure, and the conical ends are very similar to the structures

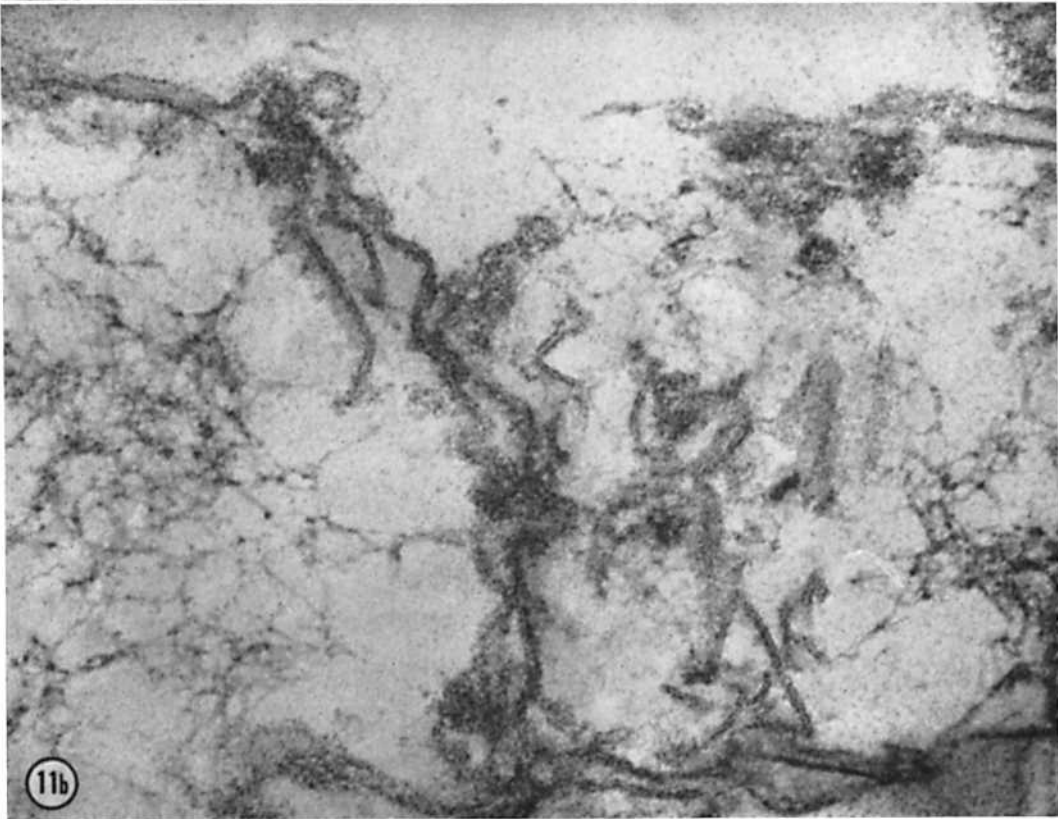
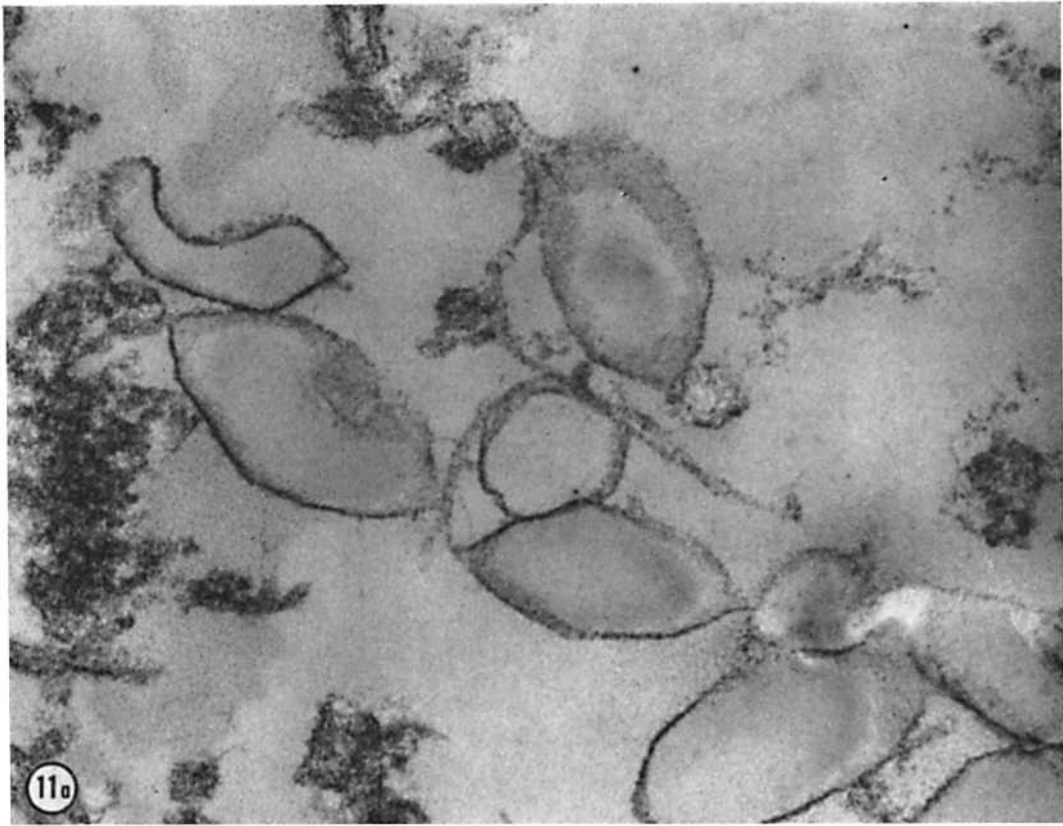
seen in *H. halobium*. The vacuoles in blue-green algae could be described as typical spindle-shaped halobacterium vacuoles in which the central part has been extended by the addition of hoops. Whether this is more than a purely formal description remains to be seen. Occasional cylindrical vacuoles can also be found in *H. halobium*. The vacuoles of blue-green algae also seem to be located preferentially in the nucleoid areas of the cell.

