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CHARACTERIZATION OF THE PLASMA MEMBRANE OF *MYCOPLASMA LAIDLAWII*

## V. EFFECTS OF SELECTIVE REMOVAL OF PROTEIN AND LIPID

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## SUMMARY

Purified plasma membranes of *Mycoplasma laidlawii* were subjected to proteolytic enzyme digestion and aqueous acetone extraction. Up to 80% membrane protein could be removed by pronase, with a concomitant decrease in density of residual banding material. This membrane residue was still organized in vesicles and showed a decreased thickness and contrast in electron micrographs. Despite degradation of all characteristic membrane proteins (as shown by detergent-polyacrylamide gel electrophoresis) some protein fragments were retained in vesicular residues. The amino acid composition of this material was indistinguishable from that of total membrane protein, arguing against any enrichment in hydrophobic protein regions. Membrane hexosamine was not solubilized by pronase or aqueous acetone extraction, and could be substantially purified by combining these techniques. Extraction with 90% aqueous acetone removed over 95% of labeled membrane lipid but left sedimentable, protein-rich membranous material with unit membrane morphology. Such lipid-depleted membranes banded in sucrose gradients at a density of 1.25 g/cm<sup>3</sup> as a single narrow band, in contrast to the isopycnic density of 1.17–1.18 g/cm<sup>3</sup> for native membranes.

At low pronase concentrations, electrophoresis experiments suggested preferential pronase attack on some higher molecular weight membrane proteins; these same electrophoretic components were preferentially released at very low concentrations of sodium dodecyl sulfate, indicating differences in the organization of proteins within the membrane. Analysis of membranes labeled with [<sup>14</sup>C]glucosamine or [<sup>14</sup>C]oleate by detergent-gel electrophoresis followed by gel fractionation and counting showed that all oleate-containing lipid moved as a single broad band, while glucosamine migrated as two bands, one extremely broad and heterogeneous.

## INTRODUCTION

Studies of the structure and chemical composition of *Mycoplasma laidlawii* membrane, with emphasis on solubilization and reassembly, have been described in

previous publications in this series<sup>1-4</sup>. In this report another technique, controlled digestion with pronase (a nonspecific proteolytic enzyme), is applied to the study of membrane structure. When analytical procedures are applied to partially digested membranes, information is made available on the localization of protein. We have also studied the effects on membrane structure of substantial removal of both protein and lipid components. In addition we have extended the chemical analysis of membrane-bound hexosamine and have characterized membrane-bound flavin. Improved methods have been applied to the study of polyacrylamide-gel-electrophoresis patterns of membrane proteins, solubilized by sodium dodecyl sulfate.

## MATERIALS AND METHODS

### *Membrane preparation*

Growth of *M. laidlawii* B cells and procedures for the isolation of purified membranes have been described<sup>1,5</sup>. For the present experiments, improved growth and membrane yield were obtained in a medium consisting of heart infusion broth (Fisher Scientific), 25 g in 900 ml of water. Autoclaved medium was supplemented with 100 ml of sterile 10% glucose, 10 ml of Bacto-PPLO serum fraction and 500 000 units of penicillin. Cells were grown for 24 h at 37° with a starting inoculum of 30 ml of a 24-h culture. To obtain membranes, cells were lysed by exposure to low ionic strength buffer, the membranes were banded in a sucrose gradient and the banded material was washed to remove sucrose.

All experiments with purified membranes were carried out in NaCl-Tris- $\beta$ -mercaptoethanol buffer<sup>5</sup> which contains: 7.8 mM NaCl, 2.5 mM Tris, 0.5 mM  $\beta$ -mercaptoethanol in deionized water, adjusted to pH 7.4 with HCl. Labeling of membrane glucosamine was accomplished by growing cells in a medium made of dehydrated heart infusion broth (Fisher Scientific), 2.5 g; Tris, 3.0 g; NaCl, 5.0 g; water, 800 ml. The medium was adjusted to pH 8.0 with HCl and autoclaved. To this medium was added 10 g of dextrose in 200 ml of water and 10 ml of Bacto-PPLO serum fraction. The final medium was dispensed in 10-ml aliquots to which were added 10  $\mu$ C of D-[1-<sup>14</sup>C]glucosamine (New England Nuclear Corp.) at an activity of about 250  $\mu$ C/mg. The tubes were inoculated with 0.2 ml of starter culture and incubated for 24 h at 37°. Membranes were then prepared by the method previously described. To check the specificity of labeling, <sup>14</sup>C-labeled membranes were hydrolyzed for 18 h at 103° in constant-boiling HCl. The hydrolysate was dried and run on a two-dimensional paper chromatogram (*sec.*-butanol-formic acid-water (70:10:20, by vol.) and phenol-conc. ammonia-water (80:0.3:20, by vol.)). The resulting chromatogram was then radioautographed on X-ray film. The position of glucosamine on this chromatographic system was determined by running the pure compound and locating the position by ninhydrin staining.

Labeling of membranes with [<sup>14</sup>C]oleic acid was carried out as previously described<sup>1</sup>.

### *Analytical procedures*

Protein was assayed by the Folin method of LOWRY *et al.*<sup>6</sup> using bovine serum albumin as a standard.

Hexosamine was assayed by the following modification of the Boas<sup>7</sup> modifica-

tion of the method of ELSON AND MORGAN<sup>8</sup>. Samples of 0.2 ml containing about 1 mg of membrane protein and 10–100  $\mu$ g of hexosamine were added to 0.8 ml of 6 M HCl and hydrolyzed for 3 h at 100°. Samples were cooled, mixed with 1 ml of 4 M NaOH and 0.05 ml of 0.5% phenolphthalein in ethanol, and titrated to a color end point with 2 M NaOH. To keep volumes and salt concentrations constant, 2 M NaCl was added in appropriate volumes to tubes requiring less NaOH. The samples were made slightly acidic with 0.5 M HCl and all tubes were brought to the same volume with water. To each sample 1 ml of 2% acetyl acetone (v/v) in 1.0 M Na<sub>2</sub>CO<sub>3</sub> was added. Samples were placed in a boiling water bath for 20 min, cooled, and 2.5 ml of ethanol was added to each, followed by 1 ml of Ehrlich's reagent (2.67% (w/v) solution of *p*-dimethylaminobenzaldehyde in a 1:1 mixture of ethyl alcohol and conc. HCl. After 30 min the absorbance was read at 530 m $\mu$  against a blank consisting of a 0.2-ml water sample carried through the entire procedure. Calibration solutions of known glucosamine concentration were used.

Amino acid analyses of membranes and pronase-digested membranes were carried out as previously described<sup>4</sup>.

Absorption spectra were run on a Bausch and Lomb Spectronic 505. Radioactive samples were dried on planchets and counted on a Beckman low-beta thin-window counter. Membrane protein digestions were carried out at 50 or 37° in a 1:20 dilution of NaCl-Tris- $\beta$ -mercaptoethanol buffer<sup>5</sup> at a membrane-protein concentration of the order of 1 mg/ml. Pronase (California Biochemical B Grade) was used in concentrations varying from 1 to 250  $\mu$ g/ml.

To extract lipids, membranes were suspended in 1 vol. of 1:20 NaCl-Tris- $\beta$ -mercaptoethanol buffer and vigorously stirred into 9 vol. of acetone at room temperature for 30 min. The resulting suspension was centrifuged at 15000  $\times g$  for 20 min and the supernatant and pellet were separated.

