JOURNAL OF BACTERIOLOGY, June 1973, p. 1058-1067

Vol. 114, No. 3
Printed in U.S.A.

Analysis of *Halobacterium halobium* Gas Vesicles[1]

MARK J. KRANTZ[2] AND CLINTON E. BALLOU
Department of Biochemistry, University of California, Berkeley, California 94270

Received for publication 21 February 1973

Gas vesicles, isolated from lysed *Halobacterium halobium* cells, gave an amino acid analysis which accounted for 78% of the weight, and the balance was mainly salt and water. One percent of tightly bound D-galactose was found, as well as 2% of phosphate that was not released by treatment which promotes β-elimination, by hydrolytic release of the galactose, by carboxymethylation of lysine, or by alkaline phosphatase digestion. Only a trace of lipid was detected, and it appeared to have a polyisoprenoid structure. The vesicles were not solubilized by extremes of pH, by agents such as urea, guanidine hydrochloride, formic acid, and detergents, or by organic solvents. Succinylation and carboxymethylation gave partial dispersion, but the products were heterogeneous and of high molecular weight. The amino acid composition of vesicles was independent of fragment size. No band was obtained by polyacrylamide gel electrophoresis, with neutral, acidic, and alkaline systems, with or without sodium dodecyl sulfate and urea, before or after chemical modification. No amino terminus was detected. Electrofocusing of a vesicle dispersion showed a major component with a pI of 4.0 and an amino acid composition of the whole vesicles, and a minor band with pI 3.4 which had an amino acid composition different from whole vesicles. Vesicle protein was resistant to digestion by Pronase, trypsin, thermolysin, and papain. The precipitin reaction with rabbit antivesicle serum was not inhibited by galactose or inorganic phosphate. Succinylated and carboxymethylated vesicles cross-reacted with antivesicle serum. Cell lysates contained material which reacted with antiserum, but it was heterogeneous and mainly larger than 5 $\times$ 10^6 daltons. Material from nonvacuolated mutants reacted weakly with antiserum, but the amino acid composition of the precipitated antigen was different from that of vesicles and of soluble cross-reacting material from vacuolated cells.

Gas vacuoles, found in certain blue-green algae and bacteria, are small membrane-bound vesicles which are filled with gas (31). The structures observed in the cell are called vacuoles, while the isolated organelles are called vesicles. The vesicle structure is probably maintained by its rigidity rather than by a positive pressure of gases inside it. The vacuoles may function for gas storage, as a float to regulate the availability of light and oxygen, or as a screen against light. *Halobacterium* gas vesicles are slightly elongated with conical ends (10), whereas those in blue-green algae are long cylinders (4). The inner and outer surfaces of the vesicle "membrane" show a series of bands with a spacing of 4 to 5 nm (29).

This report concerns the composition and structure of gas vesicles from *Halobacterium halobium*. Protein, water, and ash accounted for 98% of our vesicle preparations. Some D-galactose and monoesterified phosphate were found, but lipid was absent. The protein had a pI of 4.0, was resistant to proteolytic enzymes, and could not be dissociated into soluble subunits by solvents or chemical modification. No amino terminal was detected. Soluble precursors were not detected in wild-type cells or nonvacuolated mutants by using immunochemical techniques.

MATERIALS AND METHODS

Extreme halophiles require at least 2.5 M NaCl for growth, and most grow best in a medium containing 4 to 5 M salt (15). Reduction in salt concentration leads to cell lysis (27). Most enzymes of the extreme

[1] Taken from the doctoral dissertation of M.J.K.
[2] Present address: Department of Biology. The Johns Hopkins University, Baltimore, Md.

halophiles require high concentrations of salt for maximal activity and are denatured in dilute salt (9, (15). *H. halobium* (Delft strain) was provided by Germaine Cohen-Bazire. Cultures were grown at 37 C on a medium containing 25 g of NaCl, 1 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of KCl, 50 mg of $CaCl_2 \cdot H_2O$, 1 g of yeast extract, and 0.25 g of tryptone in 100 ml of solution. Liquid cultures were grown with slow shaking. Agar plates, prepared from the same medium with the addition of 2% agar, were inoculated with 2 ml of log-phase liquid culture to minimize any lag in initiation of growth and were incubated in closed boxes to prevent drying of the agar. Cells were harvested after 10 to 14 days.

Cells were lysed by adding to each plate 20 ml of 1 mM $MgSO_4$ containing 10 μg of deoxyribonuclease I (bovine pancreas, B grade, Calbiochem) per ml. The lysate was scraped from the agar and allowed to digest for 30 min at 25 C. Pronase (*Streptomyces griseus*, B grade, Calbiochem) was added (100 μg/ml), and the mixture was incubated 18 h at 37 C. NaCl was then added to give a 10% (wt/wt) solution, and the vesicles were collected by flotation, retaining 10% of the liquid phase. Vesicles were washed eight times with 18% NaCl, allowing 24 h for flotation between washes. The final suspension was dialyzed at 4 C against distilled water, and the vesicles (25 to 50 mg/100 plates) were lyophilized for storage. The preparations gave electron micrographs that were similar to those already published for pure vesicles (10, 31).

Residual ribonucleic acid was removed from washed vesicles (1 to 5 mg/ml) by incubation with ribonuclease IA (bovine pancreas, crystallized five times, Sigma) (50 μg) in 1 ml of 0.1 M $NaHCO_3$. After 3 h at 25 C, the digestion products were removed by dialysis.

Density gradient centrifugation was done on a 1.3 to 2.0 M sucrose gradient for 30 h at 115,000 $\times$ *g* (at R_{av}), in a Spinco model L2 ultracentrifuge with an SW-39 swinging-bucket rotor. Gradients were assayed by reading the absorbance at 280 nm.