Jost (10) was the first to describe the surface structure as seen after freeze-etching and with negative staining in a blue-green alga (*Oscillatoria rubescens*). He reports that the striae actually consist of rows of granules and are only present on the outer surface of the vacuole. We cannot confirm this for our material. Freeze-etching shows the striae pattern on the inner surface of the vacuole membrane, and neither intact nor disintegrating membranes show indications of a globular substructure. While it is, of course, possible that the structure in blue-green algae is different from that in halobacteria, neither of these two observations is very convincingly demonstrated in Jost's published electron micrographs, and the question may deserve some further work. Blue-green algae may be a better object in which to study this because the surface structure of their vacuoles seems to be more easily visible than in halobacteria.

Jost and Matile (11) have reported isolation of the gas vacuoles from *Oscillatoria rubescens* and a high lipid and carotenoid content in the purified gas vacuole fraction. Again it is, of course, possible that profound differences exist between the vacuoles of *Oscillatoria rubescens* and those of *H. halobium*. However, Jost and Matile's vacuole fraction is not too well characterized; neither the typical shape nor the surface structure of the vacuoles have been demonstrated in the purified material. Moreover, the vacuoles should have collapsed under the centrifugal forces used in

FIGURE 11 *a* Lysate from vacuolated cells of *H. halobium* embedded and sectioned to avoid exposure to hydrostatic pressure (see Material and Methods). Some vacuoles are distorted, but their characteristic spindle shape is still recognizable. $\times 124,500$.

FIGURE 11 *b* Same as Fig. 11 *a* but lysate exposed to hydrostatic pressure before embedding and sectioning. The vacuoles have disappeared from the preparation. In their place typical intracytoplasmic membranes, i.e., collapsed gas vacuole membranes are visible $\times 127,000$.