#### *Density-gradient centrifugation*

(a) Linear 4.5 ml 25–50% (w/w) sucrose gradients in 1:20 NaCl-Tris- $\beta$ -mercaptoethanol buffer were constructed, overlaid with 0.5-ml samples of control or pronase-digested membranes, and centrifuged 4 h (20°) at 37500 rev./min in an SW-39 rotor and Spinco Model L-2 centrifuge. Selected samples were centrifuged for 20 h and showed the same peak positions as comparable 4-h samples, thus indicating that equilibrium had been reached in 4 h. Gradients were unloaded in 10-drop fractions and analyzed for [<sup>14</sup>C]oleate. Selected fractions, along with control gradients subjected to identical conditions, were analyzed for refractive index with a Bausch and Lomb Abbe-3L refractometer at 20°; standard tables were used to convert these values to specific gravity. (b) Linear 4.5 ml 40–70% (w/w) sucrose gradients in NaCl-Tris- $\beta$ -mercaptoethanol buffer were used to band acetone-extracted and control membrane samples. After centrifugation at 39000 rev./min for 20 h (20°) in an SW-50 rotor, gradients were unloaded in 10-drop fractions and the refractive indices of fractions in the region of banded material were determined. (c) In some experiments, linear 4.5 ml H<sub>2</sub>O-<sup>2</sup>H<sub>2</sub>O gradients (20–100% in <sup>2</sup>H<sub>2</sub>O, 0.1 M in NaCl, and 10 mM in sodium dodecyl sulfate) were employed. Membrane samples (0.5 ml solubilized in 10 mM sodium dodecyl sulfate) were layered on the gradients, centrifuged at 50000 rev./min in an SW-50 rotor for 24 h (20°), drop-collected in 0.25-ml fractions, and analyzed for protein.

### *Polyacrylamide-gel electrophoresis*

The sodium dodecyl sulfate-polyacrylamide gel system of MAIZEL<sup>9</sup> was employed. Gels were made up in 10 or 20 cm lengths in glass tubing with an inner diameter of 5 mm. Standard gels contained 7.5% acrylamide, 0.2% *N,N*-bismethyleneacrylamide, 0.1% sodium dodecyl sulfate, 0.1 M sodium phosphate buffer (pH 7.0), 0.05% *N,N,N',N'*-tetramethylethylenediamine and 0.075% ammonium persulfate. Gel buffer (0.1% sodium dodecyl sulfate and 0.1 M sodium phosphate (pH 7)) was recirculated during each run. Samples containing 50–500  $\mu$ g protein were made up in 0.1–0.3-ml volumes in 1% sodium dodecyl sulfate, 0.01 M sodium phosphate (pH 7), and 1%  $\beta$ -mercaptoethanol; heated at 37° for 15 min or at 100° for 2 min; mixed with 0.2 vol. of 60% (w/w) sucrose; and layered over the gels. Electrophoresis was carried out at 50–90 V for 2–6 h (10-cm gels) or 70 V for 16 h (20-cm gels) with a Spinco Duostat power supply at constant voltage. In some experiments, 8 M urea was used in gels, samples, and buffer in addition to the detergent.

For staining, gels were removed from glass tubing, fixed overnight in 20% sulfosalicylic acid, stained 3 h in 0.25% Coomassie blue, and destained through several changes of 7% acetic acid. For radio-isotope counting, 20-cm gels were removed from glass tubes and fractionated in a Maizel Autogeldiver (Savant Instruments, N.Y.) into about 60 fractions which were dried and counted on planchets.

### *Electron microscopy*

**Negative staining.** Membrane samples in dilute neutral buffer were applied to the surface of 200 mesh copper grids, previously coated with formvar and carbon films and rendered hydrophilic by 15–30 min exposure to ultraviolet light immediately prior to use. After about 30 sec, excess liquid was drained and a drop of 2% sodium phosphotungstate (pH 7.4) was applied. Excess liquid was again removed and the grids dried and stored *in vacuo* until examined.

**Thin-sections.** Samples containing 0.2–1.0 mg material were pelleted and fixed in formaldehyde and OsO<sub>4</sub> as described previously<sup>3</sup>. After dehydration in acetone, samples were cut into small cubes, embedded in Epon, sectioned with a Porter-Blum ultratome, and collected on formvar-carbon coated 200 mesh copper grids. Sections were stained for 15 min in saturated uranyl acetate in 50% ethanol, washed through three changes of water, counterstained for 5 min with lead citrate<sup>10</sup>, washed in 0.2 M NaOH and water, and coated with an additional stabilizing carbon layer. For each sample, two or three blocks were sectioned and examined. No detectable differences were seen in these samples.

All specimens were examined in a Siemens Elmiskop I electron microscope at a 20000–80000 plate magnification and 60 kV operating voltage.

## RESULTS

### *Pronase digestion*

Exposure of *M. laidlawii* membranes to pronase at 50° causes a decrease in turbidity, suggesting that membrane protein is being digested. Fig. 1 illustrates some quantitative features of this digestion process in an experiment where membrane protein and lipid were assayed in supernatants and pellets of a 100000  $\times$  *g* centrifugation after digestion for different times at four different pronase concentrations. The

amount of remaining sedimentable protein falls rapidly in the first 5 min and then declines more slowly, showing little change after the first hour of digestion (Fig. 1; solid lines). After 20 h of digestion, up to 80% of the protein can be removed; addition

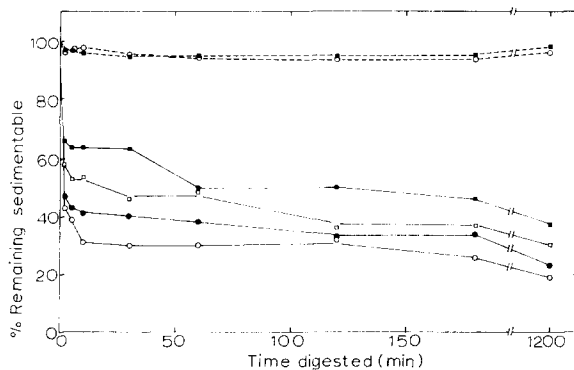


Fig. 1. Pronase digestion of Mycoplasma membranes. Identical [ $^{14}\text{C}$ ]oleate-labeled membrane suspensions containing 1 mg membrane protein/ml in NaCl-Tris- $\beta$ -mercaptoethanol buffer were incubated at  $50^\circ$ . Pronase was added to final concentrations ( $\mu\text{g/ml}$ ) of: 1 (■); 10 (□); 50 (●); or 250 (○). At indicated times, aliquots were chilled and centrifuged at  $100000 \times g$  for 30 min. Supernatants and pellets were separated and assayed for protein and lipid content.

The ordinate indicates percentage of total protein (solid lines) and lipid (dotted lines) remaining in the  $100000 \times g$  sediment. The four lipid curves were indistinguishable; only two of them are plotted here.

of more pronase at this stage has no further effect. The extent of protein digestion is a function of pronase concentration, but even at pronase concentrations as low as  $1 \mu\text{g/ml}$  about 50% of membrane protein is rapidly released from the sedimentable residue.

In contrast to the rapid digestion of protein, membrane lipid is not significantly released from sedimentable material at any of the pronase concentrations tested (Fig. 1; dotted lines). This observation argues against the possibility that some of the protein released to the supernatant upon pronase treatment may be in the form of small, nonsedimenting membrane fragments induced by pronase action, since membrane lipid would then be expected to appear in the supernatant along with protein.

In order to further characterize the chemical composition and morphologic character of residual membrane material remaining sedimentable after pronase digestion (henceforth called membrane residue), we have employed density gradients, electron microscopy, amino acid analysis, and gel electrophoresis at different stages of pronase digestion.