Samples for amino acid analysis were hydrolyzed in 6 N HCl for 24 to 72 h at 110 C. HCl was evaporated from the opened tube at 40 C, and the product was analyzed on a Beckman amino acid analyzer. Cysteine was determined as cysteic acid (20), and tryptophan was recovered in the presence of 4% thioglycollic acid (17). Serine and threonine values were corrected for destruction during hydrolysis, while those for aliphatic hydrophobic residues were corrected for incomplete hydrolysis.

Lipids were extracted with chloroform-methanol-concentrated HCl (200:100:1) for 48 h. The organic layer was freed of water-soluble materials by chromatography on Sephadex G-25 (24). Silica Gel G (Merck) thin-layer plates were developed in solvent 1 (diisobutyl ketone-acetic acid-ethanol-water [8:5:1:1]) and solvent 2 (chloroform-diethyl ether [9:1]). Lipids were visualized with a phosphate spray (5) or by charring with 50% sulfuric acid. Methanolysis was performed by refluxing lipids in 5% methanolic HCl for 3 h and extracting the lipid into petroleum ether.

Sugar was hydrolyzed from vesicles in 3.3 N H_2SO_4 for 3 h in a sealed ampoule in a steam bath, and the hydrolysate was treated with mixed-bed resin (Bio-Rad, AG 501-X8D). Sugars were detected on paper after chromatography in solvent 3 (butanol-pyridine-water [10:3:3]) by using an alkaline silver nitrate dip (1). Galactose was assayed by the Galactostat system (Worthington) or the phenolsulfuric method (6).

The release of phosphate was followed by a modification of the Bartlett method (3). Color was developed without heating, and standards were assayed in the presence of vesicle protein. Digestions with bacterial alkaline phosphatase (Worthington) and snake venom phosphodiesterase (Sigma) were carried out in 1.0 M NaCl containing 0.1 M $NaHCO_3$ for 2 h at 37 C.

Vesicle protein was assayed for substitution at serine and threonine by incubation in 0.1 N NaOH for 24 h at 25 C (22). The resulting solution was made 0.05 M in tris(hydroxymethyl)aminomethane buffer, pH 9, and 0.1 M in $NaHSO_3$, and then was incubated at room temperature for 48 h. Alternatively, 1.0 M NaOH was used for 24 h at 40 C, followed by bisulfite in the above buffer for 36 h at 40 C. After dialysis, amino acids were determined as usual.

One milligram of vesicles, 100 mg of $NaHCO_3$, and 5 mg of 1-dimethylaminonaphthalene-5-sulfonyl chloride (dansyl chloride) (8) were added to 5 ml of acetone, and the solids were dispersed by sonic oscillation. Reflux was continued for 40 h with two further additions of 5 mg of dansyl chloride. The insoluble product was washed with an acetone-water mixture. After acidification, the pellet was washed with water, and the dansyl-protein was hydrolyzed in 6 N HCl for 18 h at 110 C. Dansyl-amino acids were visualized under ultraviolet light after three-dimensional chromatography on polyamide sheets (Cheng Chin Trading Co. Ltd., Taiwan). The solvents were: solvent 4, formic acid-water (3:197) for 30 min; solvent 5, benzene-glacial acetic acid (9:1) for 60 min; solvent 6, ethyl acetate-glacial acetic acid-methanol (20:1:1) for 20 min.

Vesicles (5 mg) in 0.1 M $NaHCO_3$ were added to 50 mg of fluoro-2,4-dinitrobenzene in an equal volume of ethanol (7). The reaction mixture was shaken vigorously, and three further additions of reagent (50 mg) were made at 12-h intervals. The insoluble dinitrophenyl-protein was washed sequentially with diethyl ether, acidified water, ethanol, ether, ethanol, and finally with water. The product was hydrolyzed in 6 N HCl for 22 h at 110 C, and the dinitrophenyl-amino acids were identified by chromatography on phthalate-impregnated paper in t-amyl alcohol which was saturated with 0.1 M phthalate buffer, pH 6.0.

Succinylation of vesicles was done by refluxing in chloroform for 48 h with a 1,000-fold excess of succinic anhydride and pyridine. After the solvent was removed and the pH adjusted to neutrality in water, the product (called S vesicles) was desalted on a Sephadex G-25 column and lyophilized.

Vesicles were carboxymethylated by stirring with a 50-fold excess of iodoacetate for 48 h at room temperature in 0.1 M phosphate buffer, pH 10. The pH was adjusted to neutrality, and the product (called CM vesicles) was desalted on a Sephadex G-25 column and lyophilized.

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was carried out by the method of Shapiro

et al. (23) and in the acetic acid-urea system of Takayama et al. (28). Gels were stained with Coomassie blue or with 1% amido black in 7% acetic acid and were destained electrophoretically. Electrofocusing was done in an LKB 8100 apparatus at 300 V for 12 h, followed by 600 V for 12 h, and finally at 800 V for 40 h by using 1% carrier ampholyte with a pH range of 3 to 10. A linear sucrose gradient from 0 to 60% was used.

Titrations were done under N_2 by using a Radiometer pH meter 4. Electron microscopy was performed on a Siemens Elmiskop IA apparatus and ultracentrifuge runs were in a Spinco model E analytical ultracentrifuge with either the schlieren or absorption optical system. Mild sonic oscillation to disperse vesicles was done in a Cole-Parmer model 8845-4 ultrasonic cleaner.

Pronase digestions were done in 0.1 M $NaHCO_3$ at 37 C. Pronase, covalently bound to carboxymethyl cellulose (Miles Laboratories), was removed from the reaction by centrifugation and was washed with 2 M pyridine acetate to elute the products. Thermolysin digestions were carried out in 0.1 M tris(hydroxymethyl)aminomethane HCl at 37 C. Trypsin was preincubated in 1 mM HCl, and digestion was carried out in 0.1 M NH_4HCO_3. Papain digestions were done in 0.1 M ammonium formate after activation of the enzyme with mercaptoethanol. The reaction was followed by uptake of alkali at pH 8. Products were analyzed by descending paper chromatography in solvent 7 (butanol-pyridine-acetic acid-water [15:10:3:12]) with visualization by ninhydrin spray.

