ISOLATION AND CHARACTERIZATION OF GAS-VACUOLE MEMBRANES FROM MICROCYSTIS AERUGINOSA KUETZ. EMEND ELENKIN

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ABSTRACT

ISOLATION AND CHARACTERIZATION OF GAS-VACUOLE MEMBRANES FROM MICROCYSTIS AERUGINOSA KUETZ. EMEND ELENKIN

By

Daniel David Jones

The gas vacuoles of the unicellular, blue-green alga, Microcystis aeruginosa Kuetz. emend Elenkin, were examined. A method involving penicillin treatment was developed to lyse the cells and release the pressure-sensitive gas vacuoles intact. The gas vacuoles were purified by liquid-polymer partitioning or by macromolecular sieving and centrifugation. The degree of purification of the gas vacuoles was followed by observation in the electron microscope and by the use of C^{14}-labeled vacuolated and non-vacuolated strains of M. aeruginosa. The gas vacuole membrane is composed only of protein consisting of 10% basic, 18% acidic and 52% non-polar amino acids.

Several methods and reagents were used in efforts to solubilize the protein. It is insoluble in sodium dodecyl sulfate-urea solutions. Strongly protic solvents such as formic acid were the only reagents in which appreciable solubilization of the membrane protein occurred. End-group

analyses, tryptic digests, and gel electrophoresis at acidic pHs indicate that the protein is one species. Infrared spectroscopy reveals that the membrane protein has substantial amounts in both the alpha-helix or random coil conformation and in the beta-conformation.

Local conformational changes of the gas-vacuole membrane were investigated with spin and fluorescent labeling techniques. Studies on the temperature dependence of the electron paramagnetic resonance (EPR) spectra of spin-labeled intact vacuoles demonstrate a sharply defined transition temperature of 39°. Below that temperature the conformational change of the vacuolar membrane remains thermally reversible; above that temperature irreversible processes occur. The rate of tumbling of the spin label attached to the vacuolar surface increases as the temperature increases, indicating that in the membrane new modes of vibrations are thermally induced in the protein which narrow the line width of EPR spectra.

A suspension of the intact gas vacuoles has a milky appearance which clears upon application of hydrostatic pressure; concomitantly the EPR spectrum of the spin-labeled membrane becomes more symmetric and the area under the middle hyperfine line is reduced by approximately 25% compared to intact vacuoles. This suggests a rearrangement of the protein folding of the membrane such that the paramagnetic label is less restricted in its freedom of motion relative to the

protein surface. Ultrastructural studies show that the vacuoles collapse under pressure and consist of membranous sheets, ribs, and granules.

Intact vacuoles labeled with anilinonaphthalene sulfonate show a weak fluorescence while vacuoles subjected to pressure fluoresce strongly.

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ORGANIZATION OF THESIS

For the convenience of the reader the three parts of this thesis are covered individually, and each part is presented as an independent entity in the format of a scientific paper, with its own INTRODUCTION, MATERIALS AND METHODS, RESULTS, DISCUSSION and SUMMARY sections. However, the references for all three parts are combined at the end of the thesis.

Part One, excluding some material, has already been published under the title "Isolation and Chemical Characterization of Gas-vacuole Membranes from Microcystis aeruginosa Kuetz. emend Elenkin" by D. D. Jones and M. Jost, Arch. Mikrobiol., 70, 43 (1970).

A report of the results of Part Three has been published in Archiv. Biochem. Biophys., 135, 296 (1969) by D. D. Jones, A. Haug, M. Jost and D. R. Graber under the title "Ultrastructural and Conformational Changes in Gas-vacuole Membranes Isolated from Microcystis aeruginosa".

Part Two is also being prepared for publication.

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INTRODUCTION AND LITERATURE REVIEW

The purpose of the research described in this thesis was to elucidate the chemical composition of the gas-vacuole membranes in the blue-green alga, Microcystis aeruginosa, and to study the organization of these membranes. A discussion and review of the classical observations will first be presented before stating the objectives of this investigation in detail.

Waterblooms and the Discovery of Gas Vacuoles. The ability to float shown by certain planktonic blue-green algae (Cyanophyta) is vividly displayed in the form of dense mats called "waterblooms". Correlations between the development of these myxophycean blooms and the hydrologic conditions of the water in which the blooms occur have been difficult to determine. The blooms, however, exemplify profuse biological growth and often become a nuisance. The algal masses can make fresh water unsafe for drinking, kill fish by exhausting the oxygen supply, or even kill mammals, birds and fish by the production of toxins (23,31,60,102,103).

Strodtman (1895) first realized that the ability of the algae to float was related to the highly refractive bodies within the cells (111). Klebahn identified these unique

structures as being responsible for the buoyancy of the algae and called them gas vacuoles (54). The criteria for designating the organelles as gas vacuoles are outlined below.

Other than in certain Cyanophyta, the gas-filled inclusions have been found in only a few bacteria (e.g., Halobacterium and Pelodictyon species) (24,109).

Gaseous Content. The original experiments and arguments of Klebahn left little doubt that the vacuoles contain gas (54-57). The essential conclusions reached by Klebahn were that: (a) the contents of the vacuoles had a very low refractive index; (b) upon pressurization, the vacuoles were rapidly destroyed with an accompanying decrease in volume of the algae; (c) more gas could be extracted from algae with intact vacuoles than from algae in which the vacuoles had been destroyed by pressure and equilibrated with the surrounding medium; (d) and the volume occupied by the vacuoles was sufficient to enable the algae to float only if the vacuoles contained a gas. Molisch questioned a gaseous content because the gas vacuoles did not disappear under sustained vacuum (81). Klebahn postulated that the membrane was rigid and impermeable, however, to account for this phenomenon (55).

Recently, investigation by interference microscopy has shown that the refractive index of the vacuoles corresponds to their contents being a gas (28). Gas-filled spaces in the cytoplasm can be demonstrated directly by freeze fracturing (48).

Klebahn tried to determine the gaseous composition of the organelles. The slow absorption of vacuolar gas by alkalies or alkaline pyrogallol argued against the presence of carbon dioxide or oxygen. Klebahn also could find no flammable gases (54-57). Larsen et al. ruled out oxygen in the gas vacuoles of Halobacteria since luminous bacteria did not react to the vacuolar content (66). The gas usually favored was nitrogen, although in all cases it was found in very sparse amounts or suggested on the basis of negative evidence (54-57,66,123).

Recently, Walsby tried to determine by mass spectroscopy the gas released upon destruction of the gas vacuoles in pre-evacuated algal suspensions (122). He was able to detect only trace amounts of gas. Similar experiments performed in a modified Warburg respirometer confirmed the mass spectroscopy results. The conclusion was reached that the amount of gas present in the vacuoles was dependent on the gaseous pressure under which the material had been. Furthermore, the vacuoles remained inflated when pressurized slowly in contrast to being deflated upon rapid pressurization (122). Thus the vacuoles must be permeable to gases, and hence the gaseous composition and pressure within the gas vacuoles will simply reflect that of the gas in the environment.

The postulate of Klebahn of impermeability to account for the stability of the vacuoles under sustained vacuum is

not necessary since the turgor pressure of the cell alone will exceed one atmosphere, the pressure of the vacuolar gas (122).

Function. The role of the gas vacuoles is generally accepted as that of providing the algae with buoyancy. Assuming a gaseous content, Klebahn calculated that only 0.7% of the cell volume must be occupied by the vacuoles to enable the algae, Gleotrichia echinulata, to float. He concluded that the volume occupied by the gas vacuoles was sufficient to account for the buoyancy, since there was an 0.8% reduction in volume upon destruction of the gas vacuoles (55). Electron micrographs have now indicated that the percentage of cell volume occupied by gas vacuoles is much higher, 22 for Anabaena flos-aquae and G. echinulata and 39 for Oscillatoria agardhii (106).

The view that the gas vacuoles provide buoyancy has not gone unchallenged. Some sedimentary and mud-inhabiting organisms also possess gas vacuoles (9,24,67). It has been recognized, however, that many of these organisms may also rise to the surface, and that the volume occupied by the vacuoles is probably the critical parameter (24).

Since the gas vacuoles probably enable the algae to regulate their buoyancy, this ability would be advantageous in making possible the use of mineral nutrients from different layers of water (25).

Storage of gas for metabolic purposes (oxygen for respiration, carbon dioxide for photosynthesis, and nitrogen for nitrogen fixation) has been another suggested function of the vacuoles (67). Similarly Kolkwitz (58) theorized that the sapropelic algae, under conditions of oxygen stress, produced the gaseous contents via fermentation processes, an idea further developed by Canabaeus (11). However failure to detect any of the expected gases and the apparent permeability of the vacuolar membrane now make such a role very questionable (122).

Since algae floating at the surface of waters are subjected to high and possibly damaging light intensities, Lemmermann (1910) proposed that the vacuoles might act as a light shielding device (69). Some observations, however, do not support this idea. Vacuolation increases in low light intensities, at least in the blue-green algae, Anabaena flos-aquae (122), as well as in the photosynthetic bacterium, Pelodictyon clathratiforme (90). Furthermore, it has been shown that the optical path of light passing through a turbid sample is increased many times. Consequently, the addition of light scattering materials (e.g., $CaCO_3$, polystyrene latexes) intensifies the absorption bands of pigments in the light scattering media compared to the same absorption bands in clear solutions (10). These results would support the opposite role, namely of increasing light absorption.

Morphology. It was over half a century before the highly refractive and irregular bodies seen in the light microscope were observed in the electron microscope. The submicroscopic studies revealed that the bodies consist of packed arrays of electron-transparent cylinders (8,48). The cylinders are similar in the various species of algae; they have a diameter of about 70 nm, a variable length of approximately 100 to 1300 nm, conical or pointed ends and are bound by a membrane of about 3 nm thickness (8,48). Striations traverse the length of the gas vacuoles and appear as a stack of laterally adjacent hoops or a continuous helix. Frozen-etched vacuoles show that the hoops or ribs themselves are constructed of small distinct granules (49). The ribs of the gas vacuoles of Microcystis aeruginosa are separated by about 4 nm with an intra-rib spacing of approximately 3 nm (49).

The submicroscopic appearance of the gas vacuoles of Halobacterium halobium is similar to that of the blue-green algae, but the over-all shape of the organelle is not. They are much shorter, non-cylindrical (i.e., oblate) and are not found in the closely packed arrays typical of the blue-green algae (66,109).

Upon rapid application of pressure, the appearance of a suspension of algae changes abruptly from a pale, cloudy green to a dark, transluscent green. Pressurization of a lysate of the salt bacterium, Halobacterium halobium, likewise causes a comparable clearing of the suspension.

In specimens of compressed cells (both algae and bacteria), the intact cylinders are not seen in the electron microscope after fixation or freeze etching. Instead only short membranous elements (ca. 6 nm x 7200 nm) or "intracytoplasmic membranes" appear in regions usually occupied by the gas cylinders. The membranous elements also have the characteristic "ribbed appearance" of the intact organelles (8,109).

A recent observation on gas vacuoles isolated from Microcystis aeruginosa is that the rate of pressurization may dictate the transformation of the gas vacuoles. Slowly pressurized vacuoles appear as flattened bags, often folding at an angle of $60 \pm 8^{\circ}$. Sudden pressure changes (> 1 atm/sec) release ribs of various lengths from the membrane (49).

Development and Recovery. Canabaeus reported the first investigation on the induction of gas vacuoles (11). Blue-green algae in which gas vacuoles were normally absent were said to form vacuoles when subjected to anaerobic conditions using a hydrogen stream, or upon exposure to certain concentrations of salt like sodium chloride and ferric sulfate. These results, however, have not been confirmed (24).

Although it has been suggested that gas vacuoles formed only in unhealthy organisms growing under anaerobic conditions and in which cell division had ceased (26), others find that healthy, actively growing cells likewise form gas vacuoles (93,106).

As pointed out above, low illumination will increase vacuolation.

Working on the hypothesis that the vacuoles are filled with nitrogen, Larsen et al. failed to increase vacuole formation in the cells of H. halobium by a number of nitrogenous compounds (66). They also studied the recovery of vacuoles after destroying them by either ultrasonication or pressurization. Recovery of the vacuoles in vacuole-depleted cells could be stimulated by organic constituents of the growth media and oxygen. Uncouplers, such as 2,4-dinitrophenol, inhibited the recovery of the gas vacuoles, indicating an energy requiring process (66).

Recently Waaland and Branton have reported the induction of gas vacuoles in the blue-green algae, Nostoc muscorum, by transferring the cells from a defined medium to distilled water. It was concluded that the vacuoles developed de novo since there were no pre-existing gas vacuoles. The developing vacuoles appeared to increase in length after induction (119). As the vacuoles lengthened, certain ribs seemed to stand out suggesting that new components (individual ribs?) were added at the center (119). However, certain vacuoles may have more than one prominent rib, while in other vacuoles, there may not be any (49). Possibly the ribs that stand out may only indicate points of physical stress.

When gas vacuoles were collapsed by pressure and recovered in ca. 9 hours, they were previously thought to be reinflated by the gas pressure (24). Since it seems evident

that the vacuole membrane is, however, permeable to gas, Walsby has proposed that the gas-vacuole structure is self-assembling (122). In other words the process would be the reverse of that first suggested. An arrangement probably in the form of subunits, would be required such that a space be formed in the protoplasm and subsequently filled by simple diffusion of gas into the space.

Chemical Composition. The first cytochemical tests on the gas vacuoles were negative for sulfur, protein, resin, fat or tannins (81). Furthermore, saturated salt solutions, formaldehyde, osmic acid and alkalies did not cause any apparent change in the vacuoles. However when Klebahn found that hydrocarbons were effective in destroying the gas vacuoles, it was concluded that the vacuoles were probably lipid in nature (55). But other treatments such as boiling water, acidic solutions, and phenol were also effective in destroying the gas vacuoles (55). Therefore the composition of the gas vacuoles was unresolved.

The development of improved fractionation techniques led to direct determination of the chemical composition of the vacuoles by analysis of cell-free preparations. Density gradient centrifugation was used to separate the cylinders from the other cellular structures of Oscillatoria rubescens, and analyses of the fractions containing vacuoles indicated the presence of a β-carotene (4 keto-β-carotene) and possibly fatty acids (50). Studies on the gas vacuoles of H. halobium

found the membrane to consist of protein, RNA, and hexosamine. Lipids were not found (66,109).

Objectives. It seemed desirable to consider the gas vacuoles as model systems for biological membranes. The organism selected was the rapidly growing, unicellular, blue-green alga, Microcystis aeruginosa, which forms numerous vacuoles. The first objective of the investigation was to isolate the gas vacuoles in a highly purified, cell-free state and to determine their chemical composition. This required procedures for the lysis of the algae so that the delicate organelles could be separated from the cytoplasm. Several purification procedures were tested, and the degree of purity of the isolated organelles monitored.

The second objective was to obtain evidence for the idea that the membrane is composed of one subunit as the geometry and the morphology of the organelle suggest.

The third objective was to investigate the conformational changes of the membrane that occur when the organelles are caused to collapse. Extrinsic, molecular probes, among them paramagnetic and fluorescent molecules, were used to study the membrane conformations.

PART ONE

ISOLATION AND CHEMICAL CHARACTERIZATION OF GAS-VACUOLE MEMBRANES FROM *MICROCYSTIS AERUGINOSA* KUETZ. EMEND ELENKIN

INTRODUCTION

Many important biological phenomena and biochemical processes are mediated by membranes. The membranes of gas vacuoles, organelles found in certain procaryotic organisms, were investigated as a model system (for reviews see 24,65, 89). The gas vacuoles of the blue-green alga, *Microcystis aeruginosa* Kuetz. emend Elenkin, seem particularly simple; their membrane is an array of subunits of about 3 nm size (49). The present report describes the isolation, preparation and chemical analysis of a highly purified fraction of the gas vacuoles of *M. aeruginosa*.

A technique based on penicillin lysis of the cells was developed so that the delicate, pressure-sensitive gas vacuoles could be isolated intact. The gas vacuoles were purified by liquid-polymer partitioning or by macromolecular sieving and centrifugation. The purity of the different fractions was monitored by the use of C^{14}-labeled vacuolated and non-vacuolated strains of *M. aeruginosa*. Chemical

analyses were carried out on the purified gas-vacuole membrane fractions. According to the analyses the gas-vacuole membrane consists of protein only. Some preliminary data has been obtained on the interaction of the protein of the membrane with reagents.

MATERIALS AND METHODS

Culture. The blue-green alga, Microcystis aeruginosa, strain NRC-1, obtained from Dr. P. R. Gorham, National Research Council, Ottawa, Canada, was grown at 30 ± 2° in four-liter Erlenmeyer flasks half filled with ASM-1 medium (32). The flasks were agitated on a rotary shaker (140 rpm), and forced air (4 l/min) was bubbled through the cultures by means of gas-dispersion tubes (Kimax 12C). Illumination (600 ft-c) was furnished by a bank of cool-white fluorescent lamps. The algae had a generation time of 14-18 hours.

Harvest. Five hours before harvest, during late exponential growth (5×10^7 cells/ml), Mg^{++} and benzylpenicillin (K-salt; Sigma) were added to final concentrations of 1 mM and 200-250 U/l respectively. The algae were collected by vacuum filtration on 9 cm premoistened Millipore filters (SMWP090). The filter was placed between a medium, sintered-glass Buchner funnel (9.3 cm diameter) with a 2 cm rim and a 9.2 x 25 cm glass cylinder with a tapered end. A latex band connected the two parts. To enhance filtration, the cells were continuously removed from the filter with a rubber spatula. The concentrated material was resuspended in a Petri dish with 15 ml of the culture medium.

Lysis of Cells. The concentrate was made 1 M in glycerol and gently agitated for 15 min. The solution was then rapidly diluted with 3 volumes of 0.02 M Tris-HCl, pH 7.7. The lysate, containing about 7 mg protein/ml, was kept at 4° for 2 hours before fractionation.

Test for Gas Vacuoles. To determine the presence of intact gas vacuoles in the lysate and the following fractions, aliquots were pressurized in a plugged syringe by a sharp blow on the plunger. A decrease in light scattering indicated the presence of intact gas vacuoles.

Preparation of Vacuole Membranes

Liquid Polymer Partitioning, Method I. A two phase system was used (2). Partitioning was achieved by mixing the lysate with dextran, 20% w/w (MW 70,000 or 150,000; Pharmacia) and polyethylene glycol (PEG), 20% w/w (MW 20,000; Union Carbide Corp.) 4:1:1. The mixture formed two phases of equal volume in a separatory funnel after standing for 4 hours at room temperature. If the lysate was too concentrated ($>$ 5 mg protein/ml), causing overloading of the top phase (I), the bottom phase (II) was drained at a rate of 1 ml/min and mixed with one volume of a dextran and PEG solution (2:1). After separation phase II was again drawn off and Renografin (Squibb) added to a final concentration of 10%. Two and one-half milliliters each of 2.5% and 5%

Renografin in 0.01 M Tris-HCl, pH 7.7, were layered onto the mixture in 1.3 x 12 cm tubes and centrifuged 30 min at 2,000 x g in an IEC, CL-swinging bucket rotor. The top layer was then removed and dialyzed exhaustively against buffer at 5°. Unless stated otherwise 0.01 M Tris-HCl, pH 7.7, was the buffer used.

Centrifugation, Method II-A. The following procedures are summarized in the flow-chart in Figure 1. Five ml of buffer were layered onto 15 ml of lysate in 30 ml glass tubes, and centrifuged 10 min at 8,000 x g in a Sorvall HB-4 rotor. The top, white layer, fraction F_{A1}, was drawn off, passed through a 0.45 μ Millipore filter (HAWP047) and fractionated on a 2 x 30 cm column of Sepharose 4B (Pharmacia). The milky-white fraction, F_{A2}, was pooled, layered in portions of 2 ml onto 9 ml of buffer and centrifuged 40 min at 200,000 x g in a Spinco SW-41 rotor. The narrow white band, fraction F_{A3}, at the meniscus was removed and combined with an equal volume of 2% Triton-X-100 (Rohm and Haas) in buffer. After standing for 15 min at 25°, the mixture was rapidly pressurized to clearness in a 20 ml syringe and spun 30 min at 50,000 x g in a Spinco SW-41 rotor. The pellet was resuspended and centrifuged twice in buffer yielding the colorless gas-vacuole membrane fraction, F_{A4}.

Centrifugation, Method II-B. Fractions F_{B1} and F_{B2} were obtained as were F_{A1} and F_{A2}. Fraction F_{B2} was diluted ($A^{400} < 5$) with buffer, layered in portions of 2 ml onto 9 ml

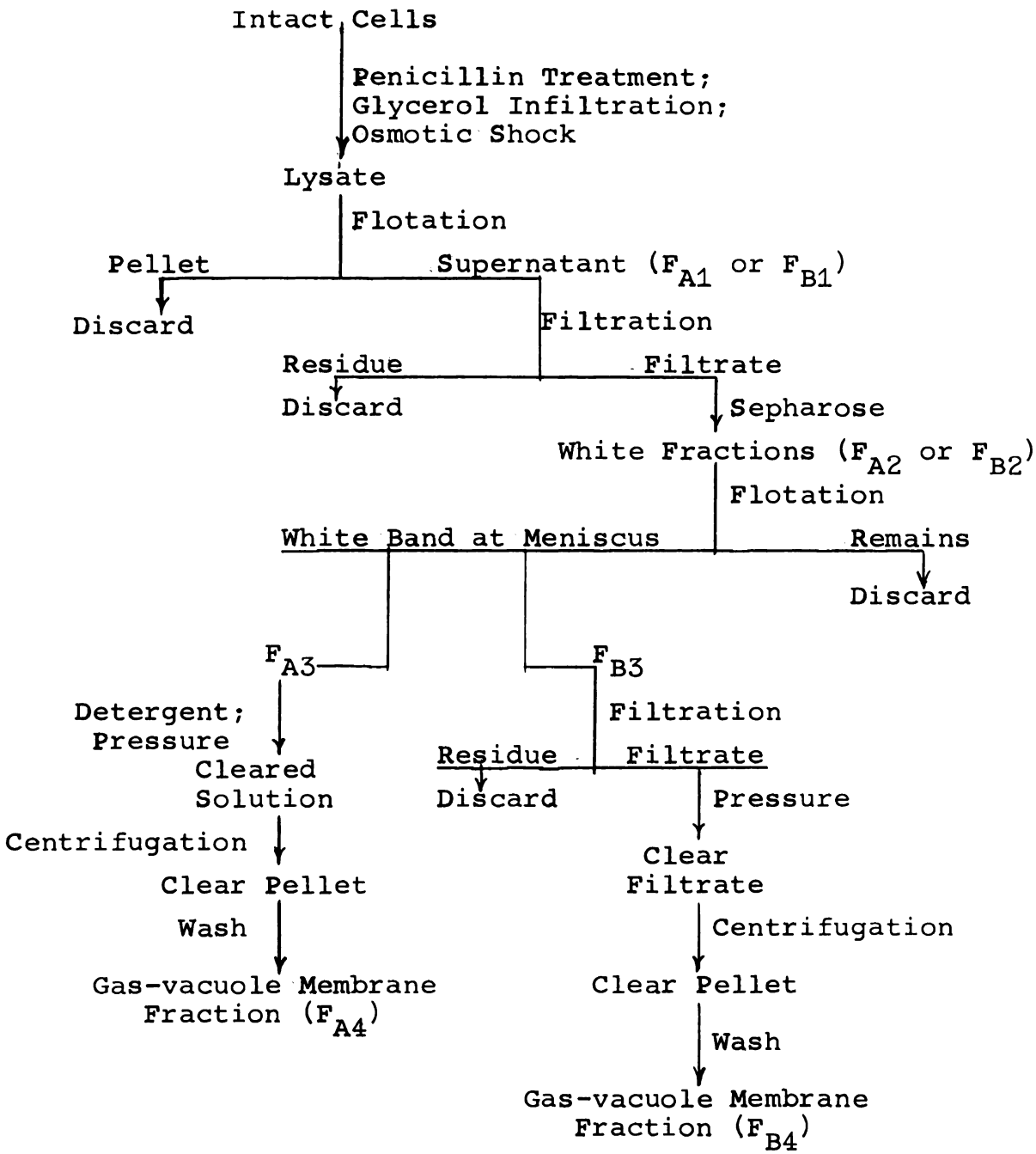


Figure 1. Fractionation Scheme of the Cell Free Preparation of Gas-vacuole Membranes.

of buffer and centrifuged for 40 min at 200,000 x g in a Spinco SW-41 rotor. The narrow white band at the meniscus, fraction F_{B3}, was passed through a 0.45 μ Millipore filter. The filtrate was rapidly pressurized to clearness, centrifuged, and washed as described for fraction F_{A3} to obtain a colorless, gas-vacuole, membrane fraction, F_{B4}.