*Density gradient analysis:* Pronase was added to a membrane suspension, aliquots were withdrawn at various times, chilled, and sedimented through sucrose gradients under conditions in which the undigested (control) membrane would band at its isopycnic density of  $1.18 \text{ g/cm}^3$  (Fig. 2a). The distribution of radioactively labeled membrane lipid across each gradient (Fig. 2) shows that the isopycnic density of membrane residue shifts upwards concomitantly with the decrease in membrane-bound protein. During the early release of protein (up to 50% of the total) there is no loss of lipid from the membrane residue, which forms a single narrow band (Figs. 2b and 2c). More extensive proteolysis leads to a new fraction of lipid-containing

material at the top of the gradient in addition to the banded material (Figs. 2d–2f). This new fraction represents either membrane fragments too small to equilibrate or material too buoyant to enter the gradient; the independent observation that over

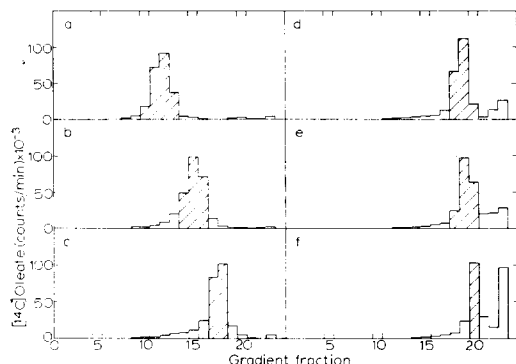


Fig. 2. Density-gradient distribution of [ $^{14}\text{C}$ ]oleic acid-labeled *Mycoplasma* membranes, digested to different degrees with pronase at  $50^\circ$ . After digestion for time indicated below, aliquots were centrifuged in 25–50% (w/w) sucrose gradients for 4 h at 37 500 rev./min at  $20^\circ$ . Gradient fractions were assayed for [ $^{14}\text{C}$ ]oleic acid and refractive index to determine density of the banded material; other aliquots, comprising the visibly banded material pooled from fractions indicated by diagonal shading, were examined by electron microscopy. Additional aliquots of the pronase-membrane solution were sedimented at  $100\,000 \times g$  for 30 min, and sedimentable protein content was assayed and compared to the control. a. Control membranes. 100% of protein sedimentable (by definition); density,  $1.18 \text{ g/cm}^3$ . b. Membranes digested 15 min with pronase/protein ratio 1:290; pronase at  $10 \mu\text{g/ml}$ . 82% of membrane protein remained sedimentable; density,  $1.15 \text{ g/cm}^3$ . c. Membranes digested 15 min with pronase/protein ratio 1:60; pronase at  $50 \mu\text{g/ml}$ . 60% membrane protein remained sedimentable; density,  $1.13 \text{ g/cm}^3$ . d–f. Membranes digested 15 min (d), 30 min (e), and 18 h (f) with pronase/protein ratio 1:17; pronase at  $173 \mu\text{g/ml}$ . 45% (d), 44% (e), and 20% (f) of membrane protein remained sedimentable. Densities: (d)  $1.12$ , (e)  $1.11$ , (f)  $1.10 \text{ g/cm}^3$ .

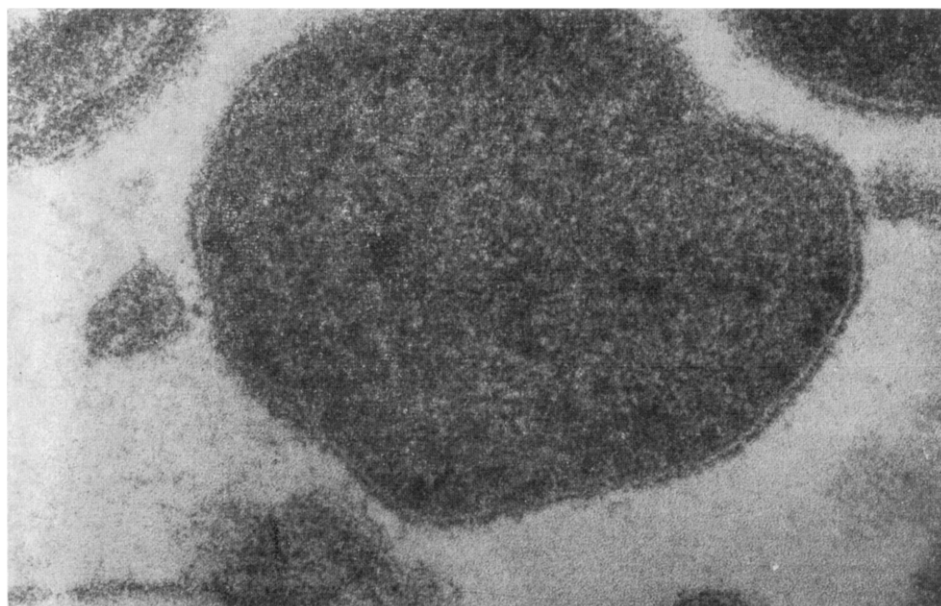


Fig. 3. *M. laidlawii* B cell from log-phase culture. The cell membrane is prominent, with no evidence of a rigid wall or internal membranes ( $\times 160\,000$ ).

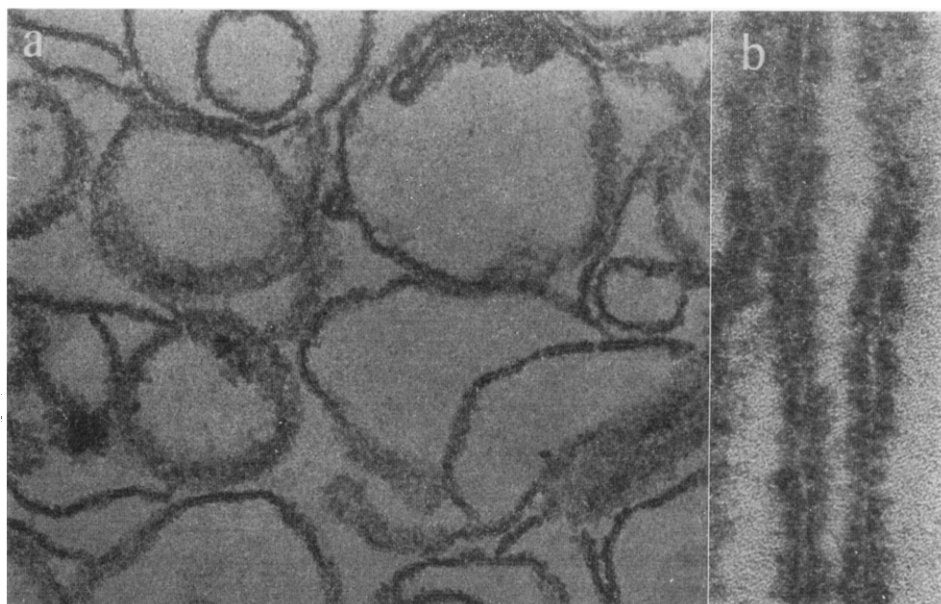


Fig. 4. a. Purified *M. laidlawii* membranes. Triple-layered structure and vesicular character are prominent ( $\times 65000$ ). b. Higher magnification of the same preparation. Membrane outer faces have a fuzzy character ( $\times 274000$ ).