A log-phase culture, 0.1 ml, was added to 0.9 ml of medium containing 1 mg of 2-aminopurine nitrate (Calbiochem). After 4 days at 37 C, the culture was added to 100 ml of fresh medium and grown for 5 h. Dilutions were spread on agar, and nonvacuolated, translucent red colonies were selected after 10 days. Vacuolated colonies had a pink, opaque appearance. Nonvacuolated colonies occurred with a frequency of 10^{-3} in the mutagenized culture, whereas the spontaneous frequency was 10^{-4}. A highly vacuolated colony, with a white opaque appearance, gave transparent colonies with a frequency of 10^{-5}. All mutant strains were treated with 2-aminopurine and examined for revertants. About 10^7 cells from each were examined, but no revertant was obtained.

New Zealand White rabbits (6-8 lb [about 2.7-3.6 kg]) were injected subcutaneously in two back sites with 4 mg of vesicles dispersed in 1 ml of a 50% emulsion of saline and complete Freund adjuvant. Four weeks later, each rabbit was given a series of three intravenous injections of 1 mg of vesicles in saline each time on alternate days. After resting the animals for 2 months, the intravenous injections were repeated, and serum was obtained by heart puncture after 1 week. Control serum was obtained before the immunization procedure was begun.

Precipitin assay tubes contained 0.1 ml of serum, vesicle suspension, and saline to give a final volume of 0.5 ml. Tubes were stored at 4 C for 48 h and then centrifuged at 2,000 $\times$ g for 20 min. The precipitate was washed twice with 1.0-ml portions of ice-cold saline, and protein was determined by the Folin procedure (16). Values were corrected for the vesicle protein which sedimented with the antigen-antibody complex.

RESULTS

Vesicles formed a single band when centrifuged to equilibrium on a sucrose gradient. No material was found at lower density characteristic of cell membrane fragments (26). Attempted centrifugation in a CsCl gradient caused the material to aggregate.

The composition of vesicles is given in Tables 1 and 2. The amino acid values were compiled from five different preparations, and vesicles isolated without the use of Pronase had the same composition. Cystine was absent, and no cysteic acid was found after performic acid oxidation, although a control analysis of lysozyme gave the expected recovery of cystine as cysteic acid. No destruction of added tryptophan occurred during hydrolysis in the presence of vesicles. Analysis of a lysozyme standard gave a 95.6% recovery as amino acids, whereas vesicles gave a 74.5% recovery as amino acids. Correction for a loss similar to that with lysozyme gave 78% as the amino acid content of vesicles. No amino sugar was detected.

Vesicles dried 18 h under vacuum over P_2O_5 at 98 C showed a weight loss of 11%. Within 5 min in the open balance the weight was regained. Elemental analysis of vesicles gave: C, 41.57; H, 6.55; N, 12.20; ash, 10.2%. Values calculated from the amino acid composition, assuming 78% protein, 12% water, and 10% ash, were: C, 41.10; H, 7.06; N, 12.45.

Vesicles contained less than 1% lipid. About 0.01 μmol of phosphate per mg of vesicles was recovered in the lipid extract. Thin-layer chromatography showed equal amounts of neutral and phosphate-containing lipids. Methanolysis converted the phospholipid to material which ran with the neutral fraction. Chromatography indicated that the neutral fraction contained several components. The infrared spectrum of hydrolyzed total lipid from vesicles was similar to that for the glycerol diether moiety of *H. halobium* lipids (14).

TABLE 1. *Composition of vesicles*

Component	Wt (%)
Amino acids	78.0
Lipids	<1
Galactose	1.5
Phosphate	2.0
Ash	10.2
Water	11

TABLE 2. *Amino acid compositions of different vesicle preparations*

Amino acid	Intact vesicles (mol/100 mol)	Succinylated vesicles		Carboxymethylated vesicles[c]	Isoelectric focusing fractions	
		Major fraction[a]	Minor fraction[b]		Major fraction[d]	Minor fraction[e]
Lysine	3.15	3.06-3.26	8.45	0.27-0.39	5.74	3.68
Histidine	1.52	1.58-1.65	1.56	0.54-0.66	3.40	1.16
Arginine	4.32	4.30-4.49	3.77	4.33-4.59	5.83	1.94
Aspartic acid	8.08	8.25-8.60	10.20	8.52-8.95	7.16	7.05
Threonine	4.42	4.76-4.95	6.25	4.73-4.86	3.53	3.96
Serine	6.18	5.98-6.04	7.63	6.47-6.68	7.58	10.40
Glutamic acid	14.11	14.30-14.54	12.75	15.10-15.40	12.32	29.80
Proline	1.66	[f]	[f]	[f]	[f]	[f]
Glycine	6.26	5.80-5.96	15.15	5.72-6.20	7.46	9.16
Alanine	13.35	14.30-14.52	12.95	13.60-14.02	13.40	10.44
Cystine	0.0	[g]	[g]	[g]	[g]	[g]
Valine	16.40	15.92-16.26	6.52	17.85-18.60	13.20	9.40
Methionine	0.42	[f]	[f]	[f]	[f]	
Isoleucine	5.94	6.60-6.77	4.32	6.42-6.98	7.07	4.05
Leucine	10.64	11.38-11.62	7.72	11.68-11.92	10.80	6.42
Tyrosine	1.50	1.19-1.32	1.10	1.10-1.41	1.23	1.13
Phenylalanine	1.58	1.23-1.64	1.56	1.50-1.85	1.28	1.46
Tryptophan	0.44	[g]	[g]	[g]	[g]	[g]
Di-ε-CM-lysine	[h]	[h]	[h]	2.2-2.5	[h]	[h]

[a] The range of analyses in fractions 19-21, 26-28, and 34-36 in Fig. 1. Moles/100 mol.
[b] The analysis of fractions 53-55 in Fig. 1. Moles/100 mol.
[c] The range of analyses in fractions 19-20, 29-30, and 34-35 in Fig. 1. Moles/100 mol.
[d] The analysis of fractions 30-33 in Fig. 2. Moles/100 mol.
[e] The analysis of fractions 38-42 in Fig. 2. Moles/100 mol.
[f] Insufficient for determination.
[g] Not determined.
[h] Not applicable.