C^{14}-Labeling, A) Vacuolated Strain. Forty μc of $NaC^{14}O_3$ were added 8 hours before harvesting to 250 ml of culture (2×10^7 cells/ml) in a one-liter Erlenmeyer flask agitated on a reciprocating shaker (115 Hz/min). Penicillin lysis and harvesting were performed as described. The lysate was pressurized in a syringe until a decrease in light scattering could be observed, and then centrifuged 10 min at 8,000 x g in a Sorvall HB-4 rotor, to obtain radioactive supernatant, fraction F_{C1}.

C^{14}-Labeling, B) Non-vacuolated Strain. The lysate was obtained as for the vacuolated cells. It was directly centrifuged at 8,000 x g in a Sorvall HB-4 rotor for 10 min to obtain the radioactive supernatant, fraction F_{C2}.

Assays

Protein. Radioactivity was determined according to Wettstein et al. (126), and protein, using bovine serum albumin as a standard, by the procedure of Lowry et al. (71). Direct gravimetric determinations agreed within 2% of the

determinations by the procedure of Lowry et al. (71) when bovine serum albumin was the standard.

Carbohydrate. Fraction F_{A4} was hydrolyzed for 1, 4, and 18 hours in 2 N trifluoroacetic acid according to Albersheim (1). The hydrolyzed sample was chromatographed on Whatman No. 1 paper using (I) ethyl-acetate, pyridine, and water (8:2:1), or (II) n-butanol, acetic acid, water (4:1:5). Alkaline $AgNO_3$ (115) and aniline phthalate (44) were used to develop the chromatograms.

Lipid Extraction. The pellets of F_{B4} were extracted with redistilled, boiling chloroform-methanol 2:1 and/or by acetone-ethanol 1:1 at 45° for at least 3 hours. The extracts were concentrated under vacuum at 30°, and chromatographed on thin layer plates (TLC) of silica gel G. The solvent systems were (1) hexane-diethyl ether-acetic acid 80:10:1; and (2) chloroform-methanol-water 75:22:3. The plates were developed by I_2 vapors or by spraying 75% H_2SO_4 and heating at 110°.

For gravimetric determinations, 2 mg or more material was dried at 105°, then extracted and the difference in weight determined.

Lipid Staining. The water soluble universal lipid indicator, slightly alkaline bromothymol blue (40 mg dye in 100 ml of 0.01 N NaOH) (107) was added to 100 volumes of F_{A3} and kept 1 hour at 25°. The material was then floated up at 8,000 x g for 10 min in a Sorvall HB-4 rotor, resuspended in buffer and refloated to inspect for staining.

Amino Acid Analysis. One mg of material from fraction F_{A4} was hydrolyzed in 6 N HCl in evacuated, sealed tubes at 110° up to 78 hours. The solution was then evaporated to dryness under vacuum at 50° and the residue suspended in the citrate buffer, pH 2.875, of Moore et al. (84). Analyses were performed on an automatic amino acid analyzer according to Technicon Instruments Company Ltd., by the accelerated method using Chromobeads C-2 (101). Separate samples were oxidized with performic acid and assayed for cysteic acid (83). Tryptophan was estimated by the colorimetric method of Opienska-Blauth et al. (88).

Density Determination. Preformed, convex, exponential CsCl gradients were prepared by the gradient maker described by Wettstein and Noll (125). The burette was filled with 50% w/w CsCl; the mixing vessel with 1.8 ml of 20% w/w CsCl. The gradient volume was 3.75 ml. Fraction F_{A4} (50 µg/0.25 ml) was layered onto the gradient and spun for 90 hours at 200,000 x g in a Spinco SW-56 rotor. The gradient was drained at approximately 0.5 ml/min and cut into 25 fractions of 0.15 ml, or the turbid band was drawn off with a micropipette. The refractive index of the fractions containing the gas-vacuole membranes, as monitored in the electron microscope (see below), was determined on a Bausch and Lomb refractometer. The density of the fractions was calculated according to Meselson et al. (78) from the respective refractive indices.

Electron Microscopy. Samples from the different fractions were placed on carbon-coated formvar grids and negatively stained with 0.5% uranyl acetate in 0.5% EDTA (neutralized to pH 7 with NH_4OH). For freeze-etching, samples mixed in 15% glycerol for 15 min were frozen in liquid Freon 12 and then transferred to liquid nitrogen. Processing was done in a Balzers-BA 510 freeze-etching apparatus (82). Etching was carried out for 1 min at -100° before shadowing at a 45° angle with Pt-C. A Siemens Elmiskop IA was used to examine the preparations.

RESULTS

Harvest and Lysis. Several methods to concentrate the cells with the gas vacuoles intact were tried. Harvesting of the algae by centrifugation posed problems. As indicated in Figure 2 the algae changes buoyancy during different phases of growth; and since a good yield is desired, only after a high cell density has been reached is it useful to concentrate the cells. In the stationary phase of growth, when the cell number is high and the cells will pellet with many of their vacuoles still intact, the sensitivity of the cells to penicillin is reduced and makes lysis difficult. Furthermore, attempts to float the cells in earlier phases of growth were also ineffective.

However, the algae can be concentrated 300 times by filtration of the culture through filters of 5 μ size. The cells can be easily resuspended and processed further. In order to obtain gas vacuoles intact in a cell-free preparation, the use of mechanical disruption of the cell and its organelles was avoided. Instead, the cell-free preparations were obtained by osmotic lysis of the cells. To induce osmotic lysis, the tensile strength of the wall component responsible for mechanical integrity must be reduced.

Figure 2. The Buoyancy of M. aeruginosa at Different Stages of Growth. The inoculum was from a culture with a density of 2.2×10^7 cells/ml. The cells were centrifuged for 10 minutes each in a 1 x 4 cm tube at 630 g in a swinging bucket rotor (Sorvall HB-4). These results are valid only under these conditions since the hydrostatic pressure increases with both the height of the medium in the tube and the centrifugal speed.

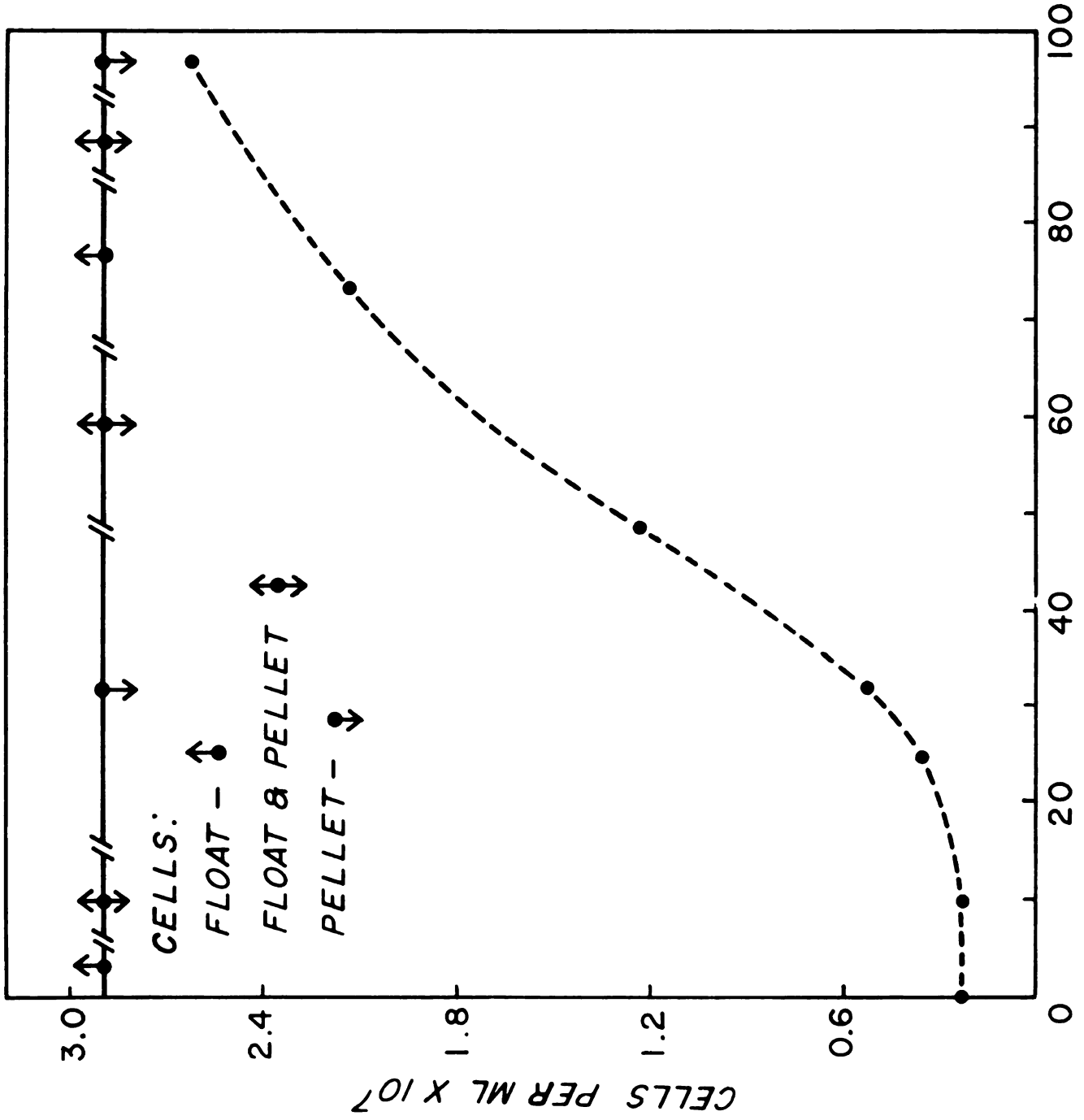

CELLS PER ML X 10⁷
3.0
2.4
1.8
1.2
0.6
0
20
40
60
80
100
HOURS AFTER INOCULATION
CELLS:
FLOAT –
FLOAT & PELLET
PELLET –

Figure 2

The effects of trypsin, lysozyme, and penicillin were looked at. Trypsin (53) did not alter the mechanical properties of the cell so as to allow lysis by osmotic shock. Lysozyme (14,20,27,42,118) left 30-50% of the cells intact; but exposure to penicillin was promising, and a method was developed to obtain complete lysis. Figures 3 and 4 show the extent of lysis of cells treated with graded concentrations of penicillin and magnesium. Figure 5 shows the lysis as a function of the concentration of glycerol, the osmoticum, and of the subsequent dilution into increasing amounts of buffer. Total lysis was achieved with 200 to 250 U/l penicillin and 1 mM Mg^{++} added 5 hours prior to harvest and infiltration in 1 M glycerol, followed by dilution with 3 volumes of buffer. Lysis reaches completion after 2 hours.

Liquid-polymer Partitioning of Gas Vacuoles. The partition coefficient of macromolecules and organelles depends on the surface area and the chemical properties of the particles involved (2). The aqueous, two-polymer phase system was used to separate the gas vacuoles from the remaining lysate. A mixture of the liquid polymers, PEG and dextran, and the algal lysate separates into two phases. Phase I with the higher percentage of PEG (ca. 3.8% w/w) was dark green; phase II with the higher percentage of dextran (ca. 3.6% w/w) was opaque blue. The gas vacuoles are in phase II as shown by negative staining and by the decrease in light scattering upon pressurization. Gas vacuoles were not

Figure 3. Growth Inhibition of M. aeruginosa by Penicillin. The penicillin was added at the onset of the logarithmic phase of growth.

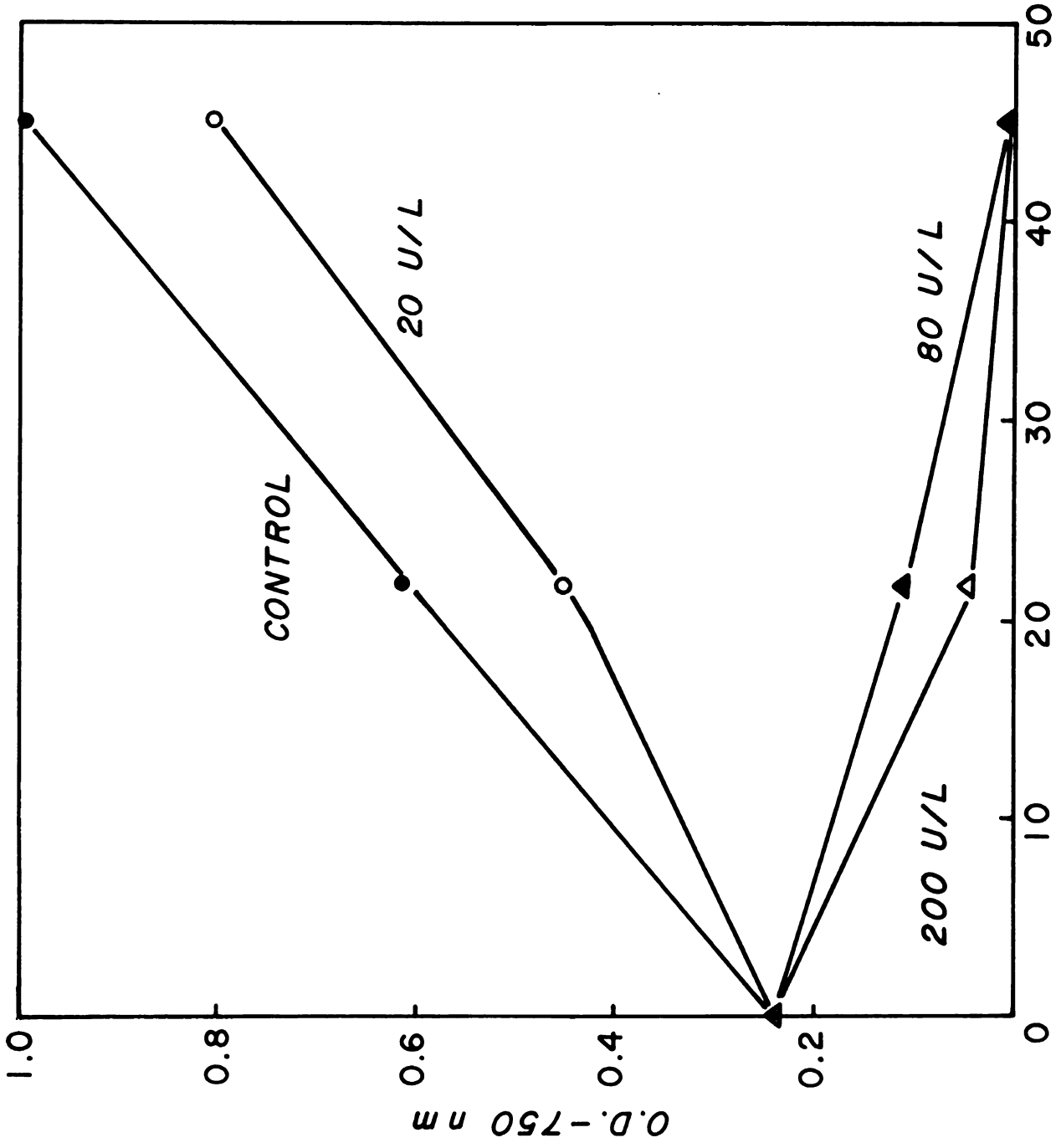

O.D.-750 nm
1.0
0.8
0.6
0.4
0.2
0
10
20
30
40
50
HOURS
CONTROL
20 U/L
80 U/L
200 U/L

Figure 3

Figure 4. Dependence of the Lysis of M. aeruginosa upon the Mg^{++} Concentration. The Mg^{++} and 200 U/l penicillin were added 5 hours before harvesting. Lysis was in 3 volumes of 0.01 M Tris-HCl, pH 7.7 after 15 minutes infiltration of 1 M glycerol. The cells remaining intact were counted after 2 hours.

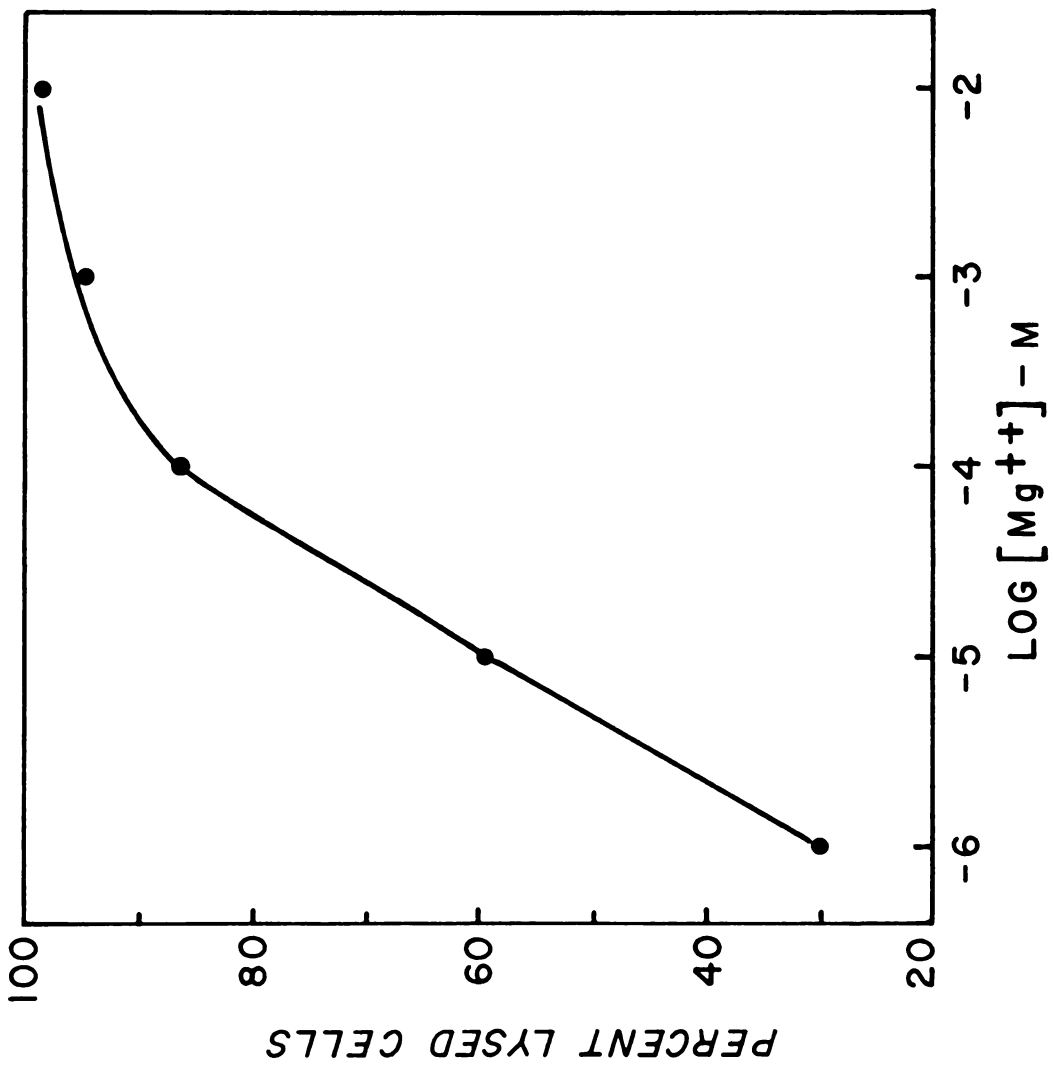


Figure 4

Figure 5. The Lysis of M. aeruginosa as a Function of the Concentration of the Osmoticum and as a Function of the Dilution with Buffer. The concentration of Mg^{++} was 1 mM and penicillin 200 U/l.

A. Filed circles: The concentrated cells were infiltrated for 15 minutes with varying glycerol concentrations and then diluted into 3 volumes of 0.01 M Tris-HCl, pH 7.7.

B. Open circles: The concentrated cells infiltrated for 15 minutes with 1 M glycerol were diluted with different amounts of Tris buffer.

The abscissa gives the dilution as multiples of the initial volume, V. The number of lysed cells was determined after 2 hours.