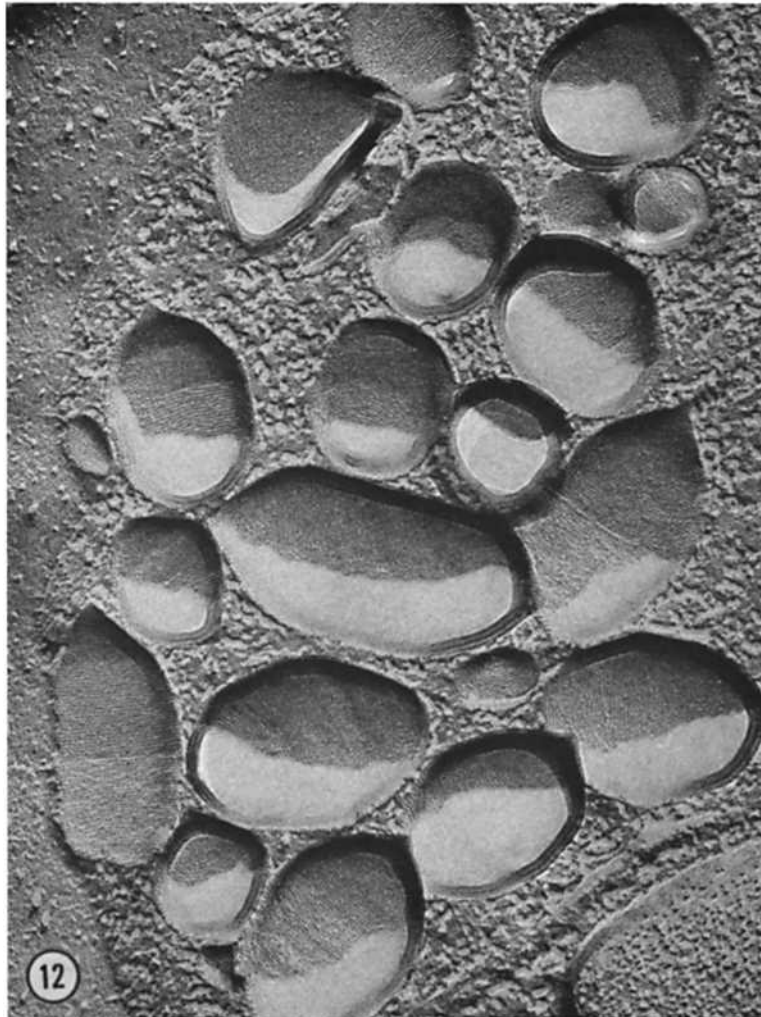


FIGURE 12 Freeze-etched preparation of vacuolated *H. halobium* cell. The gas vacuoles are easily recognized by their shape and surface structure. Shadowing direction is from bottom to top of picture. The inner concave surface of the vacuoles is exposed. $\times 105,000$.

their preparation technique, but the published electron micrograph of sectioned material shows only vesicular structures with a rather thick wall. Therefore, a reinvestigation of this fraction seems to be indicated before any definite conclusions are drawn.

The formation of stable gas vacuoles of such a small size in cells is a challenging problem far from being understood. It is obvious that the surrounding membrane must play a crucial role in the process, and it has not been found in any of the mutants studied so far that do not form gas

vacuoles. This membrane appears to differ considerably from all other cellular membranes that have been described. Since it can now be isolated free from contamination by other membranes, one should be able to gain some insight into its molecular structure and its function.