Although a nonspecific nuclease was used in the preparation of vesicles, contamination with ribonucleic acid was consistently observed. Ribose, but not deoxyribose, was obtained on acid hydrolysis of the contaminated vesicles. This contaminant was removed by a second digestion with the same enzyme. To determine whether the nucleic acid existed in a protected state, purified intact vesicles were treated with ribonuclease in 0.5 M NaCl, under which conditions they were stable. The nucleic acid was converted entirely to a dialyzable form.

Some of the phosphate in vesicle preparations was attributed to nucleic acid and lipid, but, after these had been accounted for or removed, about 0.2 μmol of phosphate per mg of protein was found. This phosphate was not released by 1 N NaOH at 100 C for 30 min, or by 1 N H_2SO_4 at 100 C for 2 h. After treatment of vesicles with alkali followed by bisulfite, no sulfonic acid derivative was recovered from the protein. Inorganic phosphate was not released from vesicles by alkaline phosphomonoesterase, alone or with snake venom phosphodiesterase. Preincubation in 0.1 N NaOH at 100 C for 15 min did not render the phosphate susceptible to alkaline phosphatase. Titration of vesicles indicated that the phosphate was in the monoester form.

Acid hydrolysis of vesicles yielded D-galactose as the main sugar. Ribose was also observed but was absent after nuclease treatment, while glucose and mannose sometimes occurred in trace amounts. Lipid extracts did not contain carbohydrate, and all of the galactose was found in the insoluble material which appeared at the water-chloroform interface. The maximal release of galactose by 3.3 N H_2SO_4 was obtained after 2 h at 100 C and amounted to 15 μg of sugar per mg of vesicle protein.

Reaction of vesicles with dansyl chloride in water yielded only dansyl-alanine. Reaction in acetone under reflux gave dansyl-alanine and a trace of didansyl-tyrosine. Dinitrophenyl vesicles yielded a trace of the alanine derivative and showed a complete loss of tyrosine and about 60% loss of lysine, indicating that reaction had occurred. Less than one amino terminal group of any kind was found per 100,000 daltons of protein.

Lyophilized vesicles were suspended in water by sonic oscillation. Examination in the electron microscope showed collapsed vesicles in a

nearly intact state and numerous fragments of various sizes. Ultrasonic oscillation reduced the particle size. Lowering the pH to 2 had no effect on vesicles, which could be detected in the electron microscope, but raising the pH to 11 seemed to reduce the number of identifiable fragments.

Column chromatography of suspended vesicles led to losses because material adhered to the column. Chromatography on Sepharose 2B of vesicles suspended by sonic oscillation showed that the molecular weight was still greater than 25 million, since the material was recovered in the void volume. Incubation of vesicles at pH 10.5 for 2 days caused some dispersion. Chromatography on Sepharose 2B indicated that the material had a reduced molecular weight, but it still appeared in the void volume of a Bio Gel A-5m column.

Treatment of vesicles with 0.1 N NaOH for 30 min at 100 C caused a significant clearing of the suspension. Gel filtration on Bio Gel A-5m indicated that some low-molecular-weight fragments were released. These fragments showed a high content of glycine, threonine, and serine, and a low content of aliphatic amino acids. Chromatography of the large material in the presence of 0.1% sodium dodecyl sulfate gave no change in the fractionation pattern.

Vesicles aggregated below the isoelectric point at pH 4.0. Treatment with 0.01 N HCl for 20 h at 110 C released aspartic acid and traces of other amino acids (21). Examination of the residue in the electron microscope, after treatment with 0.005 N HCl for 18 h at 110 C, showed numerous striated fragments which appeared to be broken vesicles. Vesicles appeared to dissolve in 12 N HCl, but the addition of an equal volume of water caused an immediate precipitation. While anhydrous formic acid (97–100%) also dissolved vesicles, the addition of 10% water caused precipitation.

Methanol, ethanol, *n*-propanol, and *n*-butanol were ineffective in solubilizing vesicles, either alone or in combination with water, as was 4 M urea saturated with *n*-butanol. Formamide, dimethylformamide, and dimethylsulfoxide did not solubilize vesicles either at 25 C or at 100 C, while acetic acid, pyridine, and phenol-acetic acid-water (2:1:1), Triton X-100, cetyltrimethylammonium bromide, and deoxycholate had no observable effect.

Succinylation of vesicles occurred in chloroform even though the protein was not soluble. While heat-disrupted vesicles appeared in the void volume on Sepharose 2B, succinylated vesicles were included and ran near the holdup volume on this gel.

Carboxymethylation of vesicles proceeded well in water at pH 10.5, and the product showed a 90% loss of lysine. About 80% of the lysine was recovered as di-ε-carboxymethyl-lysine, and none of the monocarboxymethyl derivative was found. The pattern on Sepharose 2B was similar to that of succinylated vesicles. Incubation of vesicles at pH 10.5 without iodoacetate also caused breakdown, but the product was much larger in size.

Vesicles were centrifuged in the presence of potential solubilizing agents such as 8 M urea, 8 M guanidine-hydrochloride at neutral pH and at pH 11.5, and 0.5% sodium dodecyl sulfate at pH 7.2. In all cases, the material settled at the bottom of the cell, and no peak was seen during the sedimentation velocity run. A portion of each sample was heated for 5 min at 100 C before the run, but the same result was obtained. Succinylated or carboxymethylated vesicles also sedimented without giving discrete peaks. Although formic acid seemed to give a clear solution of vesicles, no peak was observed in the ultracentrifuge and the material packed to the bottom of the cell. Sufficient time was allowed in all cases to resolve any low-molecular-weight components from the meniscus.