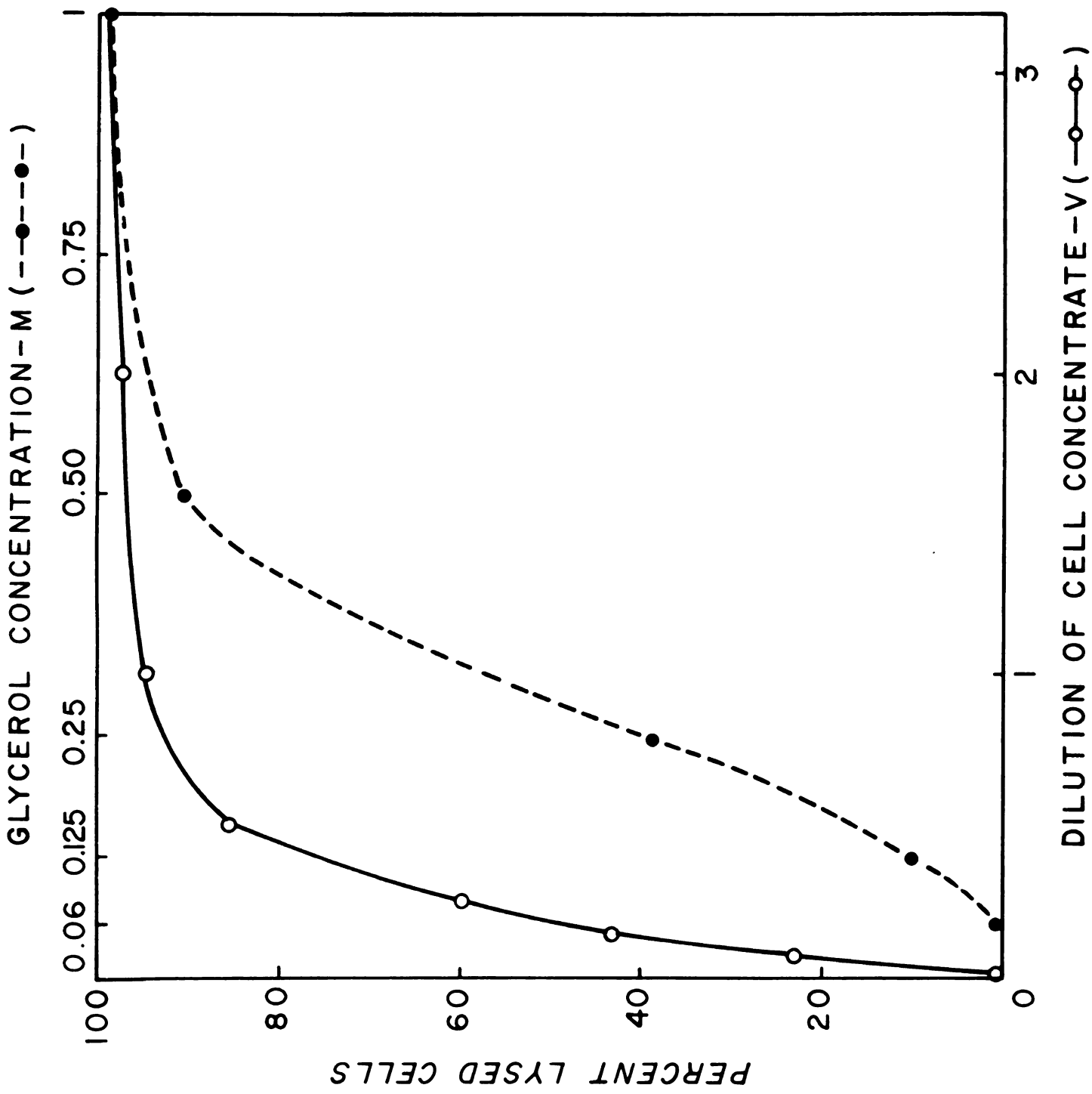

GLYCEROL CONCENTRATION-M (--●--●--)
0.06
0.125
0.25
0.50
0.75
1
PERCENT LYSED CELLS
100
80
60
40
20
0
DILUTION OF CELL CONCENTRATE-V(—○—)
0
1
2
3

Figure 5

separated from soluble proteins (e.g., blue phycobilins) by other polymers (i.e., PEG, MW 6,000; dextran, MW 2,000,000; sulfonated dextran, MW 500; 2,000) and ions (NaCl up to 0.5 M). To further purify the gas vacuoles the density of phase II was increased by Renografin. The relative buoyancy of the vacuoles was then increased, and the vacuoles floated as a white layer upon centrifugation at 2,000 x g, while the phycobilins remained in the lower half of the centrifuge tube. The enriched gas-vacuole fraction contained a high proportion of sugars to protein (estimated to be 2:1 although there was interference with the Lowry determination (71), i.e., precipitation, probably due to adhering polymers). The high carbohydrate to protein ratio and high specific surface of the vacuoles suggested that significant quantities of the polymers adhered to the vacuoles. This prevented a thorough chemical analysis of the vacuoles. However, based on the light-scattering properties of the vacuoles, about 75% were recovered from the lysate (Table 1). The high sugar content would be unusual for biological membranes; thus it was desirable to substantiate the findings by another isolation procedure.

Purification by Centrifugation and Molecular Sieving. Taking advantage of the low intrinsic density of gas vacuoles, a second procedure was developed as summarized in Figure 1. The first step, flotation, which separates the bulk of the lysate (cell walls, thylakoids, reserve bodies) from the vacuoles, yields fraction F_1. Filtration of F_1 removes most

of the remaining thylakoids. The filtrate is then separated into soluble proteins (among others, phycobilins) and vacuoles on Sepharose 4B (Figure 6). The 200,000 x g centrifugation spins out further contaminants and leaves the white gas-vacuole fraction F_3. F_3 is pressurized after addition of Triton X-100 (F_{A3}) or an additional filtration (F_{B3}), and the gas-vacuole membranes are pelleted at 50,000 x g to obtain either fraction F_{A4} or F_{B4} respectively. The electron micrograph, Figure 11, shows that this pellet consists only of gas-vacuole membranes.

Criteria of Purification. To monitor the purification two different methods were used. One is based on the typical appearance of the gas vacuoles (49). The other makes use of the specific activity (cpm/mg protein) of the fraction. The electron micrographs of the different fractions demonstrate qualitatively the increase of purity (Figures 7 to 11). Thylakoids, gas vacuoles and alpha-granules are seen in Figure 8 of the lysate. Figure 9 of fraction F_{A2} shows the enrichment of both alpha-granules and gas vacuoles. But the flotation procedure to obtain fraction F_{A3} (Figure 10) has separated the alpha-granules and thylakoids from the intact vacuoles. Figure 11 of fraction F_{A4} depicts the purified, collapsed, gas vacuoles concentrated in the pellet.

The gas vacuoles have a large specific surface. Thus, there is the possibility of material irreversibly adhering to the membrane during isolation. Therefore, the extent of

Figure 6. Separation of Fraction F_{A1} or F_{B1} into Gas Vacuoles and Phycobilins on Sepharose 4B. Fifteen ml of F_{A1} were loaded onto a 2 x 30 cm column. The absorbancy of the vacuoles at 400 nm, mainly due to light scattering (Part Three) and the absorbancy of the phycobilin chromophore at 600 nm was measured in a Beckman DB-G spectrophotometer.

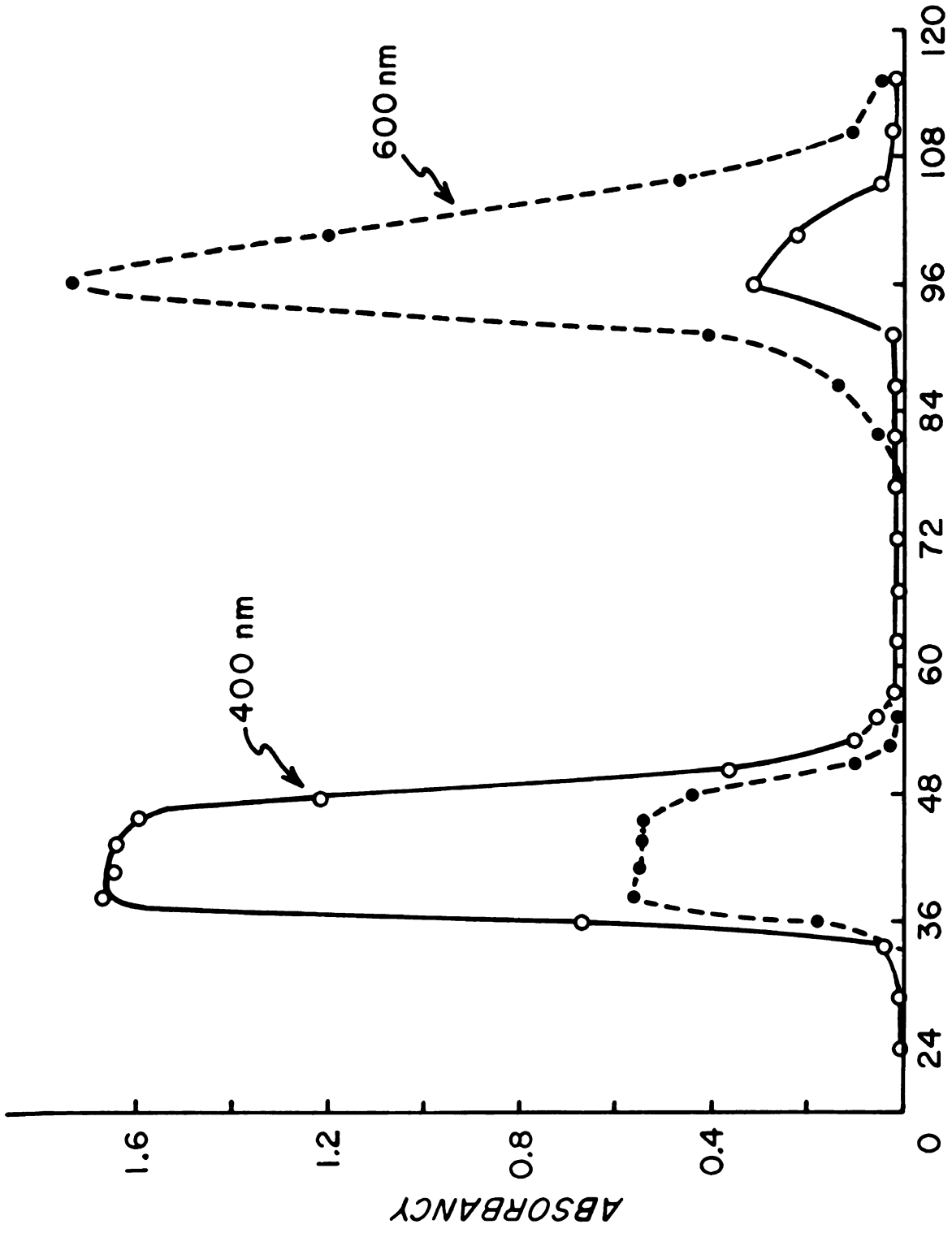

600 nm
400 nm
ABSORBANCY
1.6
1.2
0.8
0.4
0
24
36
48
60
72
84
96
108
120
VOLUME — MLS

Figure 6

Figure 7. Gas Vacuoles V in a Cell of M. aeruginosa Prepared by the Freeze-etching Replica Technique. The cylindrical organelles are fractured under different angles and lie as clusters in cytoplasm. 57,000 x

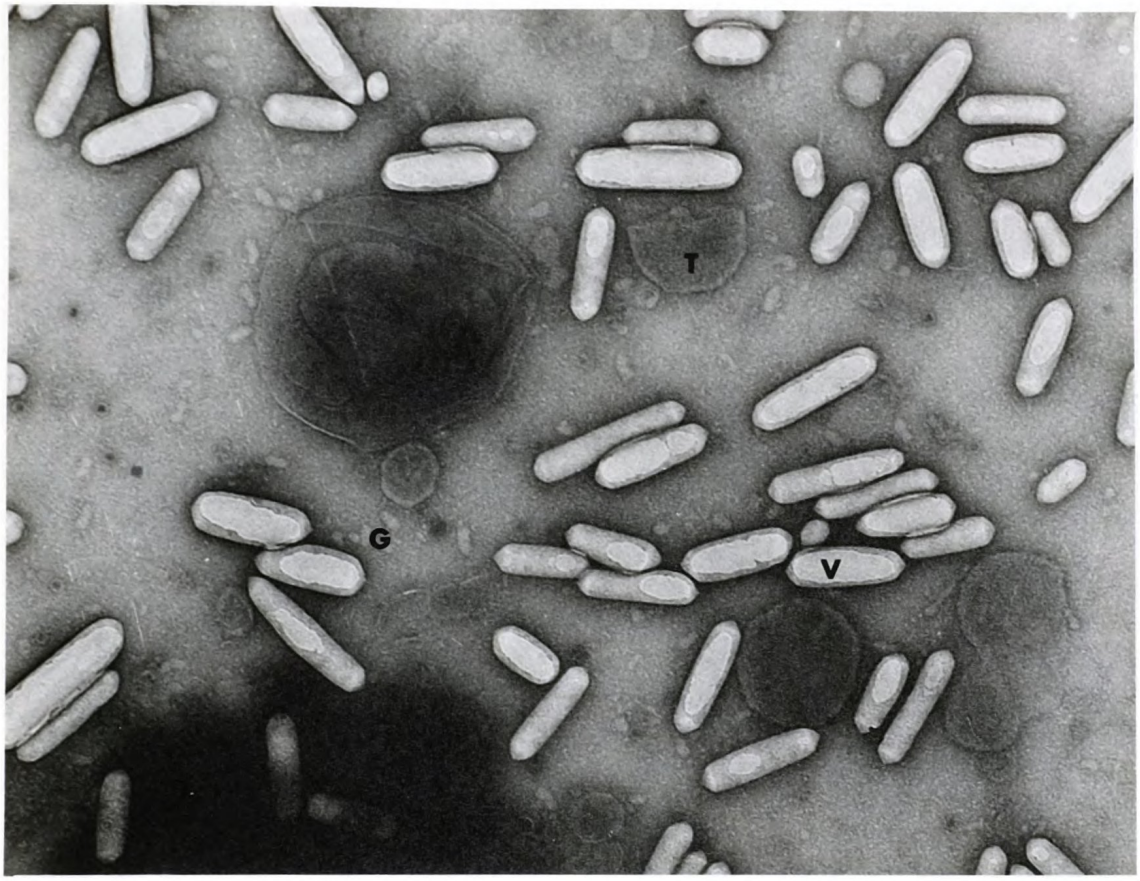


Figure 8. The Lysate of M. aeruginosa Negatively Stained with Uranyl Acetate. The cells were lysed by the penicillin technique. Intact gas vacuoles V are free in the lysate along with thylakoids T, granules G, and other cellular components. 34,000 x

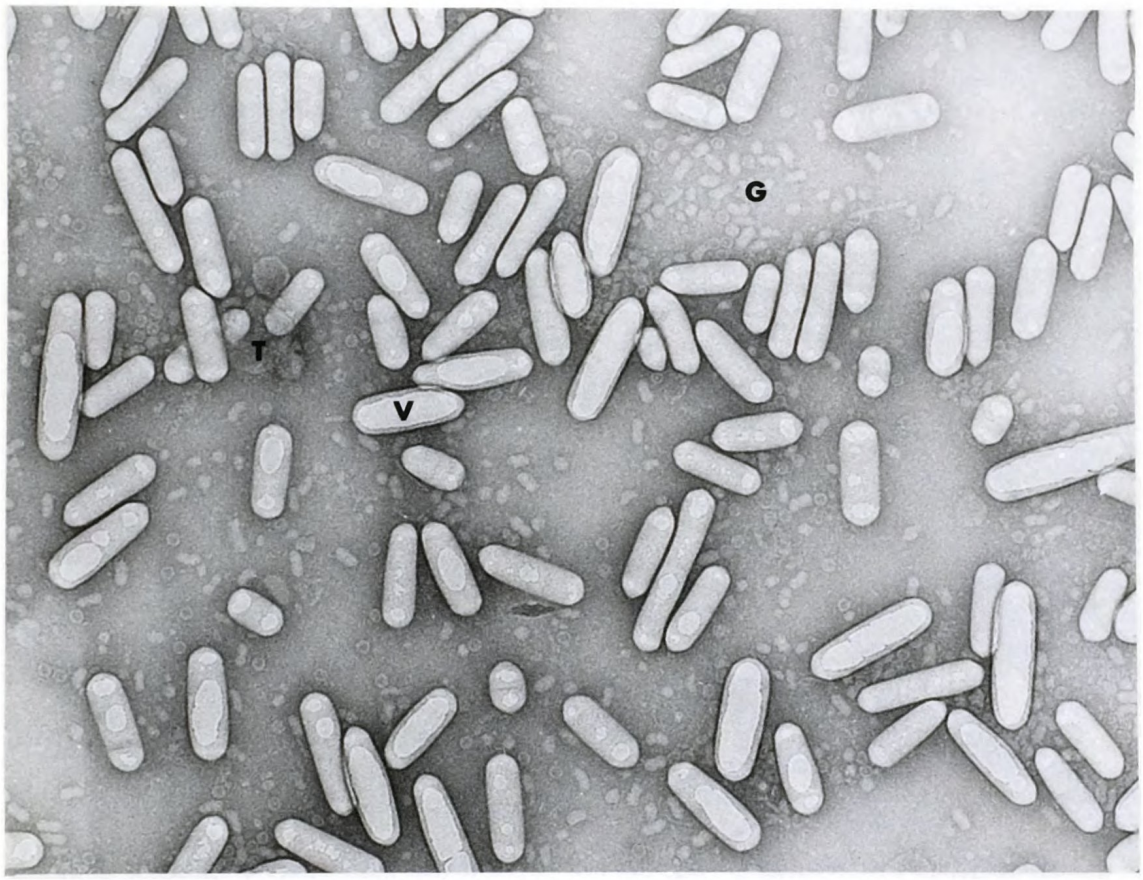


Figure 9. Intact Gas Vacuole V of Fraction F_{A2} Negatively Stained with Uranyl Acetate. Few thylakoids T were found, but many granules G are still present. 34,000 x

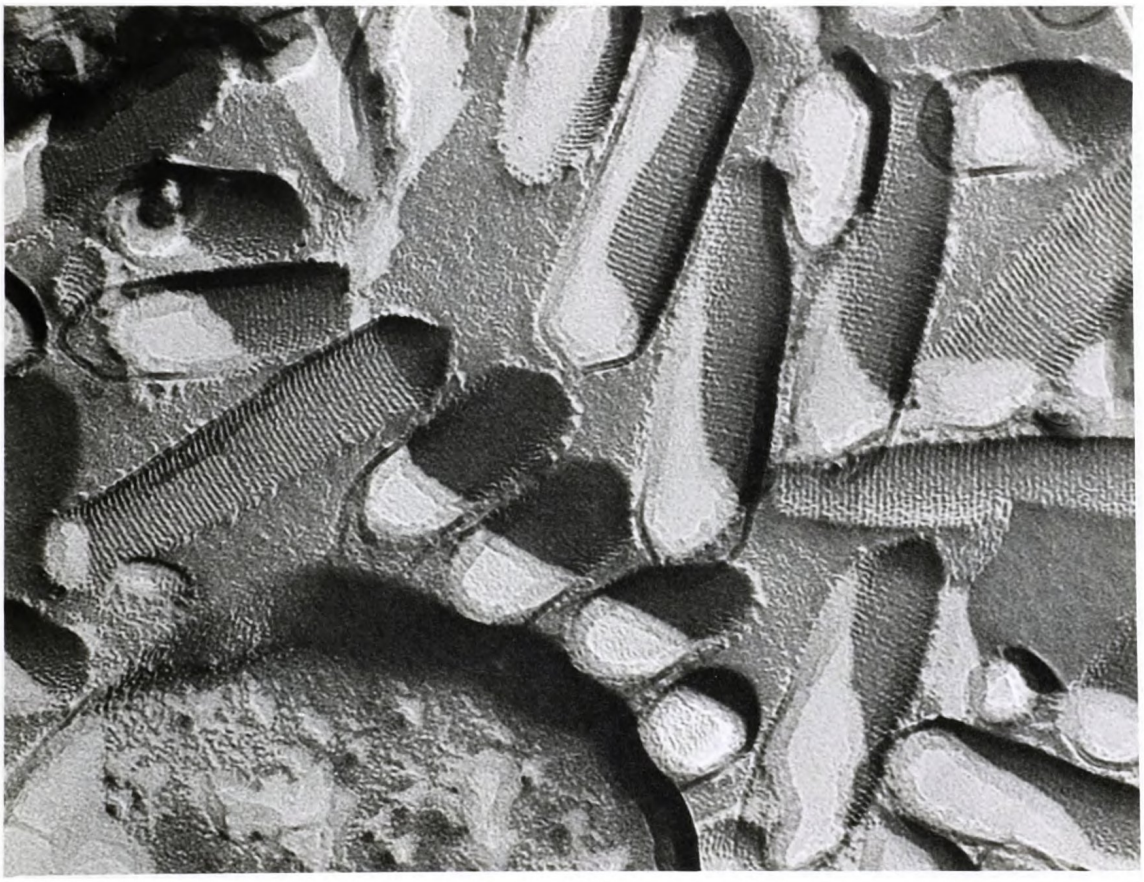


Figure 10. Frozen-etched Preparation of Highly Purified, Intact Gas Vacuoles from Fraction F_{A3} in 1.5 M Glycerol and 0.01 M Tris-HCl, pH 7.7. The surface structure on the inside of the vacuoles shows ribs consisting of protein particles. 152,000 x

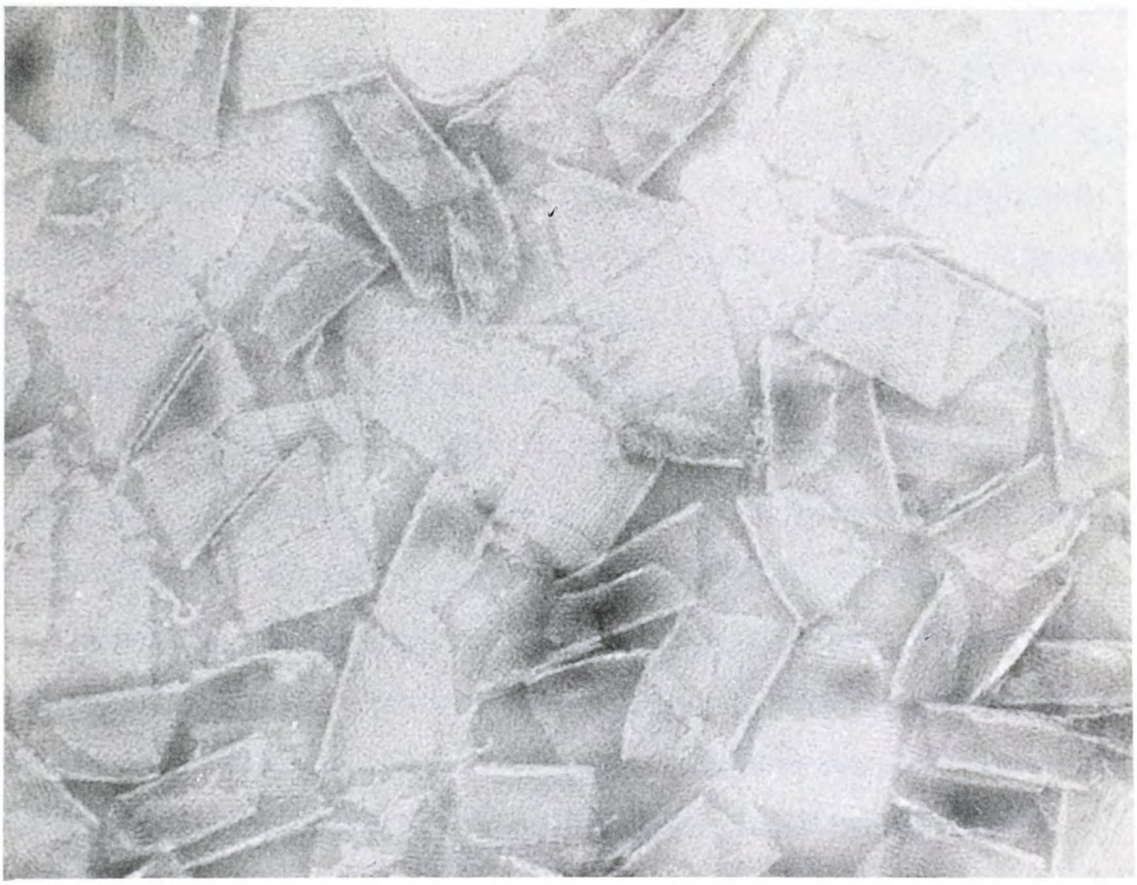


Figure 11. Purified, Collapsed, Flattened Gas Vacuoles from the Pellet of Fraction F_{A4} Negatively Stained with Uranyl Acetate. The ribs observed in Figure 4 now appear as striations. 115,000 x

purification was also based on the specific activity (cpm/mg protein) of the fractions. A radioactive fraction without vacuoles prepared from either a non-vacuolated (fraction F_{C2}) or a vacuolated strain (fraction F_{C1}) of M. aeruginosa was mixed with an unlabeled, vacuolated fraction (F_{A1}). Adsorption of material to the vacuolar surface in the lysate would be indicated by the presence of radioactive material. Table 2 shows that this is the case and the impurities are reduced in F_{B2}, F_{B3}, and F_{B4} from 78, 8 and to 4% respectively when comparing the specific activities of the fractions.