Halobacteria also offer the opportunity to disaggregate the cell envelope by a relatively mild procedure without the use of enzymes, detergents, extremely alkaline or acid conditions, or high concentrations of urea. However, when we separate the lysed envelope into a colorless fraction

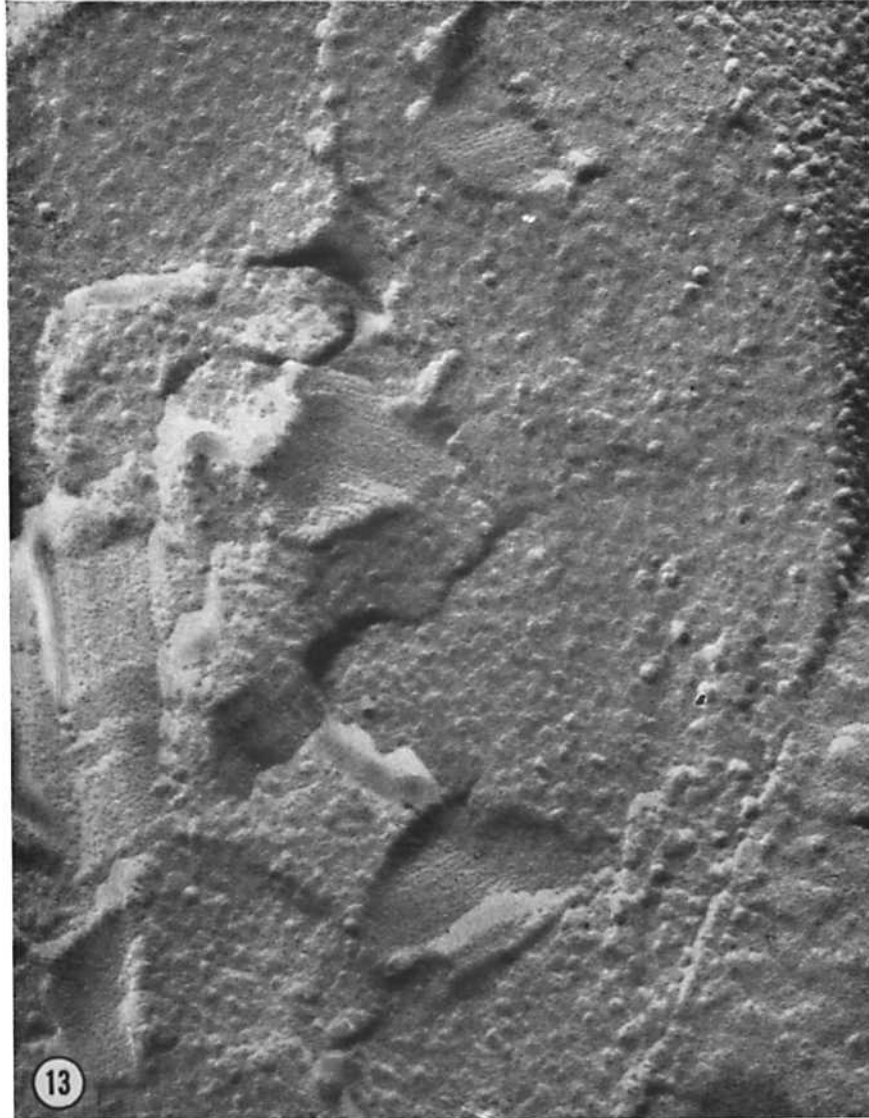


FIGURE 13 Vacuolated *H. halobium* cell that has been exposed to hydrostatic pressure before freeze-etching. The gas vacuoles are collapsed, but the surface structure of the membranes is still visible. $\times 168,900$.

and a red membrane fraction, we cannot be sure that the colorless fraction contains all of the cell wall. Even if conceptually a clear distinction between wall and membrane could be made, which is questionable, we so far have no marker for the whole wall. On the one hand, the rather high lipid to protein ratio of the red membrane fraction and its unit membrane appearance can be used as an argument that probably not too much wall material can be present. The presence

of amino sugars, on the other hand, could indicate some contamination with wall material. Unfortunately, the situation is further complicated by the existence of the purple membranes, the origin of which is still obscure. Nevertheless, the fractions are now obtainable relatively free from cross-contamination so that an investigation of the functional and structural role of their components in the intact envelope appears feasible.