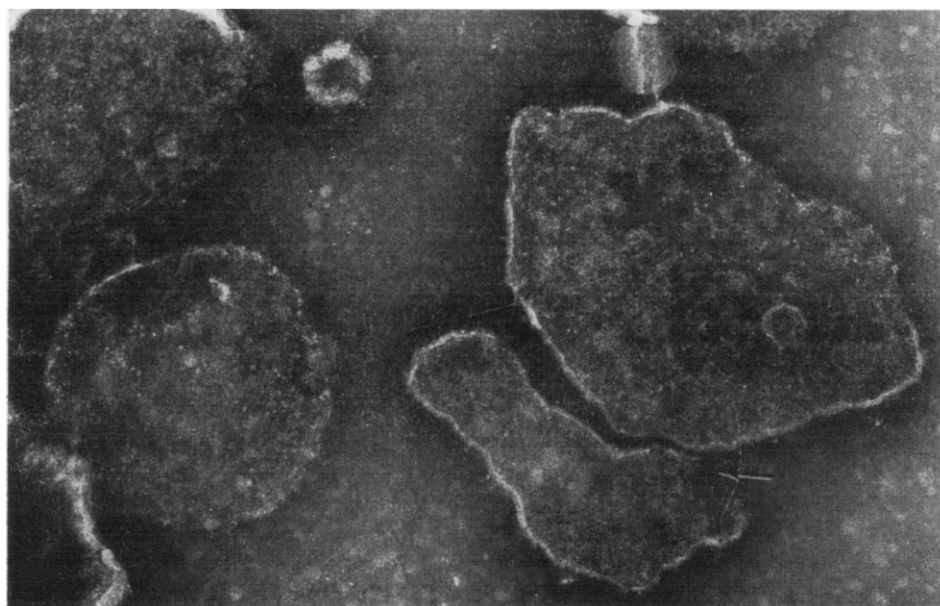


Fig. 5. Purified membranes of *M. laidlawii* negatively stained with phosphotungstate. Membranes resemble collapsed sacks and show heterogeneous surface structure ( $\times 62000$ ).



Fig. 6. *M. laidlawii* membranes depleted of over 95% membrane lipid by extraction with 90% aqueous acetone. Triple-layered structure is preserved. Membranes appear both as closed vesicles and open sheets ( $\times 68000$ ).

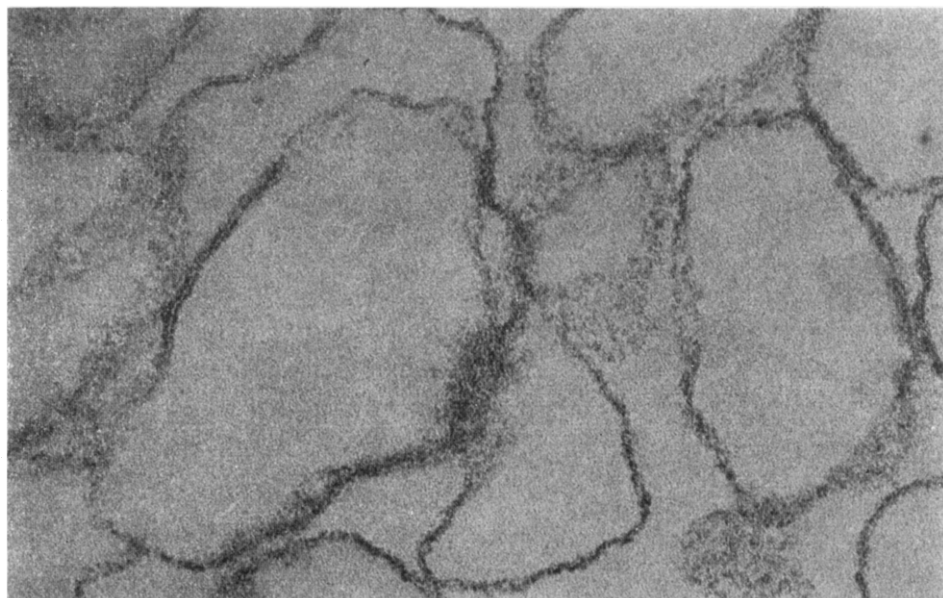


Fig. 7. *M. laidlawii* membranes digested 15 min with pronase at  $10 \mu\text{g/ml}$  (taken from gradient band in Fig. 2b). 82% membrane protein remained sedimentable in this preparation. Note loss of fuzz on membrane outer surfaces, trilaminar appearance of some areas, and thinner cross-sectional profile in other areas ( $\times 109000$ ).



95% of lipid counts from such samples are sedimentable in water at  $100\,000 \times g$  for 30 min (Fig. 1; dotted lines) suggests the latter alternative. Residual membranes seem to approach a limiting density of  $1.10 \text{ g/cm}^3$  at completion of pronase digestion.

**Electron microscopy.** The membrane residues from each of the gradients shown in Fig. 2 (representing the pooled fractions shown by hatchmarks) were washed, fixed, and examined in thin sections by electron microscopy. Control membranes are shown in Fig. 4; they have the familiar "unit" membrane appearance, with an overall thickness of 75–90 Å. These membranes show some "fuzz" along both exterior faces (Fig. 4b), compared to our previous membrane preparations<sup>2</sup>. This may be due in part to a change in culture medium (see MATERIALS AND METHODS). A number of isolated membrane profiles seen in section showed free ends, in contrast to the observation that many membrane systems form closed vesicles after mechanical breakage. Negatively stained membrane ghosts (Fig. 5) show a heterogeneous surface structure and resemble collapsed sacks with the occasional suggestion of an open, unsealed hole (Fig. 5, arrow). A representative thin sectioned *M. laidlawii* cell is shown for comparison in Fig. 3; the lack of cell wall and the absence of internal membranes are evident.

Several morphologic characteristics were prominent in membrane residues at various stages of protein depletion (representative micrographs are shown in Figs. 7 and 8): (a) All pronase-digested membranes showed a pronounced drop in electron-microscope contrast compared to the control; it was necessary for us to use both lead and uranyl stains to achieve sufficient contrast for visualization. While this impression

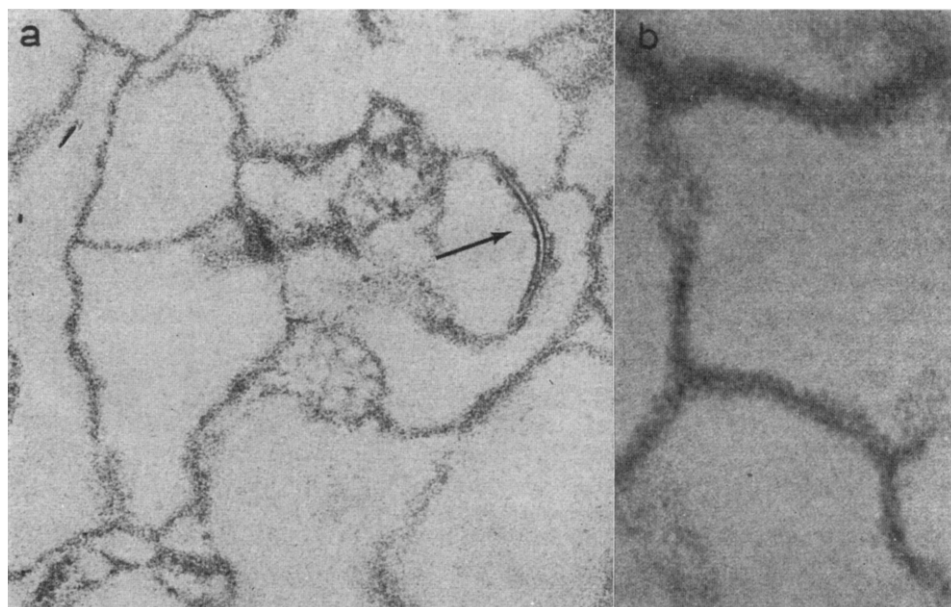


Fig. 8. a. *M. laidlawii* membranes digested 18 h with pronase at  $173 \mu\text{g/ml}$  (taken from gradient band in Fig. 2f). 20% of membrane protein remained sedimentable in this preparation. Note fusion of adjacent vesicle surfaces and thin cross-sectional profile (compared with rare areas maintaining a triple-layered profile, as indicated by arrow) ( $\times 109\,000$ ). b. Higher magnification of the same preparation. Some layered structure is visible. Membrane residues are tightly apposed at their surfaces ( $\times 211\,000$ ).

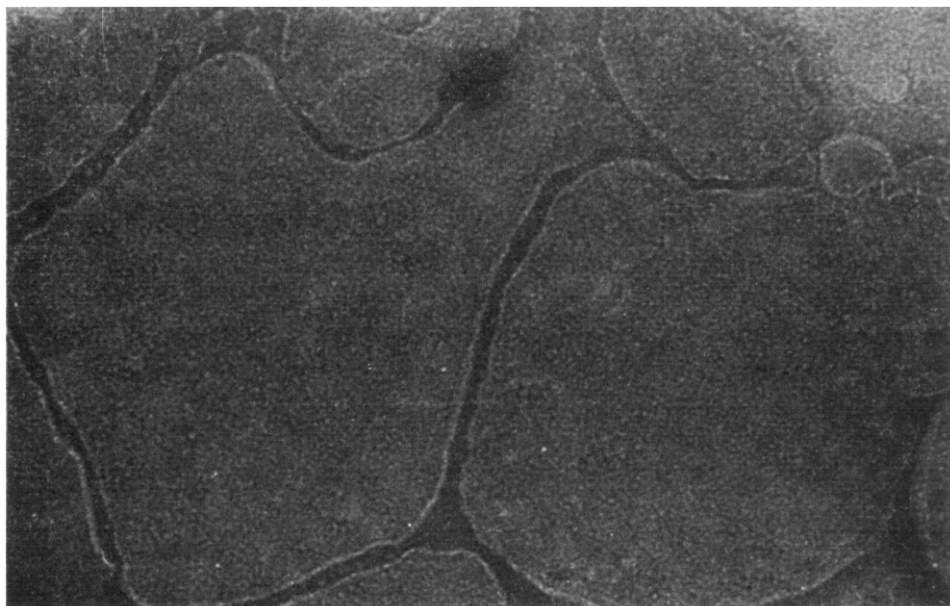


Fig. 9. *M. laidlawii* membranes were digested 5 min at  $50^{\circ}$  with pronase at  $28 \mu\text{g/ml}$  (pronase/protein ratio 1:82), chilled, and sedimented at  $100000 \times g$  for 30 min. Negatively stained portion of this material is shown here; 52% of initial protein remained sedimentable ( $\times 109000$ ).

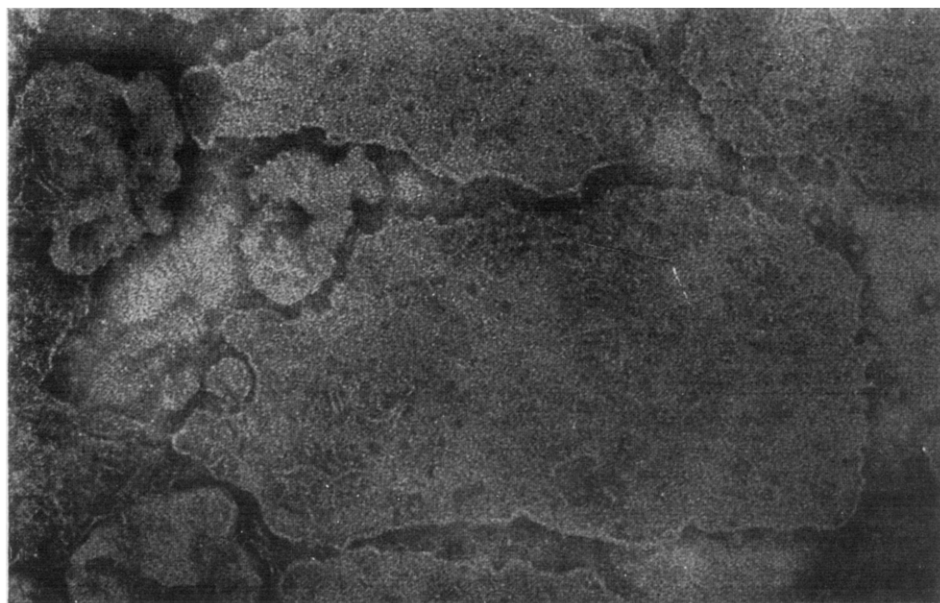


Fig. 10. *M. laidlawii* membranes were digested 4 h at  $50^{\circ}$  with pronase at  $28 \mu\text{g/ml}$  (pronase/protein ratio 1:82), chilled, and sedimented at  $100000 \times g$  for 30 min. Negatively stained portion of this material is shown here; 30% of initial protein remained sedimentable ( $\times 109000$ ).

is difficult to quantitate, it suggests a possible loss in osmiophilic membrane sites attendant on loss of protein. (b) The fuzzy character of control membranes disappeared in all pronase-digested membranes examined; membranes from Fig. 2 gradients b and c showed regions of prominent unit membrane structure (Fig. 7), but after prolonged pronase digestion the resulting vesicles (Fig. 8) showed a somewhat thinner surface profile (40–60 Å) and adjacent vesicle surfaces were generally tightly apposed (Fig. 8b). Characteristic unit membrane appearance was rarely seen after this extent of pronase digestion (Fig. 8, arrow). (c) Despite these rather drastic modifications of membrane ultra-structure, all membrane residues isolated from these density gradients retained an overall vesicular morphology; in addition, pronase-digested membranes showed relatively few open ends, suggesting that vesicle closure had followed pronase digestion.

These results were complemented by an examination of negatively-stained, pronase-digested membranes. After very limited pronase treatment, face views as shown in Fig. 9 are seen; surface structure is now quite homogeneous, in contrast to the heterogeneous character of undigested membranes (Fig. 5); this difference probably reflects loss of the external fuzzy layer seen in sections (Fig. 4a vs. Fig. 7). In addition, vesicles now lie in close apposition on the specimen support film (Fig. 9), suggesting some cohesion of vesicle surfaces as seen in section in Fig. 8. After further pronase digestion, negatively stained vesicles show some evidence of localized surface degradation (Fig. 10).

*Amino acid analysis.* The amino acid composition of purified membrane protein and the protein from membrane residue following prolonged pronase digestion until no further amino acid is released is shown in Table I. Also shown is the analysis pre-

TABLE I

AMINO ACID ANALYSIS OF PURIFIED MEMBRANE AND MEMBRANE RESIDUE FOLLOWING PRONASE DIGESTION AND PREVIOUSLY REPORTED DATA FOR WHOLE MEMBRANE

Pronase digestion for 18 h at 50° at a protein concentration of 1 mg/ml and a pronase concentration of 50 µg/ml. Correction for losses in hydrolysis were taken as 5% for threonine and tyrosine and 10% for serine. Amide content and tryptophan were not determined.

<i>Amino acid</i>	<i>Purified membrane (mole %)</i>	<i>Residual membrane protein following pronase (mole %)</i>	<i>Previous data on purified membrane (mole %)</i>
Lys	5.77	5.92	6.37
His	1.32	0.90	1.46
Arg	2.76	2.38	2.95
Cys	0.12	0.14	0.19
Asp	12.30	11.16	11.43
Met	2.15	2.22	2.33
Thr	7.90	7.96	6.77
Ser	7.44	8.34	6.40
Glu	9.59	8.10	8.23
Pro	4.29	5.23	3.60
Gly	8.31	9.38	6.95
Ala	9.62	10.45	8.23
Val	5.18	4.94	7.55
Ile	5.27	4.82	7.39
Leu	8.96	8.65	9.79
Tyr	4.36	4.70	4.81
Phe	4.70	4.66	5.40

viously reported for *M. laidlawii* membranes<sup>4</sup>. Differences exist for a few amino acids between previous determinations and the present data; these can probably be attributed to the differences in culture media for these two preparations. No significant differences exist between the whole membrane and the membrane residue obtained by enzymatic proteolysis.

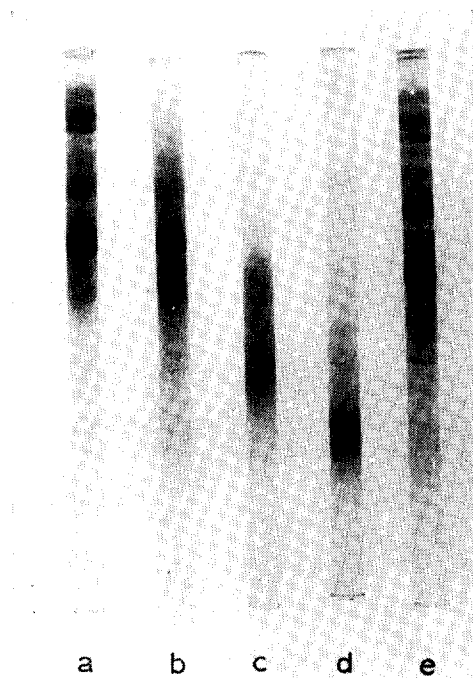


Fig. 11. Detergent-polyacrylamide gel electrophoresis patterns of *M. laidlawii* membranes subjected to different degrees of pronase digestion. a. Undigested membranes (400  $\mu$ g protein). b. Membranes digested 10 min (37°) with pronase/protein ratio 1:450, 1  $\mu$ g pronase/ml; 96% protein remained sedimentable. c. Membranes digested 10 min (37°) with pronase/protein ratio 1:45, 10  $\mu$ g pronase/ml; 84% protein remained sedimentable. d. Membranes digested 5 min (50°) with pronase/protein ratio 1:82, 28  $\mu$ g pronase/ml; 52% protein remained sedimentable. e. Undigested membranes (470  $\mu$ g protein). Each sample in 1:20 NaCl-Tris- $\beta$ -mercaptoethanol buffer was chilled after digestion for indicated time and centrifuged 30 min at  $100000 \times g$ ; pellets were taken up in 1% sodium dodecyl sulfate, 1%  $\beta$ -mercaptoethanol, 0.01 M sodium phosphate buffer (pH 7). Samples a-c and d-e were subjected to electrophoresis at 90 V for 2.5 and 3 h, respectively. Samples were applied at the top and migrated towards the anode at the bottom of the picture.

**Gel electrophoresis.** In order to determine whether any species of membrane proteins were remaining undigested after pronase treatment, we compared polyacrylamide-gel patterns of sodium dodecyl sulfate-solubilized control membrane proteins with those from membranes treated with pronase as used in the density-gradient experiments of Fig. 2. As shown in Fig. 11d, extensive pronase digestion results in complete loss of all characteristic membrane protein bands with the appearance of substantial amounts of low molecular weight material (for further discussion of acrylamide-gel results, see below).

**Sedimentation analysis.** The results of centrifuging detergent-solubilized membrane and membrane residue in a 20–100%  $^2\text{H}_2\text{O}$  gradient are shown in Fig. 12, where

we have measured protein distribution. Since this is a velocity gradient which never approaches isopycnic equilibrium, it is clear that the protein residue following enzyme digestion has an average sedimentation constant less than half that of the original membrane protein.

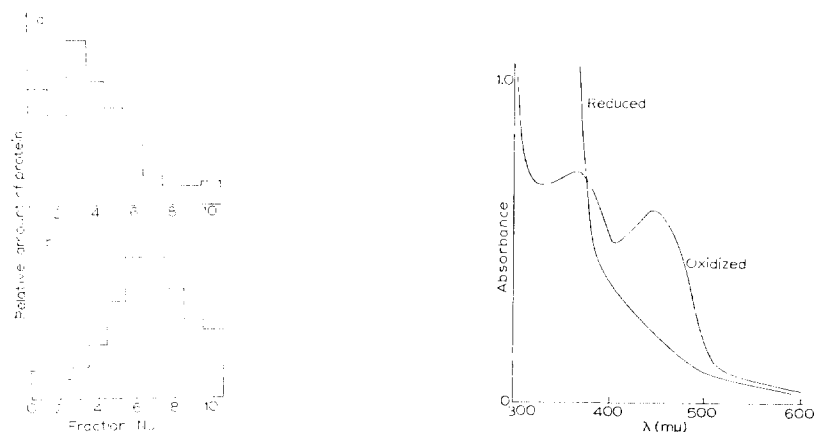


Fig. 12. Sedimentation of sodium dodecyl sulfate-solubilized protein from whole membranes and pronase-digested membranes in a 20–100 %  $^2\text{H}_2\text{O}$  gradient containing 0.1 M NaCl and 10 mM sodium dodecyl sulfate. Samples were centrifuged at 35000 rev./min for 24 h. Fractions were analyzed for protein using the Folin reaction. a. Pronase-digested membrane. b. Original membrane.

Fig. 13. Absorption spectra of oxidized and reduced flavin released from *M. laidlawii* membrane by digestion with pronase.

**Flavin characterization.** During the process of pronase digestion of membrane a yellow compound was released. In order to study this chromophore, membrane samples were digested to completion with pronase, the residual material was sedimented, and the absorption spectrum of the supernatant was examined in the range 300–600  $\text{m}\mu$ . The spectrum is shown in Fig. 13, and exhibits peaks at 366 and 445  $\text{m}\mu$ . When 10 mg of sodium hydrosulfite were added to a 3-ml cuvette containing this sample, the spectrum changed and both peaks disappeared, as indicated in Fig. 13. Bubbling air through the tube restored the original spectrum. This spectral behavior is typical of oxidized and reduced flavins and suggests that these membranes contain at least one flavoprotein.

#### Acetone extraction

The degree of lipid extraction was assayed by using purified membranes made from cells that had been grown in the presence of  $[^{14}\text{C}]$ oleic acid. Use of aqueous acetone removes over 95 % of the radioactive counts found in the whole membrane as well as releasing the yellow carotenoid pigment from the residual precipitate of lipid-depleted membrane.

Such acetone-extracted membrane preparations were pelleted, washed, and examined by thin-section electron microscopy. As shown in Fig. 6, these lipid-depleted membranes retain to a large degree both their vesicular character and their unit membrane appearance (despite some slight increase in overall thickness). Similar

preparations were sedimented to isopycnic equilibrium in sucrose-density gradients. They formed a narrow band with a density of  $1.25 \text{ g/cm}^3$ .