Protein Concentration by Light Scattering. Since the amount of protein other than the gas vacuoles in fraction F_{A3} is small ($< 10\%$) and quite constant, measuring the absorbance at 400 nm affords an easy and relatively accurate way to determine the protein concentration. Figure 12 shows the correlation between the concentration of gas-vacuole protein of fraction F_{A3} as measured by the method of Lowry et al. (71) and the absorbance at 400 nm due mostly to light scattering.

Recovery. The recovery of gas vacuoles from the lysate was followed by light scattering. Based on the scattering properties of the vacuoles a determination of their relative concentration in the different fractions was made. As seen in Table 1 only 14% of the gas vacuoles are recovered in the first centrifugation step, fraction F_1. In the other step in which vacuoles are lost, F_2 to F_3, only half of the

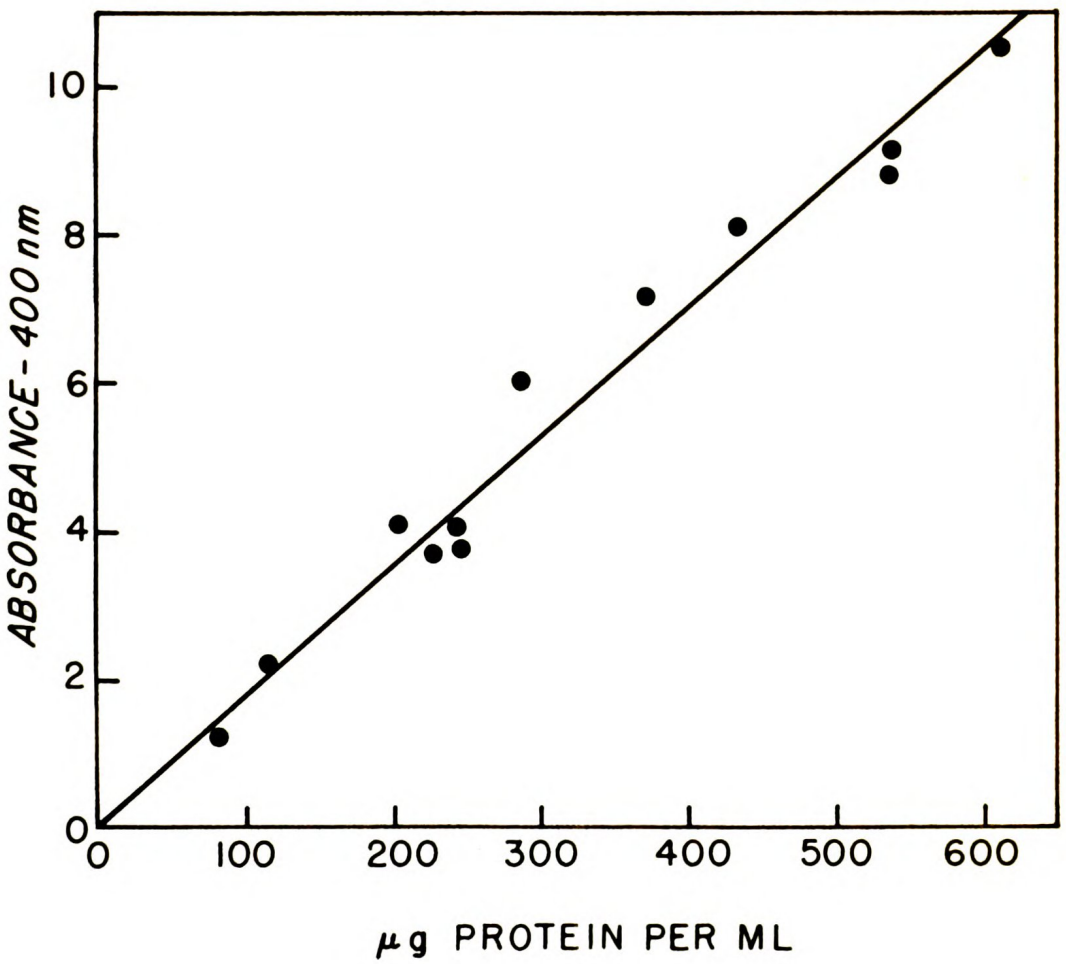


Figure 12. The Correlation Between the Concentration of Fraction-F_3 Protein and the Light Scattered as Measured by the Absorbance at 400 nm. The protein concentration was determined by the method of Lowry et al. (71), and the absorbance was measured in a Beckman DB-G spectrophotometer using a 1 cm light path. A different preparation was used to determine each point.

Table 1. Recovery of Gas Vacuoles from the Lysate

Fraction	Percent Recovery[a]
Method I	
Cell Lysate	(100)
Phase II	99 ± 1
Phase II + Renografin	99 ± 1
Floated White Layer on Phase II and Renografin	76 ± 3
Method II	
Cell Lysate	(100)
F_1 (7,000 g Supernatant of Lysate)	13.6 ± 3
F_{A2} (Sepharose 4B Fractions)	13.6 ± 3
F_{A3} (200,000 g White Meniscus)	6.8 ± 1

[a]The relative concentration of gas vacuoles was determined by light scattering under 90° in an Aminco-Bowman fluorimeter with excitation and emission at 400 nm. A plot of the scattered light versus dilution of the lysate was made. The scattered light of pressurized samples was considered background and subtracted. The light scattered by the fractions then served as a measure of relative vacuole concentration as determined from the equivalent dilution from the plot.

Table 2. The Increase in Purity of the Different Fractions as Determined by the Extent of Radioactive Cross-contamination. The fractions, F_{B1} and F_{C2}, were mixed and the increase in purity during the preparation was determined by comparison of the specific activities.

Fraction	Volume ml	Protein mg/ml	Total Protein mg	Radio-activity cpm/ml	Specific Activity cpm/mg	Percent of Specific Activity
Unlabeled Lysate (2×10^7 cells/ml)	20	3.3	66.0	-	-	-
Labeled Non-vacuolate Cell Lysate (1.5×10^7 cells/ml)	7	0.7	4.9	50,329	71,899	-
F_{B1} (Flotation; 7,000 g)	17	0.42	7.2	-	-	-
F_{C2} (Supernatant)	7	0.23	1.6	32,800	145,771	-
$F_{B1} + F_{C2}$	24	0.33	8.8	6,550	20,030[a]	(100)[b]
F_{B2} (Sieving; Sepharose 4B)	21	0.062	1.3	910	14,677	73.3 ± 3.5[c]
F_{B3} (Flotation; 200,000 g)	7	0.057	0.4	90	1,580	7.9 ± 1.5
F_{B4} (Pellet)	1	0.16	0.16	140	891	4.4 ± 0.2[d]

[a] The pooled fractions F_{B1} and F_{C2} were processed according to method IIB.

[b] The specific activity in the combined fractions F_{B1} and F_{C2} was normalized.

[c] The deviations reflect the variations of 6 different determinations made on a different batch of algae each time.

[d] The use of fraction F_{A1} obtained by method IIA and of the radioactive supernatant, F_{C1}, obtained from vacuole-containing, pressurized cells gave the same extent of contamination.

remaining intact vacuoles are recovered. Thus only 7% of the vacuoles released in the lysate are obtained.

It is conceivable that the vacuoles recovered might be preferentially isolated as a result of the fractionation procedure. However preliminary evidence based on the length of the vacuoles, a parameter which may reflect their development (119) does not indicate a selective isolation. The length of the vacuoles was determined (49) in a lysate and in the fractions F_2 and F_3 from the lysate. The values were 422 ± 100 nm (N = 259), 422 ± 96 nm (N = 244), and 443 ± 89 nm (N = 119) in the lysate, fraction F_2, and fraction F_3 respectively.

The amount of gas-vacuole protein within a cell can now be estimated. The light-scattering data indicate only 7% of the vacuoles are recovered. Furthermore, from Table 2 we see that the protein recovered in the gas-vacuole fraction (F_{B4}) from the total lysate is approximately 0.25% (i.e., 0.16 mg/66 mg). Therefore the gas vacuoles comprise about 3.6% (i.e., 0.25 x 100/7) of the total protein of the algae under the culture conditions used.

Chemical Analysis. The analyses were performed on the membrane pellet, fraction F_{A4} or F_{B4}, having less than 4.5% contamination. The fraction was found to contain protein, and its amino acid composition is given in Table 3. The protein is comprised of 52% non-polar, 10% basic, and 18% acidic amino acids. No imino amino acids or any cysteic

Table 3. Amino Acid Composition of Gas-vacuole Protein. All determinations were on fraction F_{A4}. The values are an average from at least three different batches of cells. To correct for breakdown of Thr, Ser, Asp and Phe extrapolations to zero time were made from a recovery curve.

Amino Acid	μmoles Amino Acid per 100 mg Protein[a]	Residues per 100 Residues
Aspartic acid	59.94	6.38
Threonine	48.60	5.18
Serine	87.48	9.32
Glutamic acid	108.92	11.60
Glycine	21.50	3.35
Alanine	174.80	18.61
Valine	109.03	11.61
Isoleucine	68.44	7.29
Leucine	104.94	11.18
Tyrosine	31.19	3.32
Phenylalanine	3.60	0.38
Lysine	50.56	5.38
Histidine	0.14	0.02
Arginine	45.52	4.85
Tryptophan	14.40	1.53
Proline	trace[b]	0
Half-cystine (as cysteic acid)	0	0
Ratio	$\frac{Lys+His+Arg}{Asp+Glu}$ = 0.57	

[a]The different analyses varied in the range of ± 3 to ± 4%.

[b]A trace of proline was seen in only 3 out of 8 different preparations.

acid derivatives were found. Figure 13 shows the progress of the decomposition and release of certain amino acids during hydrolysis of the protein. The slow liberation of valine and isoleucine show the need to continue hydrolysis for 48 hours or more to approach complete hydrolysis.

Using this composition the protein density was calculated to be 1.34 g/ml by the method introduced by Cohn and Edsall (12) and tested by McMeekin and Marshall (76). This is somewhat higher than the experimental value of 1.29 g/ml that was obtained from the CsCl gradients. However, the large, flocculent particles which may contain water might account for the apparent lower density.

The carbohydrate analyses of fraction F_4 showed either the presence of no carbohydrate, or only of trace amounts. Glucose (ca. 1 μg/mg of sample) was sometimes detected on the paper chromatograms as a faint spot following silver nitrate development. Such glucose may originate from polyglucoside, so-called alpha-granules. Figure 9 shows granules, sometimes seen in the preparations, with a diameter of about 350 A^{o} similar to alpha-granules (29,65). Thus the high carbohydrate content found in the gas-vacuole membrane fraction obtained in Method I is a contaminant from alpha-granules and/or partitioning polymers.

Alkaline bromothymol blue did not stain the vacuoles of fraction F_{A3}. Furthermore no extractable material could be detected either gravimetrically or chromatographically

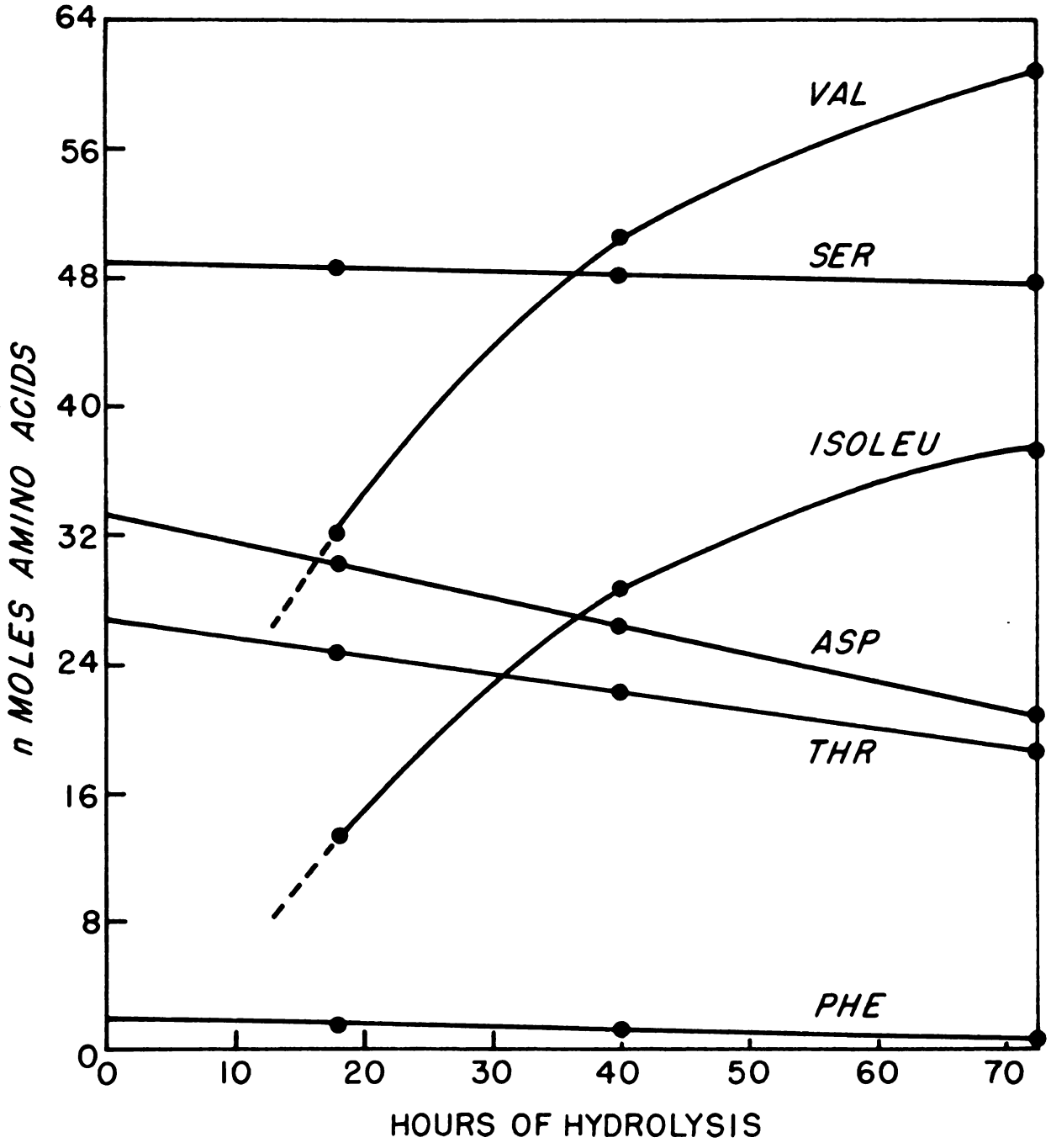


Figure 13. The Rate of Decomposition and Release of Certain Amino Acids During Hydrolysis of the Protein of F_{A4} in 6 N HCl at 105° in Evacuated Tubes.

after treatment of fraction F_{B4} with boiling lipid solvents.

An elemental analysis given in Table 4 substantiates the chemical finding that only protein comprises the gas-vacuole membrane (12). If carbohydrates or lipids were present, the percentage of hydrogen would be greater.

Chemical and Physical Treatments Causing a Decrease in Light Scattering. Since protein alone seems to make up the gas-vacuole membrane, and this protein is likely to be present as a subunit (49), the action of certain reagents on the membrane was investigated. Certain reagents make the membranes leaky and water can enter. A list of different treatments that cause the characteristic milky appearance of fraction F_3 to diminish and to become clear is given in Table 5. The mechanism of membrane permeability is uncertain for each of the different reagents, but observation of frozen-etched replicas of gas vacuoles in 80% chloroform and at pH 2 (HCl) revealed that the membranes were still mostly sheets rather than dissociated into ribs. Thus, these reagents appear to only make the membrane permeable to the surrounding media, rather than dissolving it. However, treatments which do not cause the suspended vacuoles to clear should have less effect on the integrity of the intact gas vacuoles.

Mono- and divalent ions, EDTA, detergents, disulfide reducing agents and potassium permanganate do not cause the suspended vacuoles to clear. The protein denaturant, urea, likewise did not clear the solution. However, saturated

Table 4. Elemental Analysis of Gas-vacuole Protein in Fraction F_{A4}

Element	Measured Percentage[a]	Expected Percentage[b]
Carbon	48.56	52.87
Nitrogen	12.81	16.16
Hydrogen	6.59	7.28
Phosphorus[c]	0	

[a]Performed by Spang Microanalytical Laboratories, Ann Arbor, Michigan. Only one analysis was made on a limited amount of material, viz., 1.5 mg.

[b]Calculated from amino acid composition.

[c]Phosphorus was determined by the method of Bartlett (6).

Table 5. Changes in Light Scattering (400-700 nm) after Different Physical and Chemical Treatments of Intact Gas Vacuoles. The intact vacuoles were from fractions F_{A2} or F_{A3} in buffer (0.01 M Tris-HCl pH 7.7) and diluted 4 times with the reagents.

	Decrease in Light Scattering	Treatment	Decrease in Light Scattering
Vacuum	-	Carbon tetrachloride (2.2)[c]	+
Pressure	+	Benzene (2.3)	+
Heat; 120° C	+	Triethylamine (2.4)	+
Freezing and Thawing; 5x	+	Toluene (2.4)	+
Acid; pH 3	+	Ether (4.3)	+
Base; pH 12	-	Chloroform (4.8)	+
$KMNO_4$[a]; 1%	-	1-Propanol (20.1)	+
EDTA; 0.01 M	-	Acetone (20.7)	+
NaCl; $LiCl_2$ etc.; saturated	-	Ethanol (24.3)	+
Thioglycolate[b]; 0.5 M	-	Methanol (32.6)	+
Dithiothreitol[b]; 0.5 M	-	Ethylene Glycol (37.0)	-
Mercaptoethanol[b]; 0.05 M	-	Furfural (42)	-
Glutathione; 0.5 M	-	Dimethylsulfoxide (45)	-
Urea; 8 M	-	Water (81)	-
Guanidine·HCl; saturated	+	Formamide (109.0)	-
Guanidine thiocyanate; saturated	+		
Deoxycholate; 1%	-		
Sarkosyl-NL-80; 1%	-		
Triton-X-100; 1%	-		

[a]Reduced with 1 M MSH after 3 hours; [b]Done in 0.5 M Tris-HCl, pH 8.5 to insure sufficient redox potential; [c]Dielectric constants are within the parentheses.

guanidine solutions caused the vacuolar solutions to clear; guanidine thiocyanate cleared the solution within approximately 3 minutes.

All of the reagents which had a dielectric constant of less than 35 cleared the solution, while reagents which reduced forces between charges did not cause the solution of intact vacuoles to clear. By mixing the suspended gas vacuoles with increasing percentages (v/v) of ethanol or dioxane in water, the suspension cleared in each case when the dielectric constant of the solution was about 47 (64).

DISCUSSION

The buoyancy of the cells as determined by centrifugation was found to vary with the stage of growth of the algae (Figure 2). Cells in the late exponential growth appear to have the greatest buoyancy, while cells in both earlier and later stages of growth are less buoyant. These changes in buoyancy, which were observed under defined growth conditions, may contribute to our understanding of the phenomenon of "waterblooming" observed in lakes. The seasonal fluctuation of the stratification of blue-green algae could be explained by this observation.

The shifts in buoyancy are in themselves interesting. Several parameters could regulate the effective volume of the vacuoles in different stages of growth; the size of the vacuoles, especially their length (49,119), the number of vacuoles per cell, the density of the cells, e.g., their relative content in fats and reserve bodies, and the changes in the permeability of the gas-vacuole membranes for gases.

Lysis of M. aeruginosa by the penicillin technique developed for the isolation of gas vacuoles is very efficient in releasing the organelles intact. Gas vacuoles from Halobacteria halobium were isolated by dialysis of the cells

against distilled water or by incubation in base (66,109). Methods to isolate gas vacuoles from blue-green algae have included the use of iodine prior to homogenization and mechanical grinding (50,123). However, these rough treatments seem to destroy most of the gas vacuoles.

To isolate gas-vacuole membranes sucrose gradients have been used (50,109). Attempts were also made to band a cell-free preparation of M. aeruginosa in order to isolate the gas vacuoles by gradient techniques. Exponential sucrose gradients (1.3 to 2.0 M) loaded with fraction F_1 upon centrifugation banded bluish-white material that was identified by its morphology in the electron microscope as gas-vacuole membranes. The band was rather broad, probably due to the strong aggregation of the vacuolar protein. Such aggregation occurred at less than 0.1 mg protein/ml. The blue color in the band indicated the presence of adsorbed phycobilins and probably other material. To check the purity of the fraction a radioactive non-vacuolate supernatant, fraction F_{C1}, was combined with a non-radioactive vacuolate supernatant, fraction F_{A1}. The contamination in the fraction was found to be as high as 60% on the basis of the specific activities (cpm/mg protein). Thus the large specific surface of the gas vacuoles must adsorb significant amounts of non-vacuolate material. Efforts were made to dissociate these contaminants from the vacuolar protein with detergents, salts, and urea, but the contamination was never reduced to less than 25% based on the

specific activities. Therefore other means of fractionation were resorted to since the use of sucrose gradients was not suited for this particular purpose. An isolate of less than 5% contamination was made possible by fractionation as outlined in Method II.

Gas-vacuole membranes of M. aeruginosa isolated by this method were found to have an interesting composition. They consist entirely of a protein which has a pI of approximately 7 as shown by isoelectric focusing. The protein constitutes about 3.5% of the total protein of M. aeruginosa as calculated from the recovery data, and the amino acid analyses show it consisting of 52% non-polar, 18% acidic, and 10% basic amino acids. Sulfur containing amino acids (i.e., cystine, methionine) were not found. A suggestion that disulfide bridges were absent came from attempts to dissociate the intact gas-vacuole membrane by disulfide reducing agents as thioglycolate, dithiothreitol, mercaptoethanol and glutathione at pH 7.7. Dissociation was followed by the light scattered by the vacuoles in fraction F_3; the addition of the disulfide reducing agents did not cause a decrease in scattered light (Table 5). Furthermore electron paramagnetic resonance labels which are attacked by sulfhydryl groups proved ineffective as labels of the membrane (Part Three).

Gas vacuoles from Halobacterium species have also been found to be proteinaceous (66,109). The gas-vacuole membranes of Oscillatoria rubescens were reported to consist of lipid

and carotenoids (50). The discrepancy might be the result of adding the cell-brei to the high sucrose concentrations that could cause lipids or lipoprotein structures to float with the vacuoles.

A comparison of the gas-vacuole membranes of H. halobium and M. aeruginosa shows some marked differences. The amino acid composition is especially different in the leucine, glycine, proline and cysteine contents. The membranes from H. halobium have a lower density (1.23 g/ml) (109) than that calculated (12,76) for the vacuole membranes of M. aeruginosa (1.34 g/ml) or that determined from CsCl gradients (1.29 g/ml). Furthermore the membranes of M. aeruginosa consist only of protein, as substantiated by the elemental analysis (Table 4) (12). No lipid was detected in H. halobium but nucleic acid and carbohydrate were found (109). However, this may be contamination since the isolates of H. halobium were obtained by sucrose gradiants, and our experience with sucrose gradients, as mentioned above, showed a high amount of non-vacuolate material present in the vacuole fraction.