Electrophoresis (23) did not give any bands for vesicles, S vesicles, or CM vesicles, although myosin, β-galactosidase, and lysozyme were run successfully. Preincubation of vesicles in 3% sodium dodecyl sulfate for 1 min, or changing the pH of the system from 7 to 10, gave the same result. Gel electrophoresis was also performed by using 7.5% acrylamide, 35% acetic acid, and 5 M urea (28). Again no band was observed.

Succinylated and carboxymethylated vesicles were included on Bio Gel A-5m, while collapsed vesicles, or vesicles incubated at pH 10.5 for 48 h at 25 C ran at the void volume (Fig. 1). The amino acid compositions (Table 2) were constant over the fractionation range, except for the material of low molecular weight. Lysine and carboxymethyl-lysine content were about the same for all CM vesicle fractions.

Samples of S vesicles and CM vesicles were preincubated in 0.1% sodium dodecyl sulfate at 100 C for 3 min and run on Bio Gel A-5m. The patterns were identical with those obtained in the absence of detergent. Attempts to fractionate vesicles, S vesicles, and CM vesicles on diethylaminoethyl-Sephadex A-50 were unsuccessful.

The electrofocusing pattern of vesicles is shown in Fig. 2. The isoelectric point of the main peak was 4.1, and that of the major shoulder was 3.4. The main peak had an amino acid composition similar to vesicles, but the

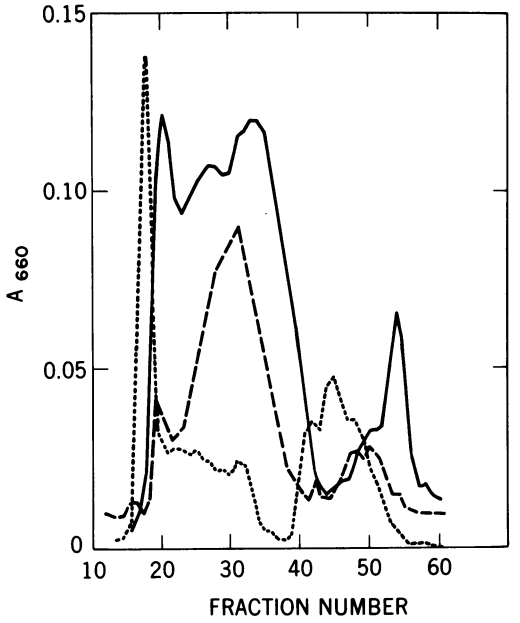

FIG. 1. *Fractionation of vesicle preparations on Bio Gel A-5m. Symbols: S vesicles (solid line), CM vesicles (dashed line), alkali-treated vesicles (dotted line). Untreated vesicles were recovered in the void volume.*

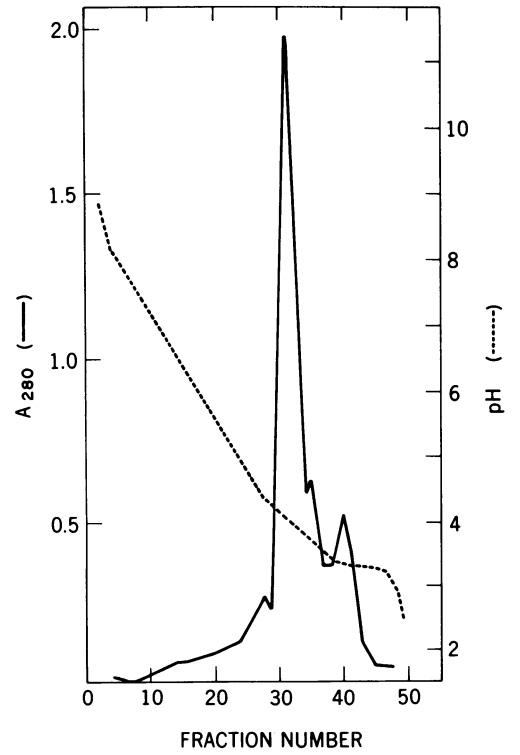

FIG. 2. *Pattern obtained by isoelectric focusing of intact vesicles.*

smaller peak differed (Table 2). The phosphate content was about the same in both (0.15 μmol/mg).

Vesicles were not attacked by trypsin, while lysozyme was readily digested under the same conditions. Paper chromatography of the reaction products formed at high enzyme levels revealed only autodigestion fragments.

Autodigestion fragments were also observed after incubation of vesicles with Pronase. When Pronase that was covalently linked to carboxymethyl cellulose was used to minimize autodigestion, no fragment was obtained even though the enzyme was active against bovine serum albumin. Vesicles and CM vesicles were incubated with Pronase for 2 days with three additions of enzyme. The mixture was boiled for several minutes before each addition of enzyme in an attempt to denature the substrate. The entire mixture was passed over a Bio Gel A-5m column, but no large fragment was found.

Thermolysin incubations were carried out with alternate boiling and addition of enzyme. No fragment was detected, either by paper chromatography or gel filtration on Bio Gel A-5m. Digestion of vesicles with papain did not release any peptides detectable by paper chromatography.

Pronase and thermolysin were also assayed for activity by using a pH stat at pH 8.0. A very slow rate of autodigestion was observed in the absence of substrate. When vesicles were used as the substrate, alkali was consumed at an unchanged rate.

Vesicles incubated at pH 10.5 for 2 days gave a precipitin curve identical to that of intact vesicles. Treatment with stronger alkali, probably with some hydrolysis, gave material with the precipitin curve shown in Fig. 3. Compared to collapsed vesicles, CM vesicles and S vesicles gave a decreased, but substantial, reaction with antibodies (Fig. 3). Precipitation of vesicles or CM vesicles was not inhibited by galactose or inorganic phosphate.