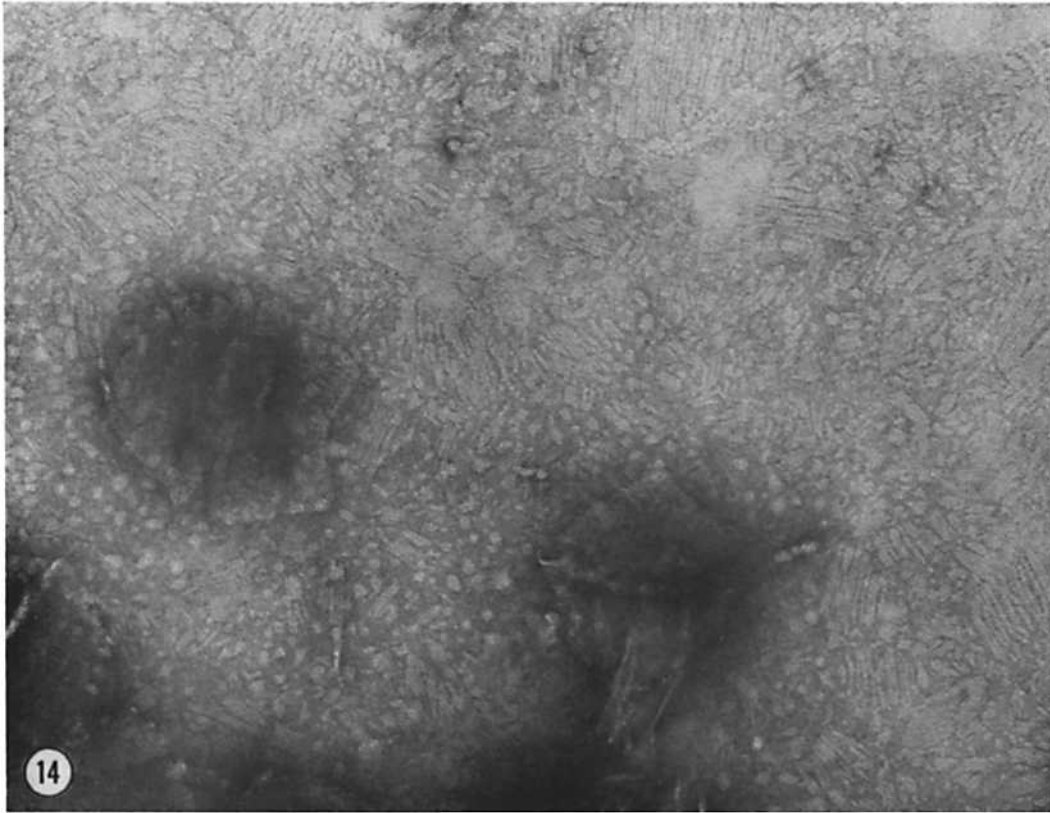


FIGURE 14 Disintegrating gas vacuole (intracytoplasmic) membranes in an old preparation from a sucrose density gradient. The membranes appear to break preferentially along the striae. $\times 123,500$.

ADDENDUM

While the manuscript for this paper was being mailed back and forth between Bochum, Germany and San Francisco, several papers on halobacteria appeared. The papers by H. Larsen, S. Omang, and H. Steensland (1967. *Arch. Mikrobiol.* **59**:197.) and H. Steensland (1967. Abstracts of the Federation of European Biochemical Societies 4th Meeting, Oslo, 49.) essentially confirm our earlier results and are in good agreement with the material presented here. C. W. F. McClare (1967. *Nature*. **216**:766.) reports separation of the envelope preparation into two fractions, one of which apparently corresponds to our purple membranes.

The other seems to contain the cell membrane and cell wall material and probably corresponds to a combination of our colorless supernatant and the red membrane fraction. We have some reservations about McClare's conclusions with respect to differences in the binding of lipid and protein in these fractions and his interpretation of the purple membranes. However, we consider our data insufficient at present to discuss this problem in detail.

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