An important parameter of the gas-vacuole protein is the molecular weight. Smith et al. (105) compared a 20,000 x g membrane fraction from vacuolate and non-vacuolate strains of Anabaena flos-aquae by gel electrophoresis. The membrane fraction was solubilized in guanidine hydrochloride, and a pronounced proteinaceous band corresponding to a molecular weight of approximately 22,000 was found for the vacuolate

strain. However, the results of Smith et al. (105) can be interpreted with difficulty only, since the two strains of Anabaena used in their experiments are not related.

Efforts were made to solubilize the gas-vacuole protein of fraction F_{A4}. It proved to be insoluble in 8 M urea with or without 0.5 M thioglycolate, 6 M guanidine-hydrochloride, lithium chloride, and extremes of pH. However in 80% formic acid and phenol-acetic acid-water (2:1:1) solubilization occurs. Preliminary results obtained by gel electrophoresis shows a single band of a relatively high mobility (MW = 14,000 with RNase and lysozyme as markers). Furthermore, the molecular weight of the particles that constitute the membrane when calculated from their dimensions of 28 x 42 x 30 A^{o} (49) and a density of 1.29 or 1.34 g/ml is 14,300 or 14,900. The values are calculated from the equation

$$\text{molecular weight} = 4/3\ \pi\ a.b.c.\ 6.02 \times 10^{-1}.\ \rho$$

where a, b, and c are the radii of the ellipsoid in A^{o} and the density expressed as g/cm^3.

Dissociation and permeability of the gas-vacuole membranes to their surrounding medium was followed by a decrease in the light scattering by the vacuoles present in fraction F_{A3}. Reagents with low dielectric constants (< 35) reduce the characteristic light scattering of a solution of cell-free vacuoles (i.e., cause clearing) as seen in Table 5. Observations in the light microscope made by Klebahn (55)

indicated that traces of fat solvents caused the refracted light due to the vacuoles to disappear. This suggested the vacuolar membrane to be of a lipid nature. But lipid solvents can also affect interactions of proteins and account for the observation (87). The conformational stability of a protein depends not only upon the forces between the protein molecules, but also on the interactions between the protein and solvent molecules. These interactions could disorganize the subunit array, make the vacuoles permeable, and cause the decrease in light scattering.

It should be pointed out that detergents which act on hydrophobic interactions do not make suspended, intact vacuoles permeable to water. Furthermore, although the reagents which lower the dielectric constant cause the solutions to clear, it has been noticed that the gas-vacuole protein from fraction F_4 remains sedimentable at 100,000 x g after 30 minutes. Although the distribution of the forces on the vacuole membrane due to hydrostatic pressure is unknown, pressurization of isolated vacuoles seems to indicate that the bonds between ribs appear weaker than the intrarib bonds (49).

The absence of lipid and an amino acid composition not notably different from many water soluble proteins (124) suggests that the hydrophobic character of the protein (i.e., water insolubility, adherence to hydrophobic surfaces) (49) is a consequence of secondary and tertiary structure.

Bowen and Jensen (8) and Jost (48) reported that gas-vacuole membranes are not preserved by $KMnO_4$. Smith and Peat (106) reported preservation of the vacuoles with $KMnO_4$. It was found that isolated vacuoles remain intact when treated with 1% $KMnO_4$. After treatment the colored ion was reduced by 1 M mercaptoethanol. Therefore the dehydration solvents to prepare the specimens for electron microscopy must destroy the vacuolar membranes.

The gas-vacuole membrane differs markedly from other biological membranes. It is comprised of 28 x 42 x 30 A^{o} subunits (49), has a high buoyant density of 1.29 (CsCl gradient) or 1.34 g/ml (calculated from amino acid composition), and no lipid association is detectable. Recent information on membranes has been compiled and has resulted in a re-evaluation of membrane architecture (35,61,68,128). Since in most membranes lipid-protein interactions are important many models based on such interactions have been proposed to account for the experimental findings that might explain the architecture of membranes. However, for the gas-vacuole membrane, protein-protein interactions are necessary, and membrane models such as the "unit membrane" (95) or any membrane model in which lipid serves as an essential backbone will therefore not suffice to describe the architecture of all biological membranes.

SUMMARY

A method involving penicillin treatment was developed to lyse osmotically the cells of the blue-green alga, Microcystis aeruginosa Kuetz. emend Elenkin, and to release the pressure-sensitive gas vacuoles intact. The gas vacuoles were purified by liquid-polymer partitioning or by macromolecular sieving and centrifugation. The degree of purification of the gas vacuoles was followed by observation in the electron microscope and by the use of C^{14}-labeled vacuolated and non-vacuolated strains of M. aeruginosa. The gas-vacuole membrane is composed only of protein consisting of 10% basic, 18% acidic and 52% non-polar amino acids.

PART TWO

CHARACTERIZATION OF THE PROTEIN OF GAS-VACUOLE MEMBRANES FROM *MICROCYSTIS AERUGINOSA* KUETZ. EMEND ELENKIN

INTRODUCTION

The interactions between lipids and proteins in membranous structures are not clearly defined. The Danielli and Davson model (17) of membranes, which satisfied most observations when proposed, depicts membranes as composed of a continuous bimolecular leaflet of phospholipid covered by protein. The bimolecular leaflet would be stabilized by Van der Waals interactions between apolar regions of the phospholipid with the protein interacting with the polar moieties.

A reassessment of the role of lipids in membranes, particularly that of a structural determinant, was prompted, however, by new findings incompatible with this model. The tripartite appearance of membranes seen in electron micrographs has been found in membranous cell structures in which lipid has not been detected or has been artificially removed. Examples of these include a cyst wall component in *Fasciola hepatica* in which no lipid has been found (77) and in lipid extracted membranes of the myelin sheath (86) and mitochondria

(21,22). Fleischer et al. showed that the tripartite appearance was not notably altered after extracting up to 95% of the mitochondrial lipid. Furthermore, possibilities of artifacts, such as fixation effects of the solvent, are very unlikely since electron transport activity is restored upon addition of lipid to the mitochondrial preparation (21,22). Protein rather than lipid was, therefore, implicated as the structural determinant of membranes.

Structural proteins have been isolated from different sources. They share characteristic properties such as insolubility under physiological conditions, strong tendencies to form polymers and to bind lipids and isogenous enzymes (13, 15,36,37,70,94,127).

To refine membrane models, determination of the parameters of these proteins, namely their composition, number, size, solubility and conformation is critical. These data are difficult to compile, however, due to the aggregating and binding properties of the structural proteins.

This part reports the further characterization of the protein of the gas-vacuole membranes from the blue-green algae, Microcystis aeruginosa. Studies were made on the solubilization of the protein in numerous solvents. The conformation of the protein cast from certain of these solvents was investigated by infrared spectroscopy. Analytical data on the solubilized material are consistent with the hypothesis

that the gas-vacuole membrane is constructed from a single protein. These data were obtained from tryptic digests, centrifugation, gel electrophoresis, and the determination of terminal amino acids.

MATERIALS AND METHODS

Gas-vacuole Membranes and Reagents. The gas-vacuole protein was isolated as outlined in Part One, and the fraction, F_{A4}, which showed less than 5% contamination, was used unless indicated otherwise. This gas-vacuole membrane protein will be referred to as either gas-vacuole protein or only protein.

Sodium dodecyl sulfate was recrystallized from water and guanidine-thiocyanate was recrystallized from ethanol. An ultra-pure grade of guanidine hydrochloride (Mann) was used. The other chemicals and solvents were reagent grade.

Solubilization. Attempts were made to solubilize the isolated gas-vacuole protein in a variety of solvents by adding 0.5-1 mg of the protein to 2 ml of solvent and stirring for 12 hours at room temperature. A serial method similar to that of Rosenberg and Guidotti was also employed (96). The protein was solubilized in a primary solvent and then dialyzed into successive solvents in which it was not initially soluble. Protein that did not sediment when centrifuged in a Spinco 65, angle-head rotor at 100,000 x g for one hour was considered to be soluble. The supernatant was dialyzed exhaustively against water to prevent interference from the

solvents with the quantitation of the protein by the method of Lowry et al. (71).

Succinylation. Succinylation, similar to that described by Hass (41), was performed on the gas-vacuole protein. Solid succinic anhydride was added with stirring to a 1% suspension of gas-vacuole protein which was either first dissolved in 80% formic acid and subsequently dialyzed into phosphate buffer, pH 8.0, with or without 8 M urea, or directly suspended in the buffer. The pH of the reaction mixture was maintained between 7.5 and 9.0 by the gradual addition of 12 N KOH. About 1000-fold molar excess (as estimated from the amino acid composition) of succinic anhydride was added. Upon completion of the reaction, the mixture was exhaustively dialyzed against distilled water or phosphate buffer, pH 8.0.

Infrared Spectra. A Perkin-Elmer spectrophotometer, Model 621, was employed. Solid films were prepared by applying membrane protein (e.g., an aqueous suspension of intact or pressurized gas vacuoles, equivalent to fraction F_{A3} (Part I), or a solution of fraction F_{A4} protein in formic acid) as a 2 cm circle in the center of an Irtran-II plate and then dried under vacuum at 30°.

Polyacrylamide Gel Electrophoresis. The apparatus used for disc polyacrylamide gel electrophoresis was similar to that described by Davis (18). The glass tubes were 0.5 cm i.d. x 8 cm long. The height of the polyacrylamide gel columns were 7 cm. No spacer or sample gels were used.

The concentration of all running gels were 7.5% (w/v).

The gel systems at 9.0 and 4.0 are modifications of those described by Zweig (129).

System at pH 9.0:

(a) 48 ml 1 N HCl, 36.3 gm Tris, 0.46 ml TEMED (N, N, N',N'-tetramethylethylenediamine Eastman 8178), 48 gm urea, and water to 100 ml.

(b) 31 gm Cyanogum-41 (E. C. Apparatus Corp., Phila., Pa.), 15.0 mg $K_3Fe(CN)_6$, 48 gm urea and water to 100 ml.

(c) 0.14 gm ammonium persulfate, 48 gm urea, and water to 100 ml.

Running gel: 1 part (a), 2 parts (b) and 1 part water, pH 8.8-9.0 were mixed and added to an equal volume of (c).

Buffer for electrodes: 0.6 gm Tris, 2.9 gm glycine, and water to 1 liter, pH 8.3.

The gas-vacuole protein was first dissolved in 90% formic acid and then dialyzed into 8M urea in Tris-HCl buffer, pH 9.0. The sample was turbid.

System at pH 4.0:

(a) 48 ml 1 N KOH, 22.4 gm glacial acetic acid, 4.6 ml TEMED, and water to 100 ml.

(b) 31 gm Cyanogum-41 and water to 100 ml.

(c) 0.15 gm ammonium persulfate per 100 ml of water.

Running gel: 1 part (a), 2 parts (b), and 1 part water were mixed and added to an equal volume of (c).

Buffer for electrodes: 2.4 gm glacial acetic acid, 17.25 gm glycine, and water to 1 liter, pH 4.0.

The gas-vacuole protein was dissolved in phenol-acetic acid-water (2:1:1, v/v/v) and adjusted to pH 4.2 with 6 N KOH.

System at pH 2.0:

The method of Takayama et al. was followed (113). Besides being dissolved in the phenol-acetic acid-water (2:1:1, v/v/v) mixture according to Takayama et al., the gas-vacuole protein was also dissolved in 90% formic acid and layered directly onto the gels.

Electrophoresis was performed at room temperature with a constant current of 5 mA per tube. On completion of electrophoresis, the gel was carefully removed from the glass tube under water by air pressure. The gel was subsequently transferred to a trough containing the dye, amido black 10 B (1 gm/100 ml of 7% acetic acid), and stained for 30 minutes (129). After rinsing in 7% acetic acid, the background dye was eluted by agitating the gels overnight in a large volume of 7% acetic acid.

Ultracentrifugation. Sucrose density gradient centrifugation was performed at 9° in 2 ml quartz tubes in a swinging bucket, Spinco SW-50 rotor with a Spinco, Model L II-B, ultracentrifuge at 35,000 rpm for 6-24 hours. Gradients were prepared by the method of Wettstein and Noll (125). The burette was filled with 20% w/v sucrose in formic acid, and the mixing vessel with 1.0 ml of 5% w/v sucrose in formic acid. The gradient volumes were 2.0 ml.

All sedimentation velocity experiments were performed at 20° in a Spinco, Model E, ultracentrifuge equipped with a schlieren optical system. Photographic plates were measured on a Nikon shadowgraph. The protein was dissolved in 88% formic acid and run in a Kel-F double-sector, synthetic cell.

Tryptic Digests. Gas-vacuole protein dissolved in 80% formic acid was dialyzed into 0.1 M NH_4HCO_3, pH 8.2, with or without 6 M urea. Two percent trypsin (Worthington) by weight of protein was added to the protein suspension and the

reaction mixture was kept at 37°. After 2 hours incubation an additional 2% trypsin was added. After 12 hours the insoluble material was removed by centrifugation and the supernatant was lyophilized. Control solutions in which the protein was omitted were incubated at 37° for 20 hours.

The lyophilized peptides were resuspended in 0.1 ml of 50% aqueous acetic acid, and descending chromatography was performed on Whatman No. 3 MM papers (46 x 57 cm). Chromatography and high voltage electrophoresis of the peptides were carried out as described by Katz et al. (51). Descending chromatography was performed in n-butanol-acetic acid-water (4:1:5, v/v/v; top phase) for 16 hours, the papers dried at room temperature, and electrophoresis performed in the second dimension in pyridine-acetate-water (1:10:289, v/v/v) at 2 KV for 1¼ hours. The papers were developed using cadmium-acetate-ninhydrin reagent (4).

Amino-terminal Amino Acid Determination. Two methods were utilized for the determination of the amino-terminal amino acids of the protein of the gas-vacuole membrane. The protein was either used directly or first dissolved in 80% formic acid before being dialyzed into 0.5 M NH_4HCO_3. Dinitrophenylation of the protein with 2,4-dinitrofluorobenzene (FDNB) was performed by the procedure first used by Sanger (99) and discussed and outlined by Porter (91). The FDNB derivatives of marker amino acids were made according to the

procedure of Thronburg et al. (114) or obtained from Sigma Chemicals, St. Louis.

The second labeling procedure utilized was the recent and more sensitive method employing 1-dimethylamino-naphthalene-5-sulfonyl chloride, usually abbreviated "dansyl chloride" or "DNS". The procedure followed was that of Gray (33).

High voltage electrophoresis as outlined in the above references was used to separate the amino acids; a solvent cooled system was used for the FDNB derivatives and a flat-bed system was used for the dansyl derivatives.

Carboxyl-terminal Amino Acid Determination. Carboxyl-terminal analyses were performed by subjecting the gas-vacuole protein to hydrolysis by carboxypeptidase A treated with diisopropyl phosphorofluoridate (Worthington) (3). The gas-vacuole protein (0.5 mg/ml) was first dissolved in 80% formic acid to denature it and then dialyzed into 0.5 M NH_4HCO_3 in which it precipitated. Carboxypeptidase A in aqueous suspension was exhaustively dialyzed at 4° against 0.5 M NH_4HCO_3 to remove any amino acids and to dissolve the enzyme. A microliter of the enzyme solution (50 mg/ml) was then added to 2 ml of the protein suspension and incubated at 37°. After 2, 4, 6, and 24 hours 0.5 ml aliquots were removed from the reaction mixture, and the reaction stopped by adjusting the pH to 3 with 2 N HCl. The gas-vacuole protein was then removed by centrifuging in a Sorvall HB-4 rotor at 5,000 rpm for 10 min at 5°. The supernatant was

frozen, lyophilized to dryness, resuspended in 0.01 ml water, and applied to an electropherogram. Electrophoresis was performed on an immersed strip system at pH 1.9 using the buffer system of acetic acid-formic acid-water (150:50:800, v/v/v). Upon completion of electrophoresis and drying at room temperature, the electropherogram was dipped in the cadmium-acetate-ninhydrin reagent (4) and developed at 70°.

Alternately, the pH of the supernatant from the reaction mixture was adjusted to 8.5 with 0.5 M $NaHCO_3$, 0.5 ml of a 2% solution of dansyl chloride in acetone was added, and the mixture was stirred for 2 hours at room temperature to yield the dansyl derivatives of the amino acids in solution. The samples were then dried *in vacuo* and the residue dissolved in 0.1 ml of 50% pyridine. The solution was spotted on Whatman No. 3 paper and subjected to electrophoresis (7 KV) in a flat-bed apparatus at pH 4.4 using pyridine-acetic acid-water (10:20:2500, v/v/v) as the buffer. The dansyl derivatives were detected by their fluorescence under an ultra-violet lamp (33).

RESULTS

Solubilization. The structural proteins of membranes are normally soluble in strongly protic solvents (16,70,96). The gas-vacuole protein is also soluble in strongly protic solvents, however in relatively small amounts, as shown in Table I. The increase in solubility of the gas-vacuole protein parallels the increase in proticity of the respective solvents (e.g., urea < guanidine thiocyanate < acetic acid < formic acid).

The low pH of formic acid limits the possibilities (e.g., column chromatography) for studying the protein. Thus a procedure which is successful in increasing the solubility of other structural proteins was employed. The gas-vacuole protein was solubilized in a strongly protic solvent before being dialyzed into a less protic solvent in which it was originally insoluble. The solubility of the protein was increased. When 500 μg/ml of protein were dissolved in 88% formic acid and subsequently dialyzed into 8 M urea, the solubility of the protein increased from 50 μg/ml to about 150 μg/ml in urea.

The structural proteins of membranes are usually soluble in urea-sodium dodecyl sulfate solutions. Various combinations of sodium dodecyl sulfate and urea were, however,

Table 1. Solubility of Gas-Vacuole Protein in Various Reagents

Reagents	µg/ml of Protein in Solution[a]
Urea (8 M)	50
Sodium Dodecylsulfate (0.2%) + Urea (4 M)	50
Guanidine-HCl (6 M)	150
Acetic Acid (66%)	200
Hexafluoroacetone (66%)	200
Guanidine Thiocyanate (6 M)	350
Formic Acid (68%)	600
Acetic Acid-Phenol-Water (2:1:1, v/v/v)	1,000
Formic Acid (88%)	7,500[b]
Acetic Acid-Phenol-Water (2:1:1, v/v/v) → Urea (8 M)	100
Formic Acid (88%) → Urea (8 M)	150
Formic Acid (88%) → Formic Acid (5%)	0

Reagents in Which Protein is Insoluble

Dimethylsulfoxide	Thioglycolate (5 mM, pH 8) + Urea (6 M)
Hexane	Sodium Dodecylsulfate (0.1%, 2%)
I_2 (10%)	Sodium Dodecylsulfate (0.1%) + Urea (0.5 M) at pH 1 (HCl) or 12 (KOH)
LiCl (7 M)	Triton X-100
Glycine (0.5%)	N-Dodecylamide (1%)
Chloroethanol (80%)	Dodecyltrimethylammonium (1%)
Thioglycolate (5 mM, pH 8)	Cetylmethylammonium Bromide (1%)

[a] The concentration was determined on the supernatant after centrifugation for one hour in a Spinco 65 rotor at 100,000 x g.

[b] The concentration was determined gravimetrically and by the method of Lowry et al. (78).

[c] The arrows symbolize dialysis into another solvent system.

ineffective solvents of the gas-vacuole protein. High concentrations of either cationic, nonionic or anionic detergents also did not solubilize the protein. Glycine, thiols, and metal ions were likewise ineffective in increasing solubility.

Succinylation, which increases the repulsive forces in protein by replacing a NH_3^+ group with a $NHCOCH_2CH_2COO^-$ function, has increased the solubility of certain proteins. Thus attempts were made to dissociate the gas-vacuole protein by this procedure. Many of the amino groups of the protein appear to be inaccessible, however, and succinylation was unsuccessful. A comparison of protein (method of Lowry et al. (71)) and amino group (ninhydrin reaction (85)) assays on aliquots of the protein solution before and after the succinylation procedure, indicated that about 90% of the amino groups were still free. This might be explained by the fact that the protein is not in solution when added to the reaction mixture.

Failure to increase significantly the repulsive forces in the membranous protein of intact vacuoles (i.e., fraction F_{A3}, Part One) after exposure to succinic anhydride was indicated by no decrease in the light scattered, as measured at 400 nm, by the intact vacuoles. The succinic anhydride must not react sufficiently, therefore, to dissociate the membrane to make it permeable to the surrounding media.

Infrared Spectra. Information on the conformation of the protein can be obtained by the position and number of bands observed in its infrared spectra. The absorption bands of particular interest are found near 1,650 and 1,535 cm^{-1} and are termed the amide I and II bands respectively. A protein with either an alpha-helical and/or random coil conformation has a characteristic absorption band in the amide I region located at about 1,652 cm^{-1} and in the amide II region at about 1,546 cm^{-1}; whereas a protein in the beta-conformation has absorption bands located at 1,630 cm^{-1} and 1,530 cm^{-1} in the amide I and II regions respectively (80,121). Thus transitions from an alpha-helix and/or random coil conformation to a beta-conformation are accompanied by the characteristic alterations in the spectra.

Figure 1 shows the amide I and II region of the infrared spectra of gas-vacuole films deposited from aqueous suspensions and formic acid. The spectra of the films cast from the intact and collapsed gas vacuoles were the same. In the region of the amide I band (C=O stretching) there are two distinguishable bands located at 1,655 cm^{-1} and at 1,623 cm^{-1}. Similarly in the region of the amide II band there are two absorption bands at about 1,535 cm^{-1} and 1,546 cm^{-1}.

The infrared spectrum of the film cast from a formic acid solution shows that in the region of the amide I band the only evidence of the band at 1,655 cm^{-1} seen in the spectra of gas vacuoles cast from aqueous suspensions is a

Figure 1. Infrared Spectra of Purified Gas-vacuole Membrane Protein Films: solid line, cast from an aqueous suspension of intact vacuoles; broken line, cast from 80% formic acid. Both spectra were of protein from the same isolate with the latter being a lower concentration after dissolved in the formic acid.