The 2,000 × *g* supernatant fluid from lysed cells gave the precipitin curves shown in Fig. 4, whereas the pellet contained little cross-reacting material. Cells in late stationary phase contained more antibody-specific material than those in early stationary phase. Antiserum adsorbed with vesicles gave no reaction with cell lysates. Antiserum adsorbed with cell lysates reacted with vesicles as shown in Fig. 5. Extracts of cells in late stationary phase inhibited the precipitin reaction to a greater extent than did extracts from early stationary-phase cells. Nonimmune serum failed to react with cell lysates.

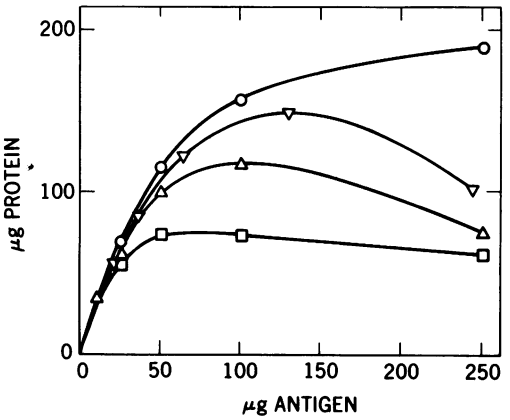

FIG. 3. *Precipitin curves of different vesicle preparations with rabbit antiserum prepared against intact vesicles. Symbols:* O, *untreated vesicles;* ▽, *vesicles treated with alkali;* □, *CM vesicles;* △, *S vesicles.*

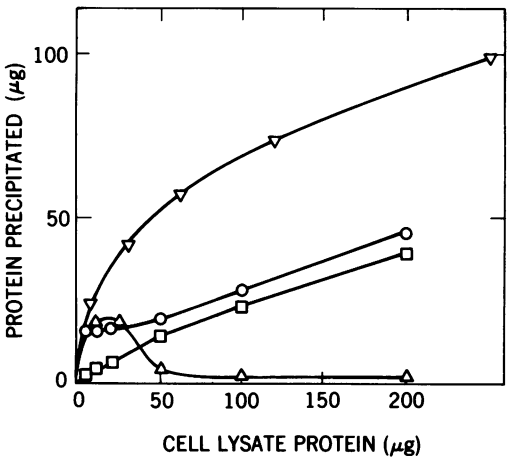

FIG. 4. *Cross-reaction of rabbit antivesicle serum with cell lysates from early* (O) *and late* (▽) *stationary-phase wild-type cells and with the cell lysate from non-vacuolated mutant cells* (△). *The reaction of early stationary-phase wild-type cell lysate with antiserum which had been preadsorbed with mutant cell lysate is also shown* (□).

Cell lysates were fractionated by differential centrifugation at 10,000 × *g*, 35,000 × *g*, and 100,000 × *g* for 1 h each. All fractions contained some reactivity toward the antiserum, with about 35% remaining in the 100,000 × *g* supernatant fluid. All antibody-specific material from cell lysates appeared in the void volume on a Sephadex G-75 column, whereas on Bio Gel A-5m most of the activity was found in the void volume, but some was distributed through all fractions except those of lowest molecular weight.

The cell lysate, from a mutant strain which had lost the ability to form vacuoles, gave very little precipitate which redissolved at higher levels of antigen (Fig. 4). The precipitin curves were identical for cells in log phase and late stationary phase. The reaction with mutant cell lysate was eliminated by adsorption of the antiserum with vesicles or with cell lysate of wild-type cells.

Antiserum which was adsorbed with mutant cell lysate reacted more strongly with the major component from electrofocused vesicles than with the minor component. Thus, fraction 2 appeared to be enriched for the antigen component in vacuole-free mutants.

The precipitin reaction of mutant cell lysate was not affected by predigestion with Pronase, and this property of Pronase-resistance was used to purify a small amount of the specific antigen. Mutant cell lysate was incubated with antiserum and the precipitate was collected by centrifugation. After being washed with saline, the complex was digested with Pronase to destroy the antibody. The digest was passed over a Bio Gel A-5m column, and the void volume fractions were pooled for amino acid analysis. The serum was recovered after reaction with the mutant antigen and was used to isolate antigen from a wild-type cell lysate. After reaction with antibody, the antigen was recovered by Pronase digestion and gel filtration as before. A sample of vesicles was also reacted with antiserum and recovered in the same manner. Vesicle membranes recovered from the antibody complex had a composition similar to unreacted vesicles (Table 3). The component recovered from lysates of wild-type cells was also similar to vesicles, but the cross-reacting antigen from mutant cells was different.

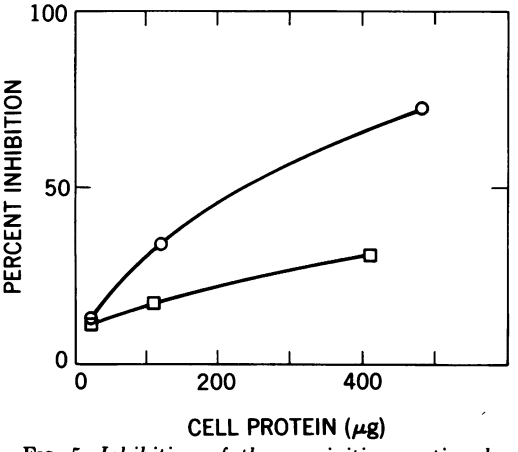

FIG. 5. *Inhibition of the precipitin reaction between vesicles and antivesicle serum by preadsorption of the antiserum with cell lysates from log-phase* (□) *and late stationary-phase* (O) *wild-type cells.*

TABLE 3. *Amino acid composition of vesicle antigens recovered from the antigen-antibody complex by digestion with Pronase*