#### *Hexosamine characterization*

The absolute hexosamine content of membranes was determined by carrying out a series of protein determinations as well as Elson and Morgan hexosamine determinations on aliquots of a purified membrane suspension. A series of eight Lowry determinations gave a protein concentration of  $730 \pm 25 \text{ } \mu\text{g/ml}$ , and eight corresponding hexosamine determinations gave a value of  $65 \pm 4 \text{ } \mu\text{g/ml}$ . This ratio corresponds to about 6 moles of hexosamine per 100 moles of amino acid and is in reasonable agreement with the ratio of 7.48 moles hexosamine per 100 moles amino acid previously reported<sup>4</sup>. From the amino acid analyzer data we calculated a galactosamine to glucosamine ratio of 4.4 compared to 3.3 in the earlier study<sup>4</sup>.

The relation of hexosamine to other membrane constituents was determined by taking six aliquots of purified membrane and subjecting them to the following treatments: (1) control; (2) aqueous acetone extraction; (3) pronase digestion (at a pronase/membrane protein ratio of 1:20) for 3 h at  $50^\circ$ ; (4) pronase followed by aqueous acetone extraction; (5) aqueous acetone extraction followed by pronase; and (6) pronase, followed by aqueous acetone followed again by pronase. At the end of each series of treatments the samples were pelleted at  $30000 \times g$  for 30 min and protein and hexosamine determined for each pellet. The results, reported as ratios of hexosamine to protein, are listed in Table II. The incomplete recovery of hexosamine is due to the difficulty in getting the material to form tight pellets under some treatments and the loss of flocculent material on glassware.

TABLE II

CHANGE IN HEXOSAMINE: PROTEIN RATIO OF SEDIMENT FOLLOWING DIFFERENT TREATMENTS OF MEMBRANE

<i>Treatment</i>	<i>Effect</i>	<i>Hexosamine/protein ratio (arbitrary units)</i>	<i>Hexosamine recovered (%)</i>
Control	—	1.0	100
90% Acetone	removes > 95% lipid	Approx. 1	100
18 h Pronase	removes approx. 80% protein	5.0	81
Pronase; acetone		6.2	63
Acetone; pronase		9.1	53
Pronase; acetone; pronase		48.5	45

The results of the treatments may be summarized as follows: (1) Aqueous acetone extraction, which removes over 95% of oleate-labeled membrane lipid, removes none of the hexosamine; therefore the hexosamine is not covalently bonded in a glycolipid. (2) Lipid extraction makes appreciably more protein available for proteolytic digestion by pronase. (3) Successive treatment by pronase, acetone, and pronase yields almost pure hexosamine which is still insoluble, indicating that it exists either in aggregated or more probably in polymerized form.

### Polyacrylamide-gel electrophoresis

We have employed the sodium dodecyl sulfate-acrylamide gel system of MAIZEL<sup>9</sup> for studies of membrane-protein components. Purified membranes show the characteristic protein pattern seen in Figs. 11a and 11e. Some 10–15 bands are clearly resolved, of which several are very intense. Comparable gel patterns for these membrane proteins are obtained whether or not reducing agent and/or 8 M urea are present in the entire sample-gel-buffer system, and whether samples in sodium dodecyl sulfate are maintained at room temperature or boiled for 2 min.

As noted above, the effect of substantial pronase digestion is to cause disappearance of all these bands, suggesting extensive protein degradation. At considerably lower pronase/membrane protein ratios, however, electrophoresis shows that the faster migrating proteins remain undigested, whereas slowly migrating proteins have been released from the membrane residue or digested (Fig. 11b).

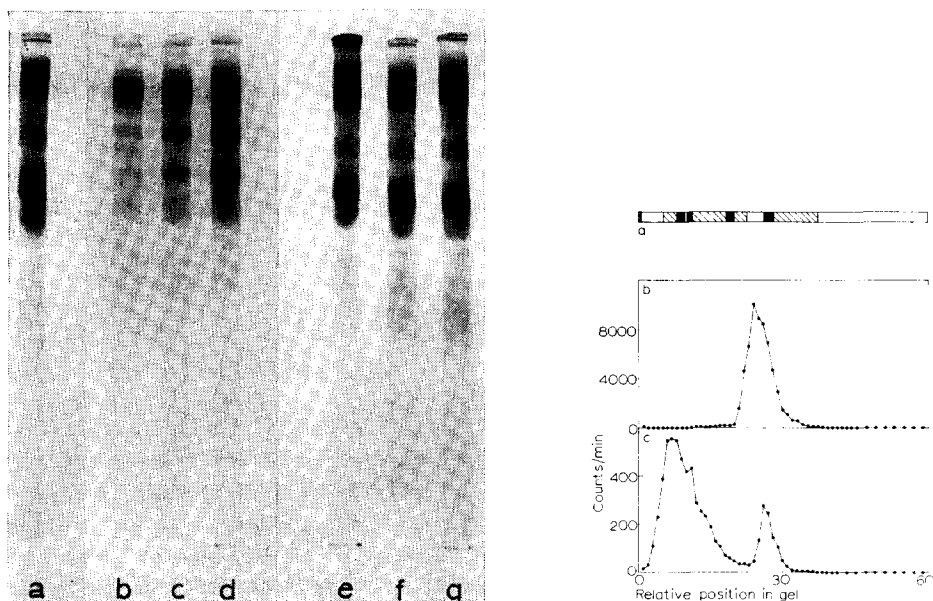


Fig. 14. Detergent-polyacrylamide gel electrophoresis patterns of *M. laidlawii* membranes exposed to different concentrations of sodium dodecyl sulfate. a. Control membranes. b–d. Protein solubilized by 0.01, 0.03, and 0.05% sodium dodecyl sulfate, respectively. e–g. Residual unsolubilized protein after 0.01, 0.03, 0.05% sodium dodecyl sulfate treatment, respectively. Membrane samples containing 750  $\mu$ g protein in 0.01 M sodium phosphate (pH 7) were brought to indicated detergent concentration, incubated 10 min at 37°, and centrifuged for 30 min at 100000  $\times$  g. Supernatants b–d and pellets e–g were separated, brought to 0.25 ml with 0.01 M sodium phosphate and 1%  $\beta$ -mercaptoethanol, and made 1% in sodium dodecyl sulfate. Electrophoresis was carried out for 4.5 h at 60 V. Samples were applied at the top and migrated down toward the anode.

Fig. 15. Detergent-polyacrylamide gel electrophoresis of *M. laidlawii* membranes, showing distribution of membrane-incorporated [<sup>14</sup>C]glucosamine and [<sup>14</sup>C]oleic acid in relation to protein migration. All three gels contained comparable membrane concentrations; samples used for gels (b) and (c) contained membrane-incorporated [<sup>14</sup>C]oleic acid and [<sup>14</sup>C]glucosamine, respectively. a. Gel stained for protein distribution. An approximate and schematic diagram of observed protein distribution is shown. b. Gel crushed and analyzed for [<sup>14</sup>C]oleic acid distribution. Yellow color of carotenoid pigments was also present in region of the radioactive peak. c. Gel crushed and analyzed for [<sup>14</sup>C]glucosamine distribution. Samples were applied at left and migrated towards the anode at right.

We have noted a similar differential effect of protein release following exposure of membranes to very low concentrations of sodium dodecyl sulfate (Fig. 14). At 0.01% sodium dodecyl sulfate a small fraction of membrane protein is not sedimented at  $100000 \times g$ ; the gel pattern for this supernatant reveals primarily the two major slowly migrating protein components (Fig. 14b). As sodium dodecyl sulfate concentration is raised to 0.03 and 0.05% more of the faster migrating proteins are rendered soluble (Figs. 14c and 14d). The corresponding pellets, however, still show all protein bands of the native membrane, despite some loss of material to the supernatant (Figs. 14e–14g).

We have also employed the detergent–gel system in conjunction with an apparatus for fractionating the gel<sup>9</sup> in order to study the distribution of radioactively labeled membrane components vis-à-vis membrane proteins. <sup>14</sup>C-labeled membrane lipids are distributed in a single broad band in the region of fast-migrating membrane proteins (Fig. 15b); this band also contains the yellow color of membrane carotenoid pigments. Membrane-incorporated [<sup>14</sup>C]glucosamine migrates in two bands of which the slower is broad and asymmetric. The fast component (containing less than 20% of the label) migrates in the region containing <sup>14</sup>C-labeled lipid and fast-moving protein components (Fig. 15c).

#### DISCUSSION

In this investigation we have sought information on the structure of a representative plasma membrane by studying how this structure is affected morphologically and chemically by the selective removal of protein and lipid components.

It is apparent from the results reported above that removal of a major fraction of either membrane lipid or protein results in material retaining a morphologic similarity to the native ghost. The detailed fine structure as seen in thin sections changes as a result of these treatments, but the vesicular pattern of organization persists.

The efficacy of 90% aqueous acetone in removing lipid components (over 95% of the lipid is extracted) and the retention of unit membrane structure by lipid-depleted membranes accord with similar observations on inner mitochondrial membranes<sup>12</sup>. In addition, we have shown that such lipid-depleted membranes band quantitatively at a higher isopycnic density (1.25 g/cm<sup>3</sup>) than native membranes (1.17–1.18 g/cm<sup>3</sup>). Although soluble proteins would be expected to band at an isopycnic density of approx. 1.33 g/cm<sup>3</sup> (the reciprocal of their partial specific volume), the buoyant density of protein crystals is substantially lower (1.23–1.30 g/cm<sup>3</sup>)<sup>13</sup>. This difference is usually ascribed to hydration of the crystals, and suggests a comparable degree of hydration for the membrane protein structure. Electron micrographs of these acetone-extracted membranes show unit membrane structure with somewhat thickened outer dark lines (Fig. 6) compared with native membranes (Fig. 4). Some lipid-depleted membranes appear as closed vesicles, while others show free ends and some evidence of fragmentation.

Pronase digestion leads to pronounced loss in electron-microscope contrast, removal of the somewhat fuzzy structure on unit membrane outer faces, a decrease in membrane thickness and loss of prominent triple-layered structure, the disappearance of free ends in sectioned membranes, and fusion of adjacent vesicle faces. Some



lipid is released from membranes during the later stages of protein release, so it is not possible to interpret the changes in the electron micrographs in terms of localization of protein. It seems, clear, however, that the bulk of membrane protein is localized on the two exterior faces and corresponds in part to the dark-staining outer dense layer, while membrane lipid is predominantly, though not necessarily exclusively, localized along the interior of the membrane. (Osmium alone provided extremely low contrast for these preparations; thus the unit membrane image shown here represents predominantly the localization of uranyl and lead ions used in staining.) These results accord generally with the suggestions put forward by ROBERTSON in his "unit membrane" hypothesis<sup>14</sup>.

We have relied on polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate for analytical separation and identification of membrane proteins; it seems worthwhile to define briefly our criteria for the interpretation of these gel results. This detergent-gel system has been shown to separate proteins predominantly on the basis of size, irrespective of charge<sup>15</sup>. Essentially all *Mycoplasma* membrane proteins are solubilized on exposure to 1% sodium dodecyl sulfate and enter the gel. In order to decide whether any bands in the complex gel pattern were the result of aggregation of smaller protein units, we subjected different membrane samples in sodium dodecyl sulfate to temperatures of 37 and 100° and to 8 M urea in the presence of a reducing agent. All gels showed indistinguishable patterns, suggesting that the protein bands seen were indeed maximally denatured and dispersed as single polypeptide chains. A comparable gel pattern for *M. laidlawii* B membrane proteins has been described by RODWELL *et al.*<sup>16</sup>, using phenol-acetic acid-water solubilization and a urea-acetic acid gel system.

The protein pattern of native membranes shows many protein species, but a few species clearly predominate (Figs. 11a and 11e). The localized increase in background staining over part of the gel seen at high protein concentration may suggest the presence of many other protein species of different sizes, present in concentrations too small to give rise to individual bands.

The observation that pronase digestion could release only about 80% of protein from these membranes in many independent experiments suggested the possibility that some protein species were buried, in whole or in part, within a lipophilic interior of the membrane and thus were inaccessible to proteolytic attack; this possibility seemed to be supported by the observation that almost all proteins in lipid-depleted membranes were pronase-sensitive (Table II). Analysis of detergent-acrylamide gels of extensively pronase-digested membranes showed, however, none of the protein bands of native membranes (Fig. 11d); also, sedimentation analysis of sodium dodecyl sulfate-solubilized membranes before and after such pronase digestion showed a substantial drop in sedimentation value of protein material (Fig. 12). Thus there are probably no proteins totally buried within the membrane interior and inaccessible to pronase. Furthermore, the amino acid composition of the 20% pronase-resistant protein is similar to total membrane composition (Table I); if this residual material comprised hydrophobic "tails" of certain proteins buried in a lipid interior, one would expect some enrichment in hydrophobic amino acid residues, which does not occur.

At very low pronase concentrations, not all protein species are equally digested; as shown in Fig. 11, brief pronase treatment at low concentration causes selective disappearance of the higher molecular weight (slowly migrating) protein species.

We also observed that these same high molecular weight species were preferentially released from membranes exposed to very low concentrations of sodium dodecyl sulfate (Fig. 14). These observations suggest the possibility that protein species may be localized differently within the membrane, with some high molecular weight molecules located more towards the exterior than other species which are resistant to release by 0.01% sodium dodecyl sulfate or digestion by pronase at low concentrations.

Our experiments on amino sugars, in combination with previous results<sup>4,17</sup>, make it clear that hexosamine is a significant constituent of the cell envelope of *M. laidlawii*. It comprises about 4% of the dry weight of the membrane for cells grown on two different media. The fact that the hexosamine remains with membrane vesicles despite extensive proteolysis argues against accidental association or adsorption. In addition it has been shown that reaggregated membranes derived from sodium dodecyl sulfate-solubilized membranes show approximately the same ratio of hexosamine to protein as the original material<sup>4</sup>. The hexosamine may appear in the membrane as a glycolipid, as a glycoprotein or as a polysaccharide.

The detailed analysis of membrane lipids by SHAW *et al.*<sup>18</sup> makes it extremely unlikely that there are hexosamine glycolipids. The argument is strengthened by the observations that the membrane hexosamine is not extracted by aqueous acetone, and the bulk of labeled glucosamine migrates independently of lipid on gel electrophoresis (Fig. 15).

The arguments against a bacterial type of cell wall have been summarized by NAIDE<sup>17</sup>. They are: absence of any wall structure in electron microscopy, resistance of cells to penicillin and cycloserine, absence of diaminopimelic acid and muramic acid.

The absence of any hexosamine release on proteolysis indicates that any attachment of hexosamine to protein must involve the pronase-resistant core protein. However, when most of this core is released in the proteolysis of the acetone-extracted membrane, the hexosamine still remains insoluble, which argues against monosaccharides or short chains.

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