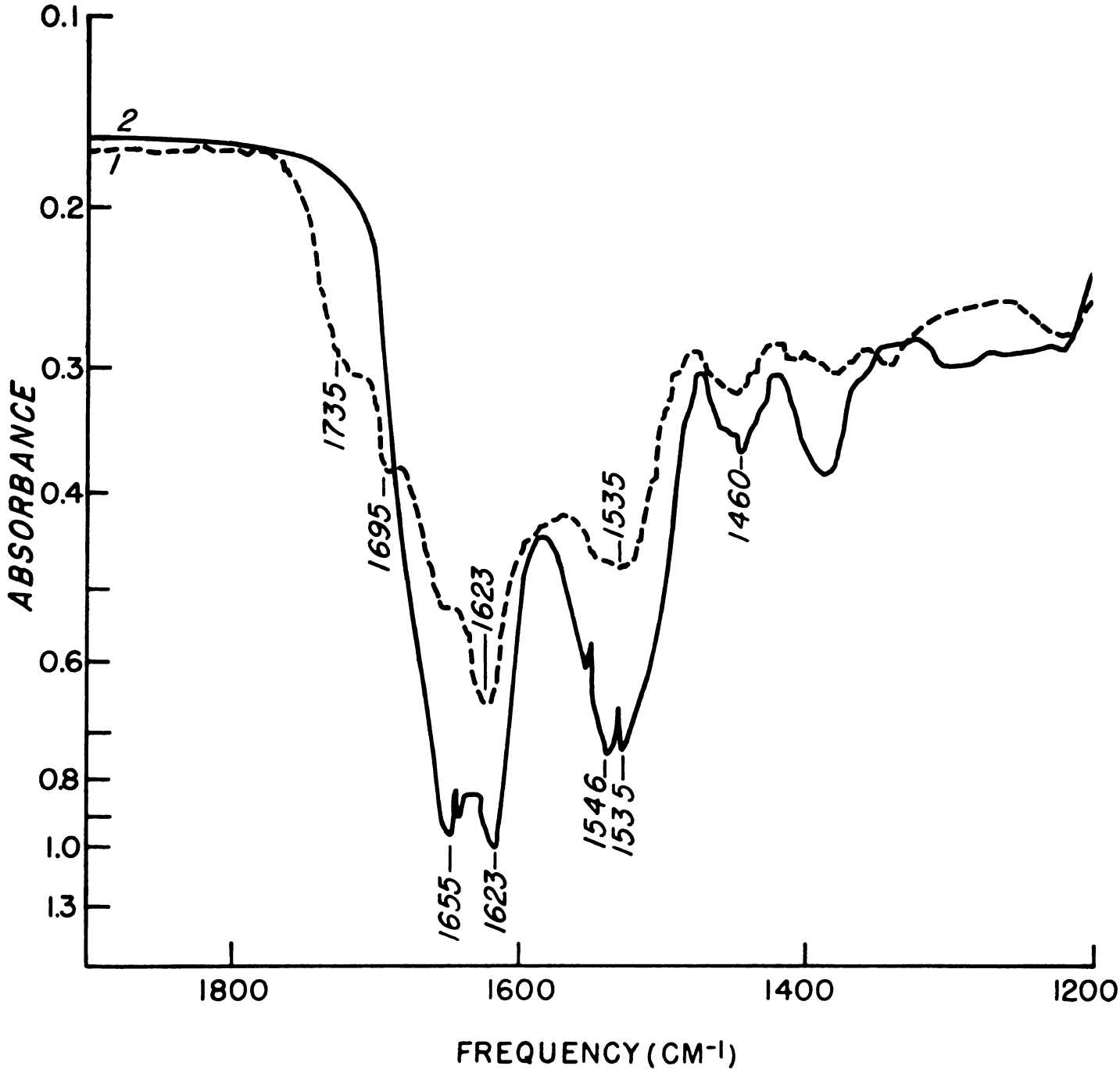

0.1
0.2
0.3
0.4
0.6
0.8
1.0
1.3
ABSORBANCE
2
1
1735
1695
1623
1655
1623
1546
1535
1535
1460
1800
1600
1400
1200
FREQUENCY (CM-1)

Figure 1

shoulder. Conversely the band at 1,623 cm^{-1} is very strong. The shoulder at 1,695 cm^{-1} is also much more prominent. In the amide II region there is no longer two distinguishable bands, but rather only one broad band at about 1,535 cm^{-1}.

Polyacrylamide Gel Electrophoresis. This technique, which effectively resolves proteins, should indicate the homogeneity of the solubilized gas-vacuole protein. The gel patterns, following electrophoresis at pHs 2, 4 and 9, are shown in Figures 2 and 3. At pH 9 the sample was turbid and remained at the origin; whereas at pHs 2 and 4, the protein is more soluble and migrated into the gel as a single band. There remained, however, protein at the origin. It was necessary to determine whether this material was representative of that which had moved into the gel or was another distinct protein. The unstained protein in the gel origin was eluted with phenol-acetic acid-water (2:1:1, v/v/v) or 90% formic acid, and the extracted protein was reapplied to another gel. The resulting electrophoretic profile was identical with that of the original sample (Figure 2).

To obtain a rough estimate of the molecular weight of the protein, co-electrophoresis was performed with RNase (MW=13,700) and lysozyme (MW=14,400). Their migration rates were about the same (Figure 3). Charge differences were, however, not accounted for, but larger proteins such as pepsin did migrate slower.

Figure 2. Polyacrylamide Gel Electrophoretic Profile of Gas-vacuole Membrane Protein. A, protein was dissolved in phenol-acetic acid-water (2:1:1, v/v/v). B, protein was extracted from the origin of an unstained gel comparable to A with phenol-acetic acid-water (2:1:1, v/v/v) and rerun. C, as B except protein was eluted from the origin with 90% formic acid. Electrophoresis was performed according to the method of Takayama et al. (113) for 1½ hours. The cathode was at the bottom.

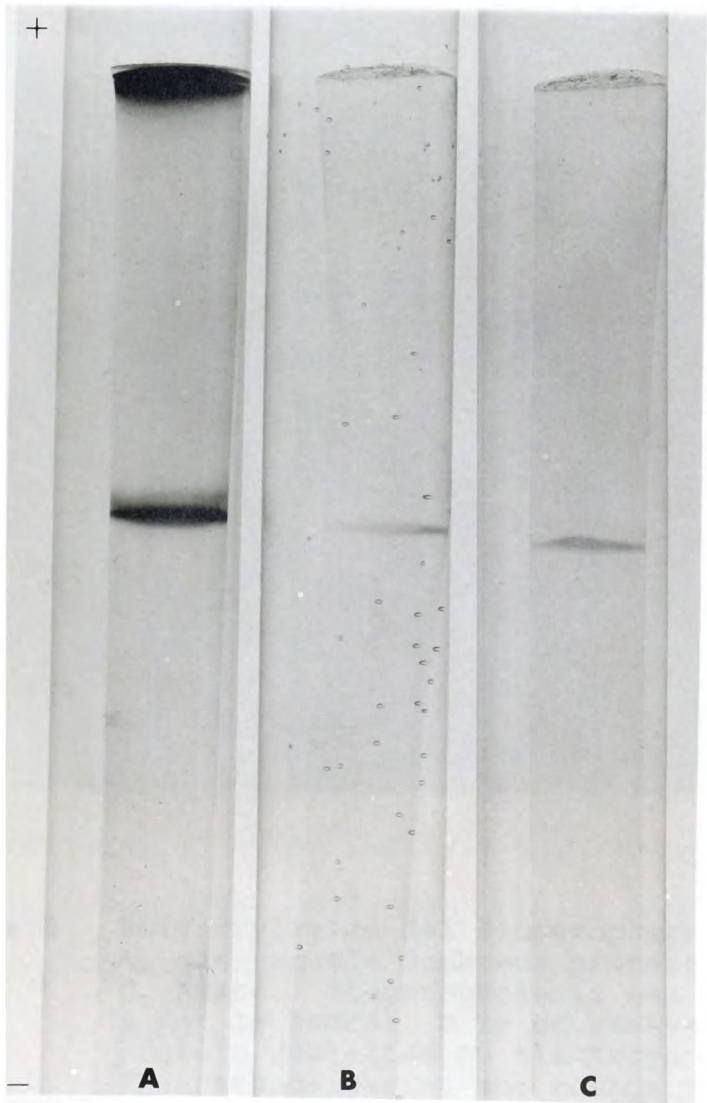


Figure 2

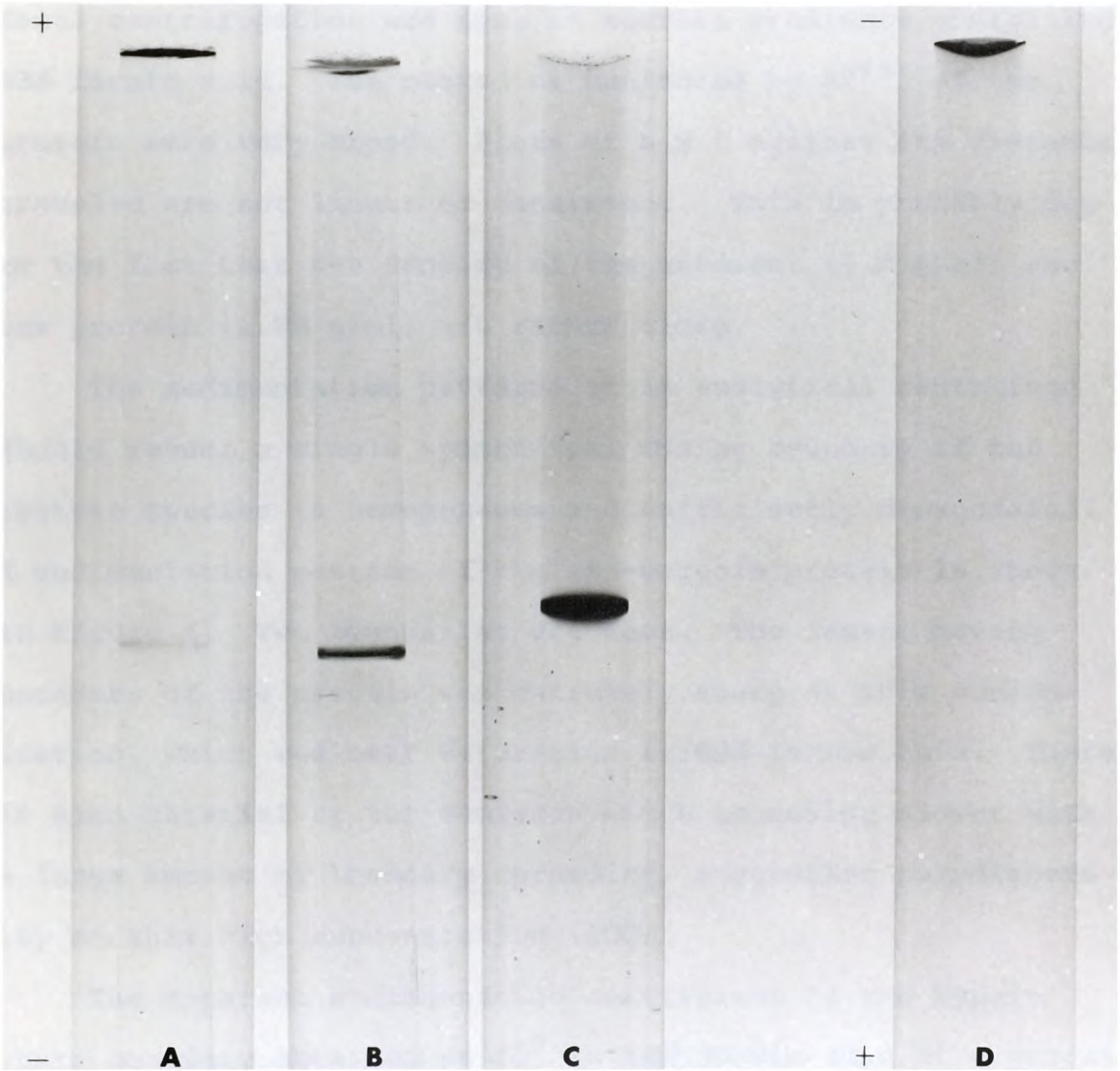


Figure 3. Polyacrylamide Gel Electrophoretic Profiles. A, gas-vacuole membrane protein; B, lysozyme; C, RNase. Electrophoresis was performed at pH 4 for 1½ hours. D is of gas-vacuole membrane protein subjected to electrophoresis at pH 9.2. The cathode was at the bottom for A-C, and at the top for D.

Sedimentation Velocity. Sedimentation velocity experiments should give a relative s value of the protein (72). Zonal centrifugation was done in sucrose gradients containing 88% formic acid. The peaks, as monitored by A^{279}, of the protein were very broad. Plots of s x t against the distance traveled are not linear or consistent. This is probably due to the fact that the density of the gradient (1.2 g/ml) and the protein (1.29 g/ml) are rather close.

The sedimentation patterns of an analytical centrifuge should reveal a single symmetrical moving boundary if the protein species is homogeneous and sufficiently dissociated. A sedimentation pattern of the gas-vacuole protein is shown in Figure 4. Two boundaries are seen. The faster moving boundary of the protein was extremely sharp at this concentration, which was near saturation in 88% formic acid. There is also material at the meniscus which is moving slower with a large amount of boundary spreading, suggesting polydispersity at this high concentration (100).

The apparent sedimentation coefficient of the hyper-sharp boundary obtained at 20° in 88% formic acid at a protein concentration of 0.75% (w/v) is $S_{20}^{app} = 3.3 \times 10^{-13}$ sec.

The hyper-sharp boundary results from the strong dependence of the sedimentation coefficient on the concentration (100). To quantitate such data, it is necessary to perform the sedimentation experiments over a broad range of concentrations and especially at low concentrations. The sensitivity

Figure 4. Schlieren Pictures of the Sedimenting Boundaries of Gas-vacuole Membrane Protein Dissolved in 88% Formic Acid at 7.5 mg/ml. Speed: 59, 780 rpm. Temperature: 20^{0}. The first picture (left one) was taken 33.1 minutes after start of run, and the following were taken at 16 minute intervals.

Figure 4

at low protein concentrations of the optical system accessible was not good enough to obtain readable schlieren patterns.

Tryptic Digests. The number of peptides expected from the arginine and lysine content of the protein with a molecular weight of 14,000 as calculated from the subunit dimensions and the protein density (Part One) would be 12. Fingerprint patterns of tryptic hydrolysates of gas-vacuole protein showed 14 spots, two of which were either extremely light or not present. A representative pattern is shown in Figure 5. The hydrolysates remained somewhat turbid, even after repeated enzymatic treatment and prolonged incubation. Controls in which gas-vacuole protein was omitted did not reveal any artifactitous spots.

Amino-terminal Amino Acids. After reacting the protein with 1-fluoro 2,4-dinitrobenzene, only DNP-ε-lysine was detected. Therefore the more sensitive dansylation method was tried. Tracings of the results obtained by electrophoresis of the products of dansyl-labeling are shown in Figures 6 and 7.

After electrophoresis at pH 4.38, ε-lysine was found on the cathode side of the origin. On the anode side, a spot could be detected immediately below the intensely blue-fluorescent dansyl-5-sulfonic acid (DNS-OH). Four dansyl amino acids, including DNS-Gly, -Ser, -Pro, and -Ala are closely grouped in or near the DNS-OH band. Proline could be

Figure 5. Tryptic Peptide Pattern of Purified, Gas-vacuole Membrane Protein. One mg of formic acid-denatured protein was subjected to 2 hours digestion by trypsin (2% by weight) followed by 10 hours digestion with fresh trypsin in 0.1 M NH_4HCO_3, pH 8.2 at 37°. Peptides not observed in every digest are indicated by the dashed circles.

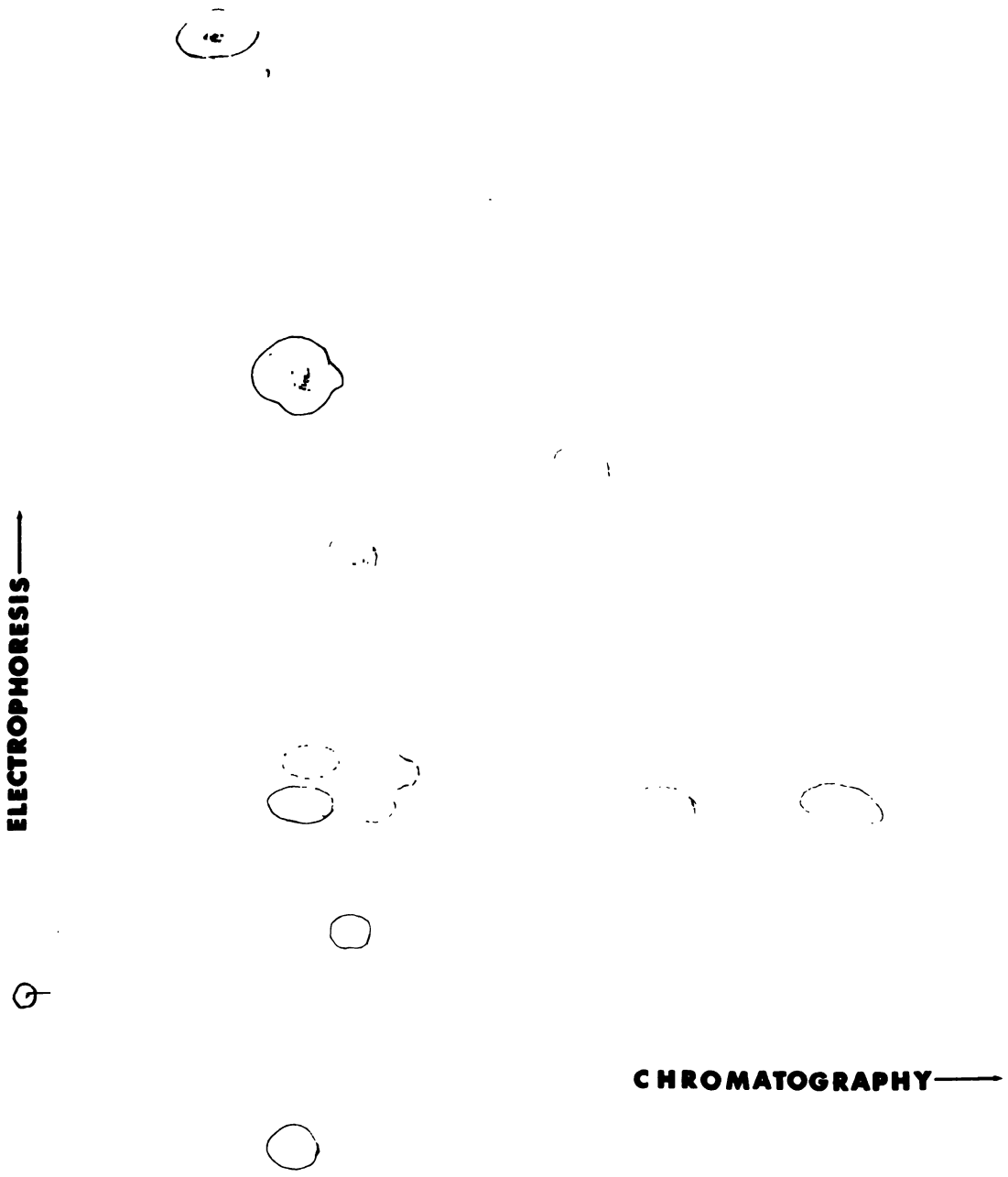


Figure 5

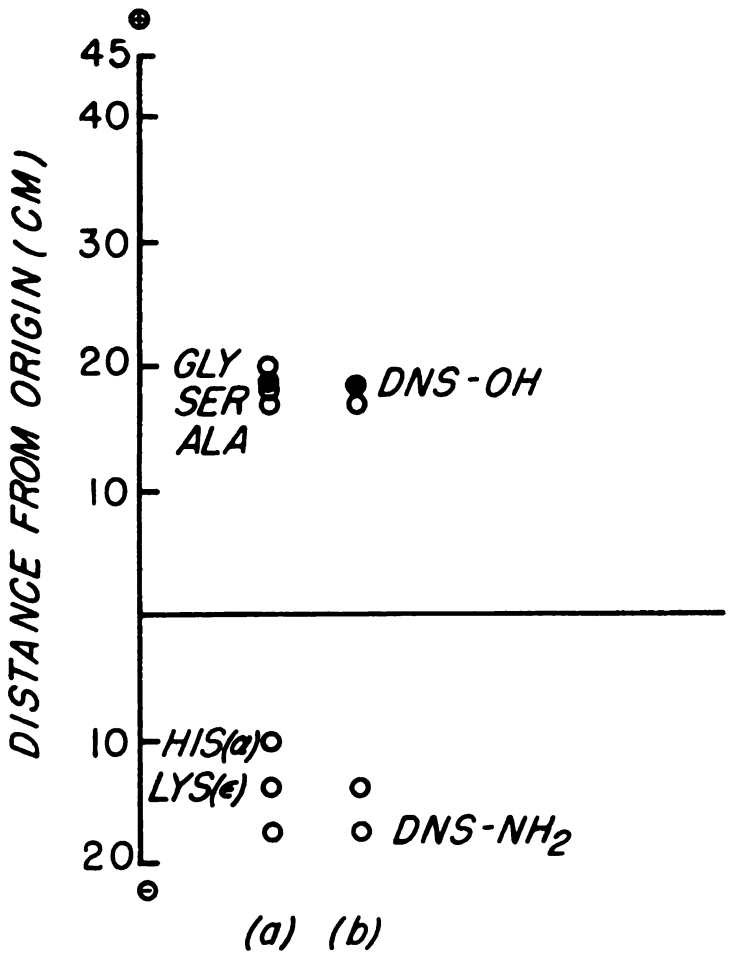


Figure 6. Electrophoretic Mobilities of Gas-vacuole Protein (b) and Standard Amino Acid Dansyl Derivatives at pH 4.38 (80 v/cm, 2.5 hours, 15°). Abbreviations used: SER = DNS-SER, etc. Filled circles represent sulfonic acid (DNS-OH) which fluoresces blue.

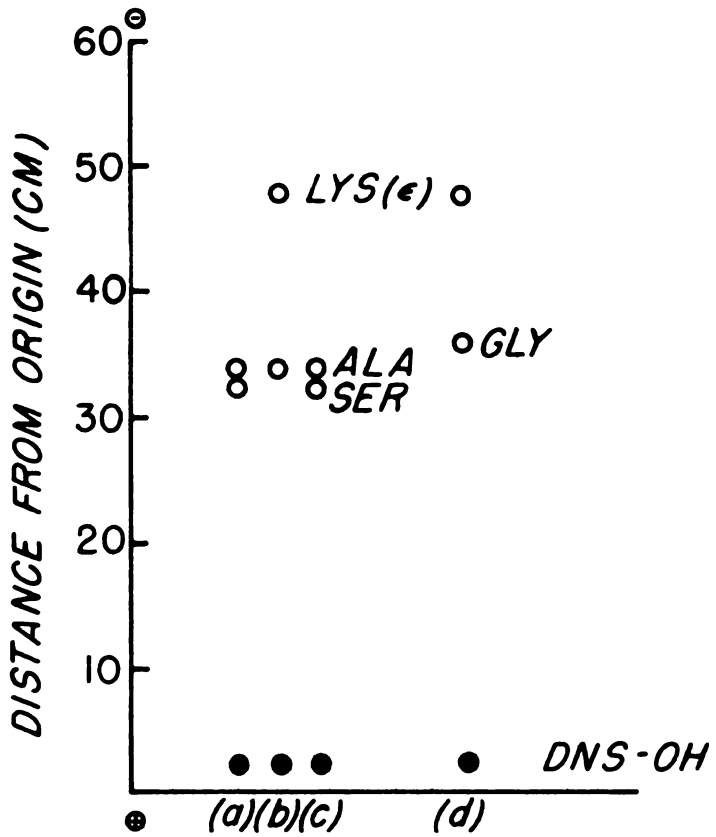


Figure 7. Electrophoretic Mobilities of Gas-vacuole Protein (b) and Standard Amino Acid Dansyl Derivatives at pH 1.9 (50 v/cm, 2 hours, 20°). Abbreviations are as in Figure 6.

excluded, however, since amino acid analysis of the protein (Part One) does not show it to be present. Glycine was excluded since no fluorescent material was found on the anode side of the DNS-OH. Thus either serine or alanine are left, with the latter being more likely since two distinct spots fluorescing blue and green were seen.

The alternative between serine and alanine was decided on by performing electrophoresis at pH 1.9. At this pH the amino acids in question move out from the DNS-OH band. The order of serine and alanine is now reversed as shown in Figure 7. The dansyl chloride derivative of the gas-vacuole protein migrates closely with the front of the dansyl-serine, dansyl-alanine standards. Alanine is, therefore, the only amino-terminal amino acid found in the gas-vacuole protein. DNS-ε-lysine is seen to correspond with its standard at pH 1.9 also.

Carboxyl-terminal Amino Acids. There was no positive evidence of any carboxyl-terminal amino acids being released from the protein after incubation with carboxypeptidase A. Development with cadmium-acetate-ninhydrin revealed no amino acid spots nor were any fluorescent dansyl chloride derivatives detected in ultra-violet light.

DISCUSSION

The aggregation of membrane proteins make their characterization difficult. The protein of the gas vacuoles of Microcystis aeruginosa is even more difficult to solubilize than proteins of other membranes. Appreciable solubilization of the gas-vacuole protein occurred only in strongly protic solvents as shown in Table 1.

Many of the solvent systems and techniques effective in solubilizing the structural proteins of eucaryotic and procaryotic membranes are of limited value in solubilizing the gas-vacuole protein. For example, treatment of serial dialysis results in large increases (quantitative data are not given) in the solubility of mitochondrial and erythrocyte structural proteins (34,70,96). Structural proteins first dissolved in 88% formic acid or phenol-acetic acid-water (2:1:1 v/v/v) will remain in solution when dialyzed into 8 M urea, 6 M guanidine hydrochloride, 5% formic acid, or even distilled water (34,70,96). However, only a 3 fold increase in solubility of the gas-vacuole protein is obtained by this procedure (see Table 1).

The method of preparation for isolation and characterization of the structural proteins (e.g., ionic concentration,

pH, presence of detergents) is important in determining their solubility. Lenaz et al. have shown that the mitochondrial structural proteins are the most soluble in their native state (i.e., as part of the membrane) (34,70). They are readily solubilized in 8 M urea or 6 M guanidine hydrochloride and in certain instances in water; whereas if the structural proteins are not present in their native state, they cannot be solubilized in the above solvents. Ordinarily the most tenacious aggregates are solubilized by a combination of sodium dodecyl sulfate (0.1 wt. of protein) and 8 M urea (16,34,36,70). The gas-vacuole protein, however, resists such treatment, though it is isolated in a state which should closely correspond to that of the in vivo or native state.

The process of protein solubilization is only partially understood, but each protein solvent probably interacts with more than one type of bond (104). Only certain inferences, therefore, of the relative importance of the different types of bonds stabilizing a proteinaceous structure can be made from the data in Table 1.

The capacity of the strongly protic solvent to solubilize the protein of gas-vacuole membranes is probably best attributed to strong solvent-solute hydrogen bonding. The increased solubility of nonpolar residues and increased, intramolecular, electrostatic interactions within the protein molecules in formic acid, which has a dielectric constant

of ca. 40 (Part One) may, however, be important in the disruption of the native structure.

In fact, the content of non-polar amino acids in the gas-vacuole protein (52%) is the same as that of the structural protein from mitochondria, in which hydrophobic interactions are considered to be the primary stabilizing forces (15,36). The relatively high content of non-polar amino acids increases the possibility that the geometry of the molecules is such that hydrophobic regions of one molecule are complementary to those on the surface of a second molecule, leading to stabilization by hydrophobic interactions.

Nevertheless, since lipophilic reagents, such as anionic detergents, in combination with urea do not dissociate the gas-vacuole protein (see Table 1), the importance of hydrophobic interactions in stabilizing this protein is apparently much less than in other structural proteins. The structural proteins from other membranes are readily dissociated by the lipophilic reagents in combination with urea.

Some evidence suggests that urea also weakens hydrophobic interactions (87, 104). This might be pertinent to the ineffectiveness of 8 M urea as a solvent of the gas-vacuole protein. Although the strong hydrogen bond-forming capacity of urea is considered to be its most important characteristic as a protein solvent.

Some information on the conformation of the protein cast from different reagents can be obtained by infrared

spectroscopy. Infrared spectroscopy is one technique in which a true solution or crystals are not necessary to study protein conformations. The membrane protein is, however, investigated as a dry film. Inspection of the infrared spectra in the regions of the amide I and II bands make possible some preliminary structural assignments. For the beta-conformation the absorption maxima are at about 1,630 and 1,520 cm^{-1}, and for an alpha-helix or a random coil conformation the maxima are at about 1,660 and 1,540 cm^{-1} in the amide I and II regions respectively (80,121).

The two infrared absorption bands in both the amide I and II regions indicate the presence of both the alpha-helix or random coil and beta-configurations in the protein of intact gas vacuoles. The existence of different conformations in the gas-vacuole protein resembles that of structural proteins from the eucaryotes, in which circular dichroism likewise shows a multiplicity of conformation states (34,116).

The fact that the gas-vacuole protein is very insoluble in the aqueous solvents is also in accord with evidence that its conformation is to a large extent in the beta-conformation or sheet structure.

Exposure to formic acid causes an almost complete transition to the beta-conformation, as seen by an increase in all of the bands characteristic of this conformation. A shoulder at 1,735 cm^{-1}, due tc C=O stretching of unionized carboxyl groups, is also produced in this solvent.

Another interesting point is the lack of any absorption band at 1,740 cm^{-1} of the intact vacuoles at neutral pH. This band, which is found in the IR spectra of plasma membrane, is due to C=O stretching in fatty acid esters and CH_2, CH_3 bending (121). After extraction of the plasma membrane with 2:1 chloroform-methanol these bands disappear as expected (121). The absence of these bands, characteristic for lipids, further substantiates the finding that lipid does not seem to be associated with the gas-vacuole membranes.

There is evidence to support the hypothesis that only one protein of a molecular weight of 14,000-15,000 makes up the gas-vacuole membrane.

The most convincing evidence is the presence of only a single band upon polyacrylamide gel electrophoresis at pHs 2 and 4. This criterion of homogeneity of a protein preparation at present is the best known. This has been clearly demonstrated by Lenaz et al. in the characterization of mitochondrial structural protein (70). Mitochondrial structural protein, appearing homogeneous by other physical and chemical criteria, is resolved into several components upon polyacrylamide gel electrophoresis at acidic pHs (70).

Knowing that lysine and arginine comprise 10% of the gas-vacuole protein (Part One), twelve peptides would be predicted upon tryptic hydrolysis if the protein is composed of a single subunit with a molecular weight of approximately 14,000. Fourteen peptides were found on the fingerprint,

however, two were very light, and were not detected in some runs. Thus, the tryptic digest results substantiate the hypothesis that the gas-vacuole membrane is composed of identical subunits. Furthermore the molecular weight of ca. 14,000 agrees with calculations based on the electron microscopic dimensions and density determinations (see Part One).

Additional evidence that supports the hypothesis was obtained from amino-terminal amino acid analyses. If the gas-vacuole protein is a single protein species, a single amino acid residue would be expected to appear at the amino end of the molecule and another at the carboxyl terminus of the chain.

The dansyl-chloride reaction products indicate that the gas-vacuole protein has only alanine as its amino-terminal amino acid.

Carboxyl-terminal amino acids could not, however, be detected. This could be the consequence of several difficulties. First, the specificity of the enzyme, carboxypeptidase A, might be the reason. For example, the carboxyl-terminal amino acid could be arginine which is not cleaved by the enzyme (3). In addition glycine and the acidic amino acids are released only very slowly. Also, the presence of other chemical groups might block access of the enzyme. A second major difficulty could be a result of the extreme insolubility of the gas-vacuole protein. The arrangement of molecules or their aggregates could prevent enzymatic

hydrolysis by some steric configuration which denies the enzyme ready access to the end of the chain. Nevertheless, the fact that no hydrolytic products were detected would favor a single protein species (15).

The success of determining the sedimentation coefficient of the smallest subunit of a protein depends on the ability to produce a subunit that will be stable long enough to measure it with accuracy. At the relatively high gas-vacuole protein concentration, more than one moving boundary is observed in the analytical centrifuge suggesting a certain degree of polymerization. This phenomenon is known to occur when aggregates are present (30). Quantitative estimates of heterogeneity are precluded by the self sharpening of the protein boundary which is due to a strong dependency of the sedimentation coefficient on concentration (100). It seems, therefore, that the strong aggregation of the gas-vacuole protein has not been overcome sufficiently to produce a stable subunit. Further experiments must be performed at very low concentrations, using optical systems of greater sensitivity, before the question of heterogeneity and subunit size can be answered by ultracentrifugation.

The chemical resemblances between structural proteins from different membrane systems (e.g., erythrocyte ghosts, sarcoplasmic reticulum, chloroplasts, mitochondria) have been sufficiently close to suggest that these proteins are members of a distinctive class of proteins (34). However, the

structural proteins isolated from procaryotes, namely from Hydrogenomonas facilis (62), and the flagellins from different species of bacteria (73) and the gas-vacuole protein do not appear to be very similar. The molecular weights, amino acid compositions, peptide maps, and solubilities are all different.

SUMMARY

The purified protein which constitutes the membranes of the gas vacuoles from the blue-green alga, Microcystis aeruginosa Kuetz. emend Elenkin, was partially characterized. Several methods and reagents were used in efforts to solubilize the protein. Strongly protic solvents as formic acid were the only reagents in which appreciable solubilization of the membrane protein occurred. End-group analyses, tryptic digests, and gel electrophoresis at acidic pHs indicate that the protein is a single species. Infrared spectroscopy reveals that the membrane protein has substantial amounts of both the alpha-helix or random coil conformation and of the beta-conformation.

PART THREE

ULTRASTRUCTURAL AND CONFORMATIONAL CHANGES IN GAS-VACUOLE MEMBRANES FROM *MICROCYSTIS AERUGINOSA* KUETZ. EMEND ELENKIN

INTRODUCTION

A biological membrane is qualitatively described by models such as bimolecular leaflets or as lipoprotein subunits. The bimolecular leaflet model is thought to be a protein-lipid-protein sandwich (17,19); whereas the subunit model puts more emphasis on the protein-protein interactions (7). In both models, the structure and function of the membrane proteins depend on the molecular interaction forces between proteins and lipids.

Biological membranes may act as transducers, establish cell compartments, act as amplifiers, or provide basic structural units. Our understanding of the genesis, architecture and function of cell membranes at the molecular level is fragmentary.

Information on cellular membranes has been obtained with a variety of techniques such as electron microscopy and X-ray analysis. Recently, spectroscopic techniques have been applied to study the structure and conformational changes of

membranes. In addition to optical rotatory dispersion (47, 117,121) and nuclear magnetic resonance relaxation (79,120), electron paramagnetic resonance spectroscopy (spin labeling) has been introduced.

McConnell (40,110) developed the spin-labeling techniques for biological applications. This method is based on the observation that a radical is sensitive to its environment. Examining the electron paramagnetic resonance (EPR) spectrum of such a molecular label, which has been covalently attached to the otherwise non-paramagnetic biological material, provides information on structural and functional changes of the membranes and biological macromolecules. As spin labels, nitroxide containing organic molecules are presently used (39) since the electron is preferentially localized at the nitrogen, which has a nuclear spin-quantum number of unity; the line shapes of the EPR spectra of these nitroxide radicals may show a symmetrical three line pattern, or they may be anisotropic, depending upon the environmental factors which influence the tumbling frequency of the organic radical. If the rotation is restricted the line shapes become broader and the tumbling frequencies are of the order of 10^8 Hz. The tumbling rates of freely rotating labels are of the order of 10^{11} Hz.

Spin labeling has been applied to the study of ATP dependent conformational changes in the sarcoplasmic reticulum (63), uptake of mitochondrial lipids in Neurospora crassa (52),

conformational transitions due to oxidation of electron transport particles from bovine heart mitochondria (59), conformational changes in erythrocyte membranes induced by phenothiazine drugs (98), and conformational changes in nerve membranes (46,75).

Similar to paramagnetic tags, fluorescent molecules may also be used to study protein conformations (74,108). Such fluorescent probes are covalently attached to the macromolecule and sense environmental alterations via noncovalent interactions with the macromolecule. Fluorescent probes have been used to investigate energy dependent structural changes in fragmented membranes from beef-heart mitochondria (5).

I have investigated conformational changes of a membrane by the spin and fluorescent labeling techniques.

This membrane is the one which constitutes the cylindrical gas vacuoles found in certain blue-green algae; the organism used was Microcystis aeruginosa. The gas vacuoles of this organism have a constant diameter of 69 nm and a length of 360 ± 90 nm. The thickness of the membrane is about 3 nm. The membrane consists only of proteins (Part One); it has been highly purified and exhibits a well defined ultrastructure (49,105).

Gross alterations of the gas vacuoles were observed with the electron microscope. More subtle and partially irreversible conformational changes were induced by the application of pressure, enzymatic digestion, pH and temperature variations.

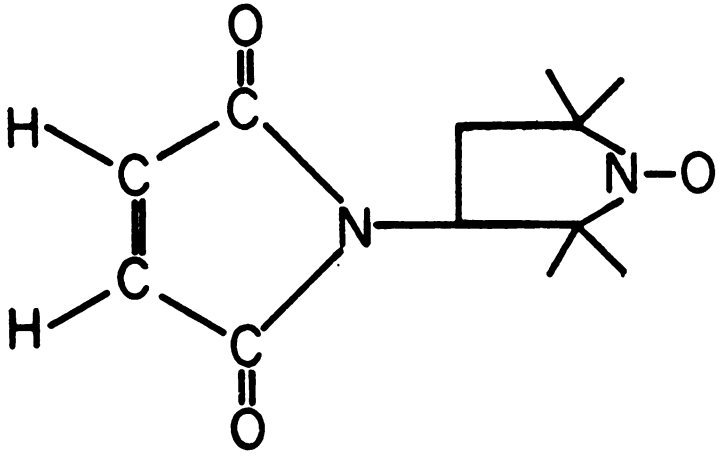
I have applied the spin labeling technique to the study of conformational alterations of gas vacuoles which are not readily studied by methods like optical rotatory dispersion because of the light scattering characteristics of intact organelles.

MATERIALS AND METHODS

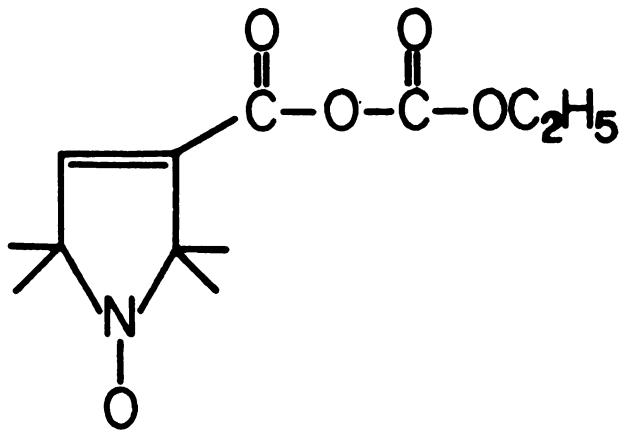
Membranes and EPR Labels. The vacuolar membranes were isolated from the blue-green alga, Microcystis aeruginosa, as described in Part One. The paramagnetic labels (Figure 1) N-(1-oxyl-2,2,5,5-tetramethylpyrrolidinyl)-maleimide and N-(1-oxyl-2,2,5,5-tetramethylpyrrolidinyl)-ethyl anhydride were synthesized according to procedures of Rozantsev and Krinitzkaya (97) and Griffith et al. (38).

EPR Labeling. The membranes were labeled by mixing about 2 mg of membrane protein in 3 ml of 0.1 M phosphate buffer, pH 7.5, with 0.2 mg of the spin label and then stirred for approximately 4 hours at 4°. The free, hydrolyzed label was removed by exhaustive dialysis for 20 hours. All samples were stored at 4° and used within 2 to 3 days. Electron micrographs show that only non-aggregated vacuoles were present at the concentrations employed, namely, 0.3 mg of protein/ml, corresponding to approximately 5×10^{11} vacuoles/ml (unpublished data, M. Jost).

EPR Spectra. The spectra of spin-labeled vacuolar membranes were recorded with a Varian 4502-15 X-band electron-paramagnetic resonance spectrometer which employs a Mark II magnetic field regulator and a 100 kHz field modulation unit.



A



B

Figure 1. The Spin Label N-(1-oxyl-2,2,5,5-tetramethyl-pyrrolidinyl)-maleimide (A) and N-(1-oxyl-2,2,5,5-tetramethyl-pyrrolidinyl)-ethyl anhydride (B).

The melting curves of the spin-labeled vacuolar membranes were measured in a flat quartz cell (volume about 0.1 ml) which could be inserted into the variable temperature accessory of the EPR spectrometer.

Fluorescent Labeling. The vacuolar membranes were labeled as in the case of spin labeling. The magnesium salt of 8-anilino-1-naphthalene sulfonic acid (ANS) (K and K Laboratories, Hollywood, Calif.) was recrystallized twice from hot water. The fluorescent spectra were measured with an instrument, modified for fluorescent experiments, which has been recently described (92).

RESULTS

Optical Spectra of Isolated Gas Vacuoles. Intact vacuoles in suspension have a milky appearance. The spectrum, mainly due to light scattering, was plotted from 230 to 700 nm (Figure 2). Upon application of hydrostatic pressure of about 1 atm and more, the solution clears up and displays a slight opalescence. The main absorption is now in the region of the aromatic amino acids (Figure 2).

Electron Microscopy of Intact and Pressurized Vacuoles. Frozen-etched samples of intact vacuoles show that the vacuoles are closed cylinders having a membrane made up of ribs with a spacing of 4 nm. These ribs consist of small granules with a spacing of approximately 3 nm (Figures 3 and 4) (49). Gradually pressurized gas vacuoles transform into flattened-out bags of roughly rectangular shape (Figure 5), sometimes folding under an angle of $60 \pm 8^{\circ}$ (49). Sudden pressure changes of a rate larger than 1 atm/sec releases ribs of various length from the membrane (Figure 6). The width of the vacuolar cylinder is rather consistent with a diameter of 69 ± 3 nm; the length of the vacuoles varies, namely, 360 ± 90 nm (49).

EPR Spectra of Spin-labeled Vacuolar Membranes. The EPR spectra of the free ethyl-anhydride label in phosphate

Figure 2. Absorption Spectra of Intact (dashed curve) and Collapsed (solid curve) Vacuoles.

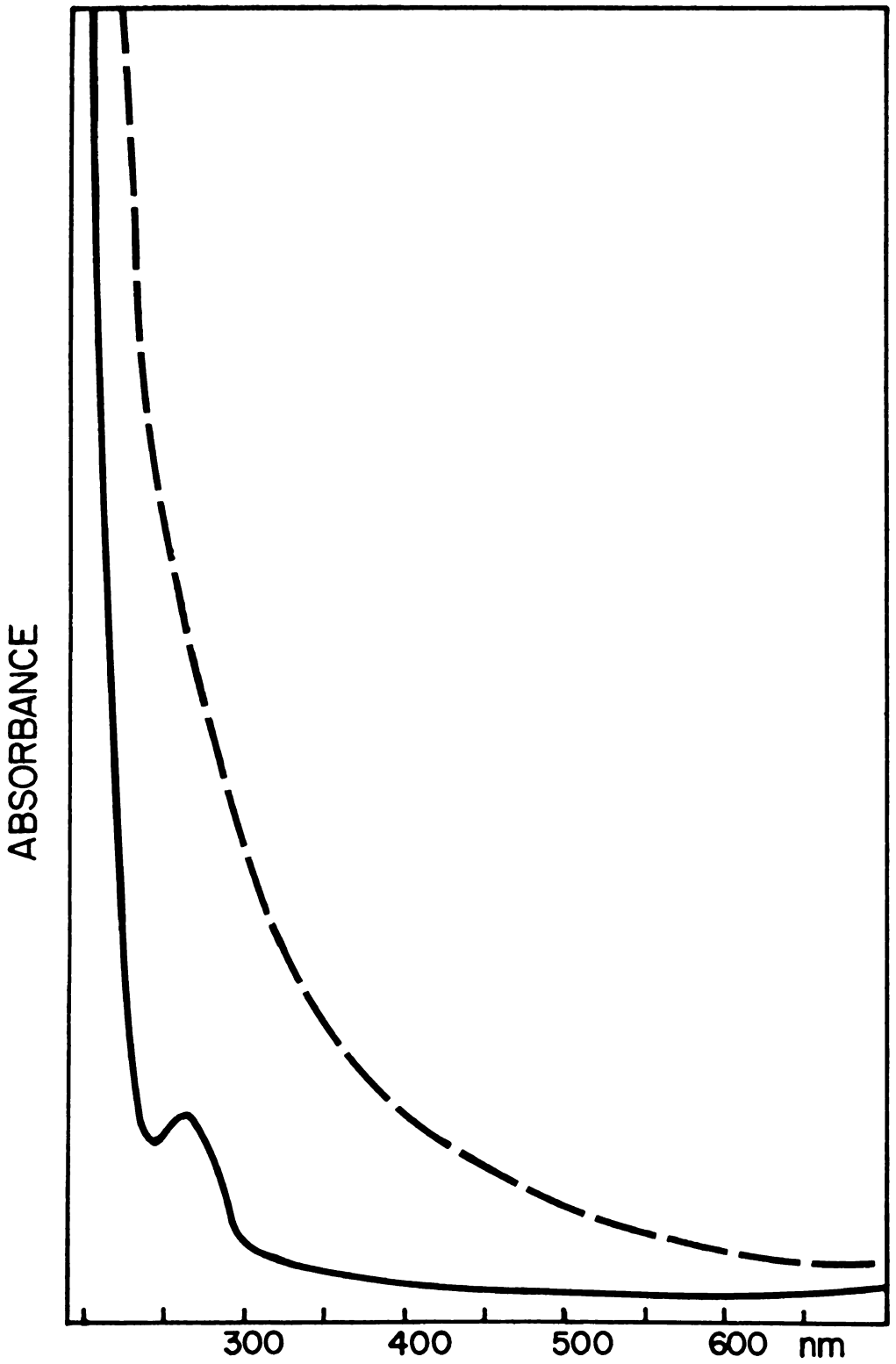


Figure 2

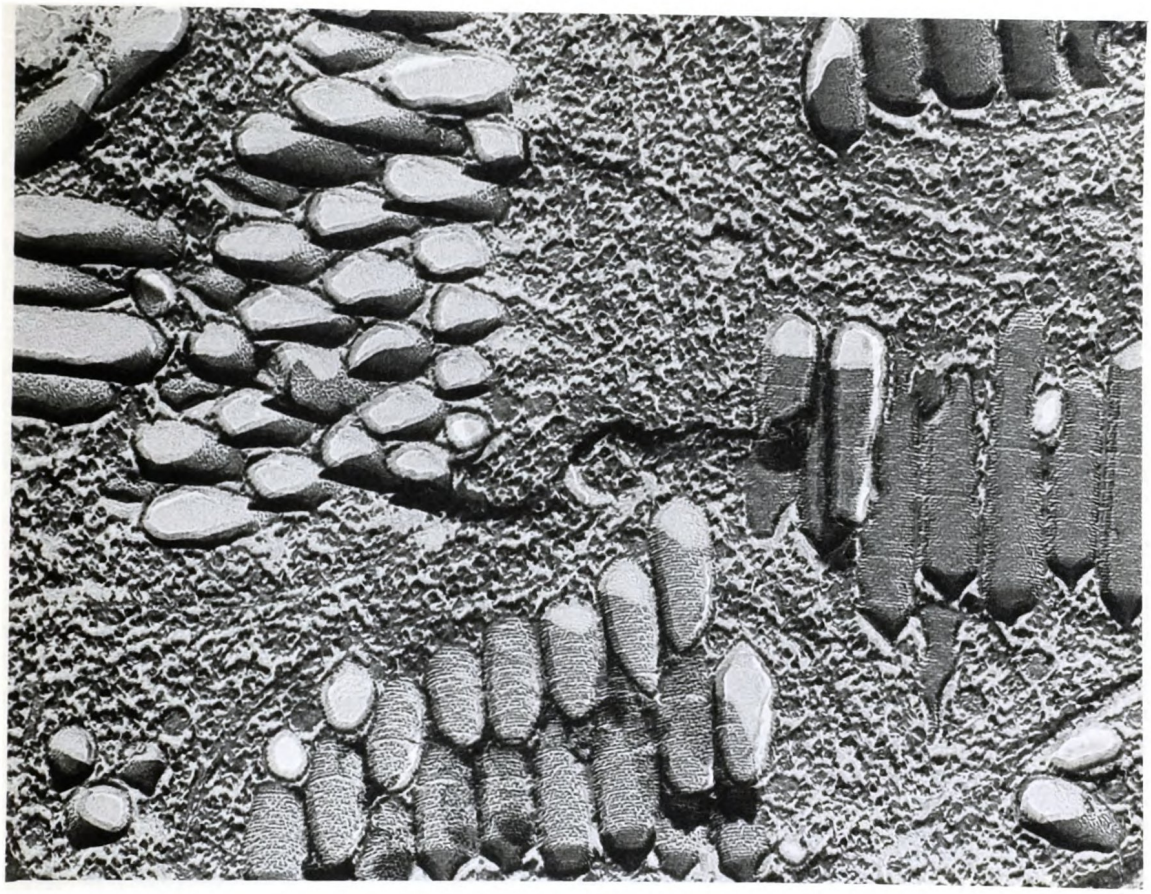


Figure 3. Gas Vacuoles in a Cell of Microcystis aeruginosa Prepared by the Freeze-etching Replica Technique. Shadowing is with platinum-carbon. The cylindrical organelles are fractured under different angles and lie as clusters in the cytoplasm. The freeze-etching preparation was done after infiltrating the cells for 15 minutes in 1.5 M glycerol. 57,000 x

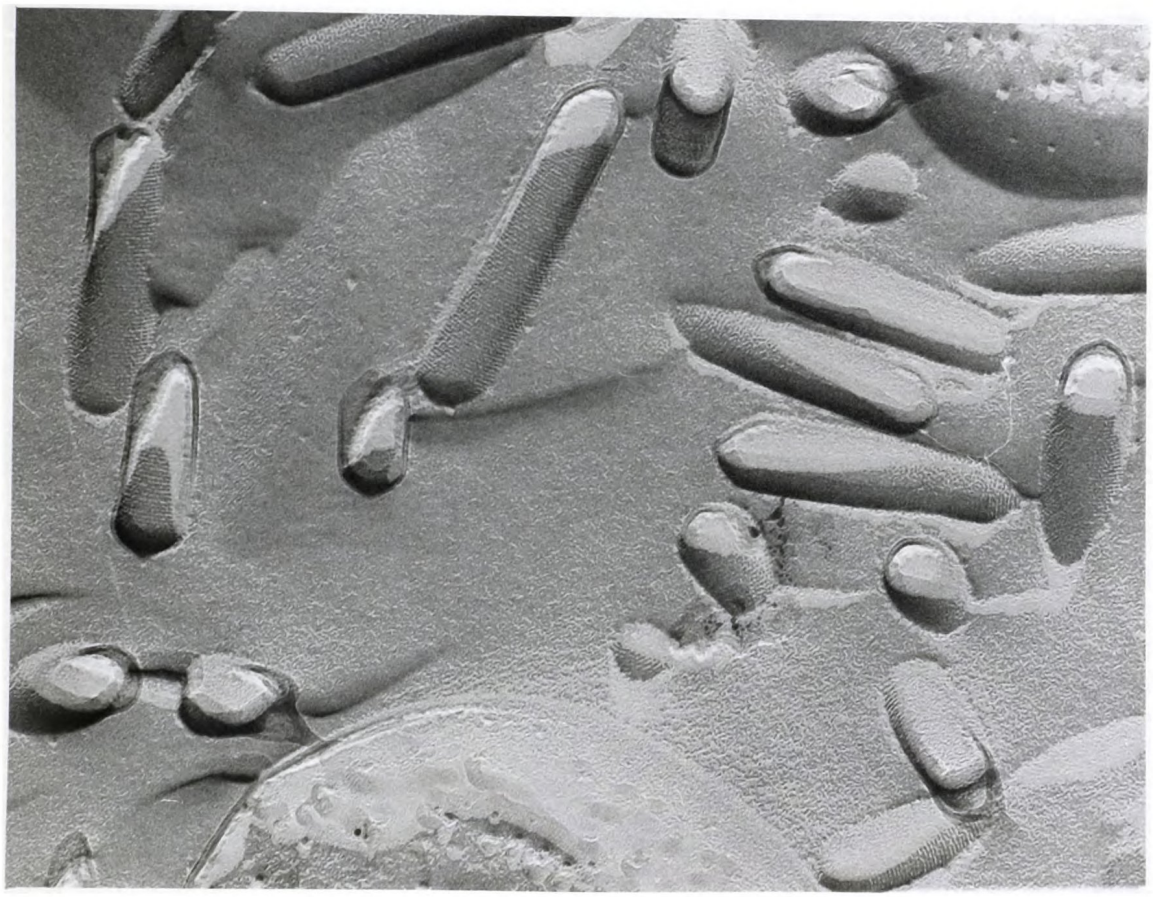


Figure 4. Highly Purified, Intact Gas Vacuoles in 1.5 M Glycerol and 0.01 M Tris-HCl, pH 7.7. The surface structure on the inside of the vacuoles shows ribs consisting of protein particles. 86,000 x

Figure 5. Isolated Gas Vacuoles Subjected to Pressure. Shadowing was done with platinum-carbon under approximately 10°. 76,000 x

Figure 6. Vacuoles Ripped Open by Surface Tension. The ribs released resemble strings of beads. 145,000 x

buffer at neutral pH and of the labeled, intact gas vacuoles at room temperature are shown in Figure 7. Compared to the free label, the spectrum of the bound label exhibits a broadened three-line pattern. With increasing temperature (Figure 7) the line width dimenishes and the signal height increases concurrently, e.g., from 0° to about 80° the intensity of the middle hyperfine line becomes larger by a factor of 2 to 3, varying from one preparation of vacuolar membranes to another. With an increase in temperature the EPR spectra show a more symmetric hyperfine line pattern.

Qualitative Mechanical and Chemical Alterations of Spin-labeled Gas Vacuoles at Room Temperature. As already mentioned, the milky suspension of intact gas vacuoles clears upon application of hydrostatic pressure. Such a treatment causes the middle hyperfine line in the EPR spectrum to decrease in height by about 20 to 30% compared to its height for intact gas vacuoles; the two outer hyperfine lines maintain approximately their original height (Figure 7).

Adding chloroform to a suspension of intact gas vacuoles (1:1) clears the suspension and yields a more symmetric hyperfine pattern as compared to intact vacuoles in buffer.

At 4° or at 25°, and upon standing for 4 days and more the hyperfine lines of the EPR spectrum of labeled vacuoles become more intense and more symmetric.

Except for a decrease in signal height of about 10%, the EPR spectra of spin-labeled vacuoles did not change

Figure 7. Electron Paramagnetic Resonance Spectra: of (a) the free spin label N-(1-oxyl-2,2,5,5-tetramethyl-pyrrolidinyl)-ethyl anhydride at room temperature, (b) of spin-labeled, intact gas vacuoles from Microcystis aeruginosa at 0^{o}, (c) 25^{o}, (d) 82^{o} at neutral pH in phosphate buffer. Applying pressure to gas vacuoles alters spectrum (c) to spectrum (e). All spectra have the same vertical and horizontal scale. The scan rate of the magnetic field was 100 gauss/25 minutes and the field increases from left to right in the spectrum.

their pattern when the pH was lowered from neutrality to 3.

Addition of guanidine-HCl to the milky suspension, to a final concentration of 6 M, caused the suspension of intact vacuoles to clear; the middle hyperfine line increased by 20%, and the outer lines increased by a factor of about two, as compared to the spectrum of intact vacuoles. Additional storage for several hours at room temperature does not alter the signal height.

If labeled intact vacuoles are incubated with trypsin for 20 hours the hyperfine lines of the EPR spectrum increase in intensity almost three times and the lines sharpen. The suspension remains milky, but clears upon application of a very slight pressure. Such an EPR spectrum indicates a high mobility of the free radical, with a tumbling rate of the order of 10^{10} Hz. Being attached to the second amino group of lysine or arginine, the label contains more freedom of motion relative to the protein membrane since trypsin splits preferentially peptide bonds formed by basic amino acids.

Labeling vacuoles with the maleimide label instead of the ethyl or phenyl anhydride label yields rather poor EPR spectra. This is consistent with the finding that the vacuolar membrane contains no detectable thiol groups (Part One). The maleimide label is known to react preferentially with thiol groups, whereas the anhydride label reacts covalently with the amino groups of lysine and arginine.

Amino acid analysis showed that the vacuolar protein contains about 10% of these amino acids (Part One).

Temperature Dependence of the EPR Spectrum of Spin-labeled, Intact Gas Vacuoles. As mentioned, heating of the vacuoles from 0^{o} to about 80^{o} results in a more symmetric hyperfine line pattern with sharper lines (Figure 7). Plots of the ratio of the signal height of the middle and the high field hyperfine line as a function of temperature are given in Figure 8. This graph demonstrates a pronounced hysteresis effect resulting from irreversible processes in the membrane material. The signal height of the free spin label increases by about 20% when the temperature varies from 0^{o} to about 80^{o}, as compared to the more than 200% increase of the bound label.

There exists a rather sharp transition temperature of 39 ± 1^{o}; hysteresis is only found above that temperature.

This melting point was determined in the following manner. The EPR spectrum of a sample was first measured at 1^{o}, then the sample was warmed up to some higher temperature, maintained at that temperature for 15 min, and the spectrum recorded. Then the sample was cooled down to the starting temperature of 1^{o}, the EPR spectrum measured and compared with that recorded before warming the sample.

A different suspension of vacuoles was then treated as above except warming it to a higher temperature than the previous sample.

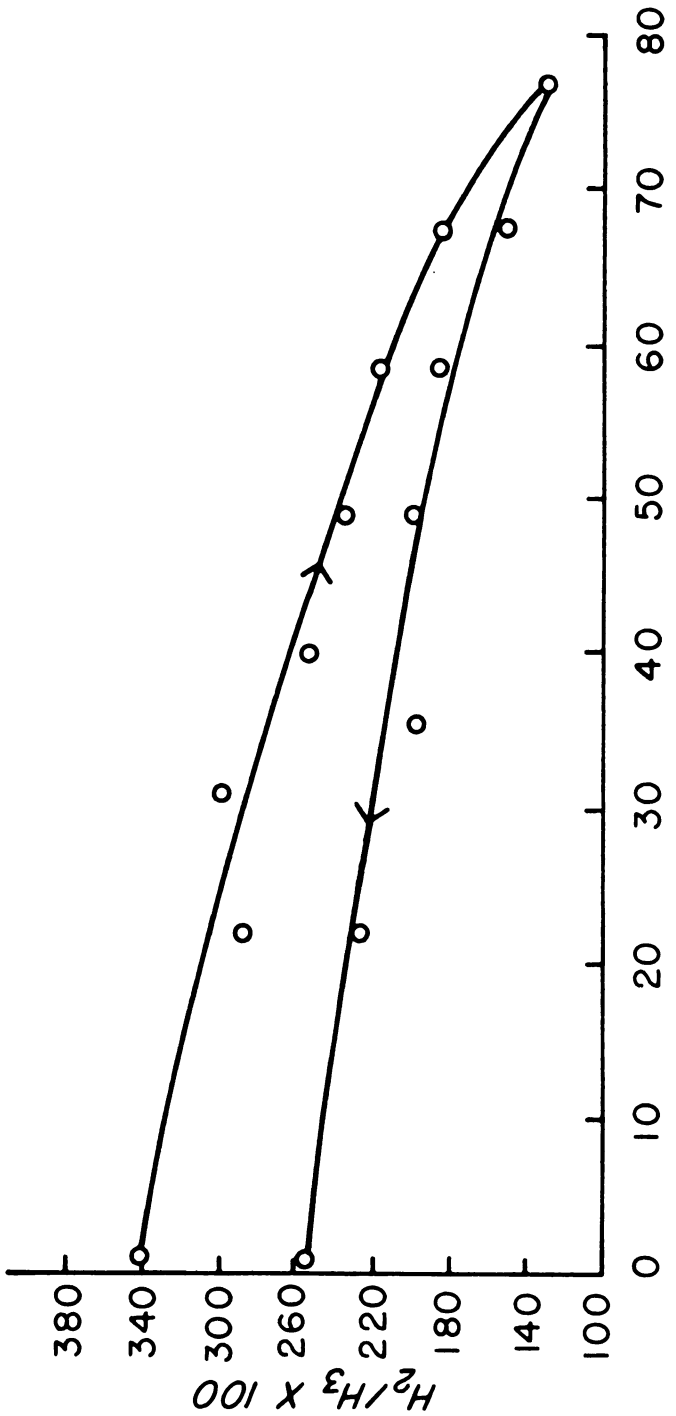


Figure 8. Temperature Dependence of the Ratio H_2/H_3 of the Signal Heights of the High Field (H_3) and the Middle Hyperfine Line (H_2) of the EPR Spectrum. All measurements were performed with one and the same sample, first warmed (upper branch), then cooled (lower branch). The temperature was maintained for 15 minutes at the temperatures indicated in the graph.

Up to 39 ± 1°, the spectra at 1°, before and after warming, could be superimposed independent of whether the spectra were from the same sample or from different samples. From these data it can be concluded that thermally induced changes of the membrane proteins are reversible.

Fluorescent Labeling Experiments with Vacuolar Membranes at Room Temperature. Intact gas vacuoles were labeled with the fluorochrome ANS at neutral pH and excited at 365 nm. Whole vacuoles show only an indication of a fluorescent shoulder; whereas there is a large increase in the fluorescent intensity of vacuoles which have been subjected to pressure (Figure 9). The strikingly enhanced fluorescence of vacuolar membrane sheets has a maximum at 465 nm.

Figure 9. Fluorescent Spectra of Gas Vacuoles Labeled with the Fluorochrome ANS at Neutral pH and Excited at 365 nm. Dotted line: intact gas vacuoles. Solid line: gas vacuoles collapsed by pressure.

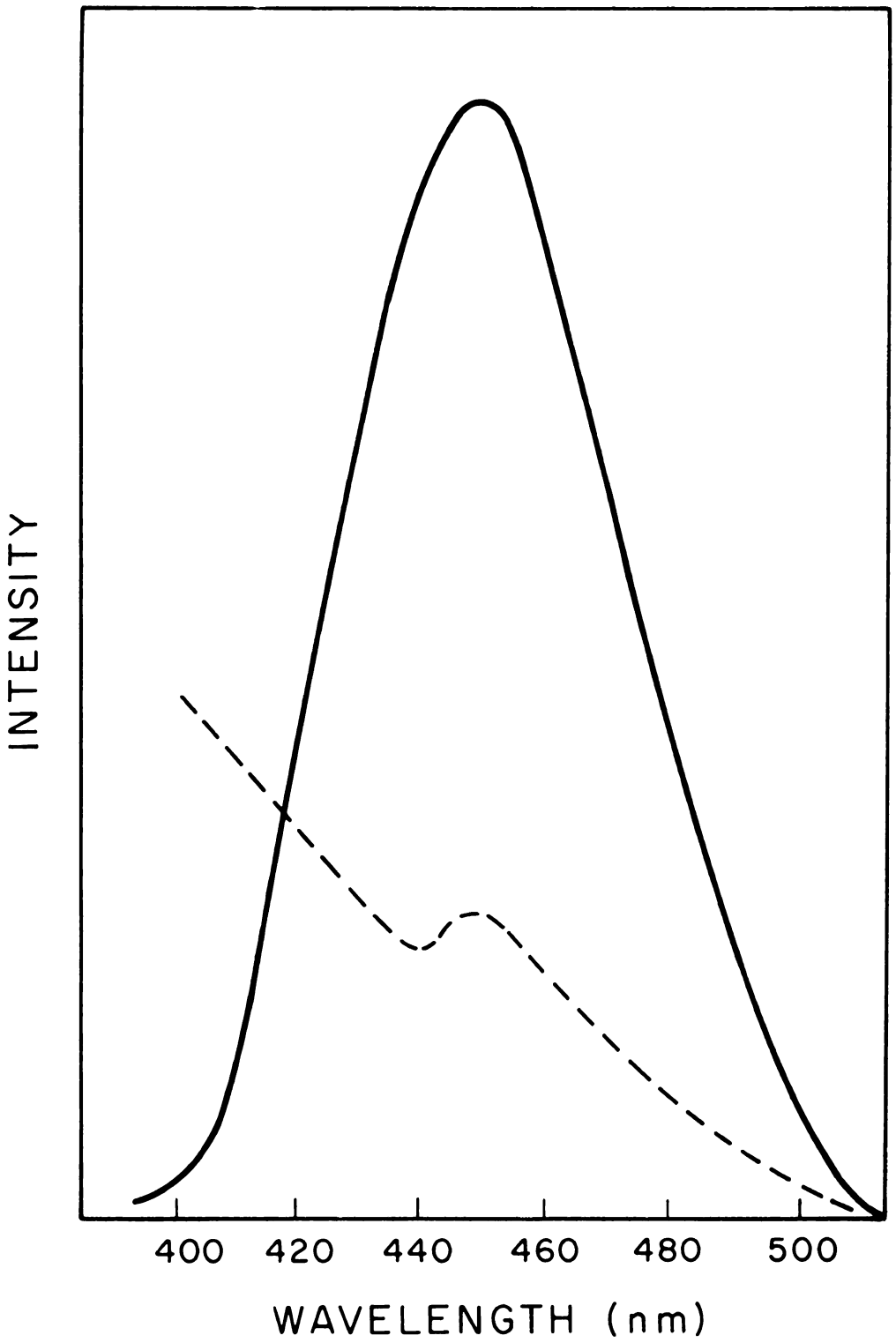


Figure 9

DISCUSSION

Electron micrographs show that the cylindrical gas vacuoles transform into flattened bags and break down into ribs when the vacuoles are pressurized. These ribs apparently confer rigidity to the cylindrical structure of the gas vacuole. Gas permeability studies of algal gas vacuoles demonstrate that the pressure inside of the gas vacuoles is about one atmosphere (122).

Application of pressure to collapse intact gas vacuoles results in an enhanced fluorescent intensity which reflects a pronounced change in the surroundings of the binding site of the ANS label. The effective radius (45) of such a site is in the order of 0.5 nm. Stryer (112) demonstrated that ANS is covalently attached to the hydrophobic heme crevice of apomyoglobin. The stronger fluorescent intensity of compressed gas vacuoles probably arises from a trapping of the ANS label in a hydrophobic environment such that the label becomes isolated from free water molecules.

Collapsing intact vacuoles by means of pressure transforms the typical EPR spectrum of intact gas vacuoles to a more symmetrical pattern. The latter spectrum reflects a weakly immobilized spin label in a more isotropic environment, probably due to a reorientation of protein chains.

The line width and signal heights of the EPR spectra of spin labeled vacuoles are thermally reversible for temperatures below 39°; this in turn indicates thermally reversible conformational alterations of the membrane. These reversible changes could not be detected by electron microscopy or by light absorption. Similarly, subtle changes may be important for the biochemical and physical functions of a membrane. The hysteresis effect above 39° reflects a progressive denaturation of the protein of the membrane since new modes of vibrations of the protein are thermally created. These new modes can disrupt bonds between neighboring protein chains. For spin labeled transfer RNA a sharp melting point has been found which depends on the ionic strength of the medium (43).

With increasing temperature, the EPR spectrum increases in intensity and three lines become narrower and of nearly equal height. Such a spectrum is typical of a free label rapidly tumbling in a nonviscous solvent such as water.

Such a temperature dependence is to be expected when the spin label attached to secondary amino groups obtains a higher degree of rotational freedom relative to the protein surface. This is also consistent with preliminary electron microscopic data which seem to indicate that intact gas vacuoles swell and become rounder, when a suspension of vacuoles is heated to 80° followed by negative staining at room temperature. At low temperatures the spin label is partially buried in hydrophobic regions of the membranes. However, with increasing

temperatures these areas become unfolded because of the deformation of the protein structure.

However, there is insufficient information to determine what type of interactions are involved in maintaining the structure of the vacuolar membrane. The surface of intact and pressurized gas vacuoles seems to be hydrophobic, since gas vacuoles adsorb preferentially to hydrophobic surfaces (49). The fluorescent intensity is practically independent of pH changes in the range from pH 2 to pH 8, which may indicate that ionized groups do not account for the quenching of ANS-labeled intact vacuoles. When beta-lactoglobulin (74) is labeled with TNS, an analog of ANS, the fluorescent quantum yield decreases with decreasing pH; this has been interpreted in terms of a group in the binding region which altered its ionization state.

The interaction forces within the rib itself appear stronger than those between ribs (49). Thus, it is reasonable to assume that the intercostal regions of the vacuolar membrane are more accessible to alterations induced by temperature, pressure and chemicals.

SUMMARY

Highly purified, intact gas vacuoles were isolated from the blue-green alga, Microcystis aeruginosa.

Local conformational changes of the gas-vacuole membrane were investigated with spin and fluorescent labeling techniques. Studies on the temperature dependence of the electron paramagnetic resonance (EPR) spectra of spin-labeled intact vacuoles demonstrate a sharply defined transition temperature of 39^{o}. Below that temperature the conformational change of the vacuolar membrane remains thermally reversible; above that temperature irreversible processes occur. The rate of tumbling of the spin label attached to the vacuolar surface increases concurrently with the temperature, indicating that in the membrane new modes of vibrations are thermally induced in the protein which narrow the line width of EPR spectra.

A suspension of the intact gas vacuoles, which has a milky appearance, clears upon application of hydrostatic pressure, while the EPR spectrum of the spin labeled membrane becomes more symmetric and the area under the middle hyperfine line is reduced by approximately 25% compared to intact vacuoles. This suggests a rearrangement of the protein of the membrane such that the paramagnetic label is less

restricted in its motion relative to the protein surface. Ultrastructural studies reveal that the vacuoles collapse under pressure and consist of membranous sheets, ribs, and granules.

Intact vacuoles labeled with anilinonaphthalene sulfonate show a weak fluorescence while vacuoles subjected to pressure fluoresce strongly.

Irreversible conformational changes of the membrane were induced also by guanidine-HCl, chloroform, and trypsin digestion.

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