Amino acid	Vesicle antigen (mol/100 mol)	Wild-type lysate antigen (mol/100 mol)	Mutant lysate antigen (mol/100 mol)
Lysine	3.42	3.57	3.54
Histidine	1.70	1.35	0.73
Arginine	4.50	4.72	3.75
Aspartic acid	8.62	8.85	12.20
Threonine	4.66	5.73	7.94
Serine	6.87	6.93	6.52
Glutamic acid	12.00	10.92	8.10
Glycine	6.12	7.72	14.50
Proline	[a]	[a]	[a]
Alanine	13.52	13.18	13.02
Valine	17.14	17.75	7.85
Methionine	[a]	[a]	[a]
Isoleucine	6.83	5.47	4.27
Leucine	10.62	9.28	8.96
Tyrosine	1.76	2.24	3.81
Phenylalanine	2.28	2.24	4.83
Glucosamine[b]	[c]	7	30
Galactosamine[b]	[c]	6	24

[a] Insufficient for determination.
[b] Moles per 100 mol of amino acid.
[c] Not present.

DISCUSSION

Gas vesicles of *H. halobium* are composed entirely of protein, which differs considerably in its properties from other proteins. It is more hydrophobic, more resistant to solvents, denaturing agents, and chemical treatments, and is unusually resistant to solubilization and to attack by enzymes. Unlike other membranes, vesicles contain no lipid.

Valine, leucine, and isoleucine make up 33% of the residues of gas vesicles, whereas the value for vesicles from blue-green algae is 30% (11). Stoeckenius and Kunau reported 20% for their preparation from *Halobacterium* (26), but they isolated collapsed vesicles by sucrose density gradient centrifugation and found an amino acid composition different from that obtained in this study. Jones and Jost (11) reported that vesicles isolated from blue-green algae by this technique were highly contaminated. Several membrane proteins have values of about 20% (18). Thus, our *Halobacterium* vesicle protein is more hydrophobic than other membrane proteins but is similar to a membrane-bound enzyme from *Staphylococcus aureus* which has over 50% hydrophic amino acids, and 38% of valine, leucine, and isoleucine (19).

Gel electrophoresis of membranes from two strains of *Anabaena flosaquae* gave similar patterns, but a protein band of molecular weight 22,000 was seen only in the vacuolated strain (25). Apparently soluble subunits with a molecular weight of 14,000 were obtained from *Microcystis aeruginosa* vesicles (11). Although phenol-acetic acid-water dissolved the *Microcystis* vesicles, it had no effect on the vesicles in this study. Succinylation and carboxymethylation led to partial solubilization of vesicles, giving polydisperse products. All other treatments were ineffective, and heterogeneous products of high molecular weight, as assessed by gel filtration, were obtained. Amino acid compositions of the different fractions were similar, indicating that no one protein had been selectively solubilized.

CM vesicles and S vesicles, heated in sodium dodecyl sulfate at 100 C and subjected to gel filtration, electrophoresis, or centrifugation, all in the presence of detergent, gave patterns identical with those observed before treatment. Covalent cross-links between protein molecules could account for the apparent aggregation, but disulfide bridges could not be involved because cysteine was absent. No unusual or modified amino acid was detected, but it is possible that the few lysine residues which did not react with iodoacetate or fluoro-2,4-dinitrobenzene were involved in the cross-linking of subunits.

The resistance of vesicle protein to digestion by proteolytic enzymes is another indication of its unusual structure. Incubation of the cell lysate with Pronase during vesicle isolation did not affect the amino acid composition of vesicles and did not expose reactive amino groups.

The nature of the association of D-galactose with vesicles is unclear. The amount of this sugar was constant in all preparations, and the rate of its release on acid hydrolysis indicated that the sugar was tightly attached. The trace of lipid found in vesicle preparations, which was mainly polyisoprenoid in structure (12, 14), was probably a contaminant. Jost and Matile (13) found lipid in *Oscillatoria rubescens* vesicles, but their preparation may have been misidentified (25).

The phosphate of nucleic acid-free vesicles was not removed by electrofocusing or gel filtration, even after carboxymethylation. The rate of hydrolysis indicated that it was tightly bound. The resistance to alkaline-catalyzed elimination rules out a linkage to protein through serine or threonine, while acid hydrolysis released only 15% of the phosphate. Titration of vesicles suggested that most of the phosphate was in the monoester form. Jones and Jost reported that the vesicles from a blue-green algae lacked phosphate (11) and that the protein had a pI of 7 and a ratio of basic to acidic residues of 0.57.

The pI indiated that most of the acidic residues were in the amide form. For *Halobacterium* vesicles we found a ratio of basic to acidic residues of 0.41 and a pI of 4.0. The amide content was not determined, but the low pI may reflect the phosphate content.

The origin of vesicles is uncertain, and one suggestion is that they could arise by the self-assembly of a viral coat protein produced by the gene of a defective virus (25). This interpretation would explain the high frequency of spontaneous and induced loss of vacuolation and the tendency of vesicles to bind nucleic acid. That a second nuclease digestion removed the ribonucleic acid from purified intact vesicles suggests that the nucleic acid was bound to the outside of vesicles. However, an amber mutation in the maturation protein of bacteriophage R17 has been described (2) which leaves the coat protein intact but renders the phage ribonucleic acid susceptible to ribonuclease digestion.

Antivesicle serum was readily obtained by subcutaneous injection of rabbits. Neither galactose nor phosphate, both of which are present in vesicles, inhibited the vesicle-antibody reaction. Material in cell lysates cross-reacted with antivesicle serum, but it was polydisperse and had a high molecular weight. This material may have been vesicle fragments or vesicles in early stages of assembly. Waaland and Branton (30) have shown that, from the earliest stages of development, the vesicles in blue-green algae have closed conical ends, and they concluded that new units were added at a growing point in the center of the vesicle. Such a unit might be of low molecular weight and soluble, but we observed no antigen of this type.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grants AM 884 and TI-GM31 from the National Institute of Allergy and Infectious Diseases and the National Institute of General Medical Sciences, respectively, and by National Science Foundation grant GB-19199.

LITERATURE CITED

1. Anet, E. F., and T. M. Reynolds. 1954. Isolation of mucic acid from fruits. Nature (London) **174**:930.
2. Agetsinger, J. E., and G. N. Gussin. 1966. Intact ribonucleic acid from defective particles of bacteriophage R17. J. Mol. Biol. **21**:421-434.
3. Bartlett, G. R. 1959. Phosphorous assay in column chromatography. J. Biol. Chem. **234**:466-468.
4. Bowen, C. C., and T. E. Jensen. 1965. Blue-green algae: fine structure of the gas vacuoles. Science **147**:1460-1462.
5. Dittmer, J. C., and R. J. Lester. 1964. A simple, specific spray for the detection of phospholipids on thin-layer chromatograms. J. Lipid Res. **5**:126-127.
6. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350-356.
7. Fraenkel-Conrat, H., J. I. Harris, and A. L. Levy. 1955. Recent developments in techniques for terminal and sequence studies in peptides and proteins, p. 359-425. *In* D. Glick (ed.), Methods of biochemical analysis, vol. 2. Interscience Publishers, New York.
8. Gray, W. R. 1967. Sequential degradation plus dansylation, p. 469-475. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 11. Academic Press Inc., New York.
9. Holmes, P. K., I. E. D. Dundas, and H. O. Halvorson. 1965. Halophilic enzymes in cell free extracts of *Halobacterium salinarium*. J. Bacteriol. **90**:1159-1160.
10. Houwink, A. L. 1956. Flagella, gas vacuoles and cell wall structure in *Halobacterium halobium*; an electron microscopic study. J. Gen. Microbiol. **15**:146-150.
11. Jones, D. D., and M. Jost. 1970. Isolation and chemical characterization of gas-vacuole membranes from *Microcystis aeruginosa* Kuetz. emend. Elenkin. Arch. Mikrobiol. **70**:43-64.
12. Joo, C. N., and M. Kates. 1969. Synthesis of the naturally occurring phytanyl diether analogs of phosphatidyl glycerophosphate and phosphatidyl glycerol. Biochim. Biophys. Acta **176**:278-297.
13. Jost, M., and P. Matile. 1966. Zur Characteriisierung der Gasvacuolen der Blaualga *Oscillatoria rubescens*. Arch. Mikrobiol. **53**:50-58.
14. Kates, M., B. Palameta, C. N. Joo, D. J. Kushner, and N. E. Gibbons. 1966. Aliphatic diether analogs of glyceride derived lipids. IV. The occurrence of di-O-dihydrophytyeglycerol ether containing lipids in extremely halophilic bacteria. Biochemistry **5**:4092-4099.
15. Larsen, H. 1962. Halophilism, p. 297-342. *In* I. C. Gunsalus and R. Y. Stanier (ed.), The bacteria, vol. 4. Academic Press Inc., New York.
16. Lowry, O. H., N. H. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
17. Matsubara, H., and R. M. Sasaki. 1969. High recovery of tryptophan from acid hydrolysates of protein. Biochem. Biophys. Res. Commun. **35**:175-181.
18. Mazia, D., and A. Ruby. 1968. Dissolution of erythrocyte membranes in water and comparison of the membrane protein with other structural proteins. Proc. Nat. Acad. Sci. U.S.A. **61**:1005-1012.
19. Sandermann, H., and J. L. Strominger. 1971. C_{55}-isoprenoid alcohol phosphokinase: An extremely hydrophobic protein from the bacterial membrane. Proc. Nat. Acad. Sci. U.S.A. **68**:2441-2443.
20. Schram, E., S. Moore, and E. J. Bigwood. 1954. Chromatographic determination of cystine as cysteic acid. Biochem. J. **57**:33-37.
21. Schultz, J. 1967. Cleavage at aspartic acid, p. 255-263. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 11. Academic Press Inc., New York.
22. Sentandreu, R., and D. H. Northcote. 1968. The structure of a glycopeptide isolated from the yeast cell wall. Biochem. J. **109**:419-432.
23. Shapiro, A. L., E. Vinuela, and J. V. Maizel. 1967. Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. Biochem. Biophys. Res. Commun. **28**:815-820.
24. Siakotos, A. N., and G. Rouser. 1965. Analytical separation of nonlipid water soluble substances and gangliosides from other lipids by dextran gel column chromatography. J. Amer. Oil Chem. Soc. **42**:913-919.
25. Smith, R. V., A. Peat, and C. J. Bailey. 1969. The isolation and characterization of gas-cylinder mem-

branes and α-granules from *Anabaena flos-aquae* D124. Arch. Mikrobiol. **65**:87–97.
26. Stoeckenius, W., and W. H. Kunau. 1968. Further characterization of particulate fractions from lysed cell envelopes of *Halobacterium halobium* and isolation of gas vacuole membranes. J. Cell Biol. **38**:337–357.
27. Stoeckenius, W., and R. Rowen. 1967. A morphological study of *Halobacterium halobium* and its lysis in media of low salt concentration. J. Cell Biol. **34**:365–393.
28. Takayama, K., D. H. MacLennan, A. Tzagaloff, and C. D. Stones. 1966. Studies on the electron transfer system. LXVII. Polyacrylamide gel electrophoresis of the mitochondrial electron transfer complexes. Arch. Biochem. Biophys. **114**:223–230.
29. Waaland, J. R., and D. Branton. 1968. Ultrastructure of gas vacuole membranes in blue-green algae and halophilic bacteria. Amer. J. Bot. **55**:709.
30. Waaland, J. R., and D. Branton. 1969. Gas vacuole development in a blue-green algae. Science **163**:1339–1341.
31. Walsby, A. E. 1972. Structure and function of gas vacuoles. Bacteriol. Rev. **36**:1–32.