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*MEMBRANE SUBUNITS OF MYCOPLASMA LAIDLAWII AND THEIR ASSEMBLY TO MEMBRANELIKE STRUCTURES**

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Mycoplasma (pleuropneumonialike organisms, PPLO) are bounded by a thin lipoprotein "unit membrane."[1] The high sensitivity of *Mycoplasma laidlawii* to osmotic lysis[2, 3] allows the isolation of cell membranes in large quantities and in a rather pure state. Previous analysis of *M. laidlawii* membranes[4] showed them to be composed of 47–60 per cent protein, 35–37 per cent lipid, 4–7 per cent carbohydrate and small amounts of RNA and DNA. Subsequent improvements in isolation and washing procedures of the membranes yielded material composed almost exclusively of protein and lipid. These lipids contain yellow-colored carotenoid pigments, which serve as a convenient marker for membrane material.[4, 5] The high purity of the isolated membranes of *M. laidlawii* prompted us to use them as models for the study of the "unit membrane" substructure.

The sensitivity of *M. laidlawii* membranes to dissolution by detergents, such as sodium lauryl sulfate,[1] suggested the use of detergents for disaggregation of the lipoprotein membrane to smaller units. It was found that detergents disaggregated *M. laidlawii* membranes into rather uniform subunits composed of lipid and protein, and that these subunits could be reaggregated in the presence of di- or multivalent cations to form structures which by the criteria of electron microscopy were very similar to the original membranes.

Materials and Methods.—Organism and growth conditions: Mycoplasma laidlawii strain B was used throughout the experiments reported here. The organism was grown in tryptose broth consisting (per liter) of: bacto-tryptose, 20 gm; NaCl, 5 gm; Tris (hydroxymethyl) aminomethane, 5 gm; bacto-PPLO serum fraction, 10 ml; penicillin G (crystalline) 50,000 units. The final pH of the medium was about 8.3 without adjustment. Growth was carried out in 1-liter vol of medium dispensed in 2-liter flasks incubated statically at 37°. The organisms were harvested at the end of the logarithmic phase of growth (16–24 hr incubation) by centrifugation at 9000 × *g* for 15 min. The sedimented cells were then washed twice in β-buffer[6] (NaCl, 0.156 *M;* Tris, 0.05 *M;* 2-mercaptoethanol, 0.010 *M* in deionized water, adjusted to pH 7.4 with HCl).

Isolation of cell membranes: The sedimented washed organisms (about 0.5 gm wet weight) were osmotically lysed by resuspension in 40 ml of β-buffer diluted 1:20 in deionized water.[6] The suspension was incubated at 37° for 30 min and then centrifuged at 37,000 × *g* for 40 min to sediment the membranes. The membranes were washed twice in 1:20 β-buffer, resuspended in a small volume of the dilute buffer, and kept at −20° until used. Storage of the membrane suspensions at −20° for several days did not affect any of their properties studied in the present investigation.

Assessment of membrane dissolution by detergents: Various amounts of the tested detergents were added to 3 ml membrane suspension in 1:20 β-buffer. The degree of membrane dissolution was estimated by measuring the decrease in optical density of the membrane suspension at wavelengths 400–700 mμ, using a Bausch and Lomb Spectronic 505 spectrophotometer.

Ultracentrifugal analysis: For every ml of a concentrated membrane suspension (10–20 mg membrane protein) in 1:20 β-buffer, 0.15 ml of 0.1 *M* sodium lauryl sulfate (SLS) solution was added. This amount of SLS sufficed to clear the turbidity of the suspension. Similar preparations were made by adding 0.3 ml of 2% sodium deoxycholate or 2% Lubrol W (Imperial Chemical Industries, Ltd., Manchester, England) to 0.7 ml of membrane suspensions. The dissolved membrane preparations were centrifuged at 44,770 rpm in a Spinco model E analytical ultracentrifuge at 20°. Sedimentation peaks were observed by use of Schlieren optics.

Reaggregation of membrane subunits: The SLS-dissolved membrane preparation was dialyzed against a minimum of 1000 vol of 1:20 β-buffer containing 0.01 *M* $MgCl_2$ for 1–3 days at 4°. The contents of the dialysis bag were then centrifuged at 37,000 × *g* for 2–4 hr. The yellow translucent sediment was washed twice in deionized water and tested for enzymic activity, prepared for electron microscopy, or was freeze-dried for chemical analysis.

Analytical methods: The chemical composition of the original membranes and the reaggregated material was determined by the methods outlined by Razin, Argaman, and Avigan.[4] In summary, lipid was extracted from the freeze-dried material by chloroform-methanol (2 + 1, by volume). Protein was estimated by the Lowry procedure using crystalline bovine plasma albumin as standard.[7] Total carbohydrate was determined according to Dubois *et al.*,[8] with glucose as standard. Nucleic acids were extracted according to Schneider.[9] RNA was determined by the method of Drury,[10] and DNA according to Burton.[11] Phospholipids in the extracted lipid were estimated by acetone fractionation.[4] Cholesterol in lipid was separated by thin-layer chromatography[4] and determined by the $FeCl_3$ reaction.[12] Carotenoids were assessed by optical density of the lipid extract at 440 mμ.[6] Reduced nicotinamide adenine dinucleotide ($NADH_2$) oxidase activity of membranes and reaggregated material was determined spectrophotometrically by measuring the decrease in optical density at 340 mμ of the reaction mixture after addition of $NADH_2$.[6]

Density measurements: Sucrose solutions of various densities were made up in 1:20 β-buffer. Plastic centrifuge tubes were filled with 4.5 ml of the buffered sucrose solutions, and 0.2 ml of membrane suspension or reaggregated material was layered on top of the sucrose. The tubes were then centrifuged at 30,000 rpm for 30 min at 20° in a Spinco model L-2 centrifuge using an SW 39 rotor. Material less dense than the sucrose solution banded at the interface between sample suspension and sucrose solution, while denser material formed a pellet at the bottom of the tube.

Electron microscopy: Cells, membranes, or reaggregated material were centrifuged at 37,000 × *g* for 20 min and the supernatant was decanted. Fixation[13] was achieved by adding 6.25% glutaraldehyde in 0.1 *M* sodium cacodylate buffer, pH 7.4 and incubating for 1–2 hr at 4°. The material was then rinsed in 0.1 *M* sodium cacodylate, cut into thin blocks (~1 mm^3), and postfixed for 1 hr at 4° in OsO_4 in veronal acetate buffer.[14] The material was rinsed briefly in distilled water, dehydrated through a graded series of alcohols and propylene oxide, and embedded in Epon[15] (Shell Chemical Co., N. Y.). Sections were cut with an LKB Ultratome. Sections giving a silver interference color were collected on uncoated 1000-mesh copper grids and stained for 15 min in saturated uranyl acetate in 50% ethanol. The grids were examined with a Phillips EM 200 microscope, and pictures were taken at plate magnification of 23,000.

Results.—Dissolution of membranes by detergents: Sodium lauryl sulfate and sodium deoxycholate caused a nearly complete loss of turbidity of *M. laidlawii* membrane suspensions, while Lubrol W caused only a partial clearing. After adding detergent the optical density usually reached a constant value within a few minutes at room temperature. Figure 1 shows a series of lysis curves obtained with different quantities of membrane material. The amount of SLS required for a given fractional decrease in the optical density of the suspension varied monotonically with the concentration of membrane material, indicating a possible stoichiometric relationship between SLS and binding sites in the membrane. Examination of the completely dissolved membrane preparation in the analytical ultracentrifuge demonstrated a single symmetrical schlieren peak characterized by an uncorrected sedimentation constant of 3.3 (Fig. 2). When the amount of detergent added to the

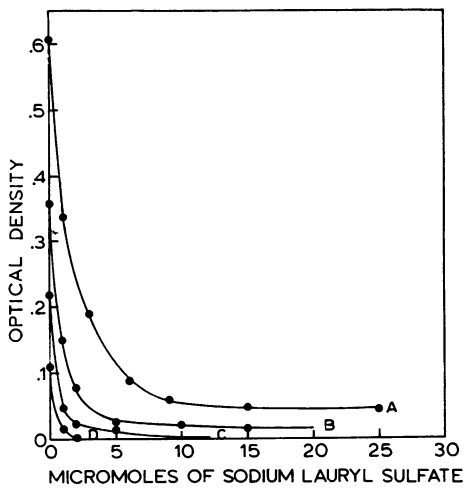

FIG. 1.—Lysis of membranes by sodium lauryl sulfate (SLS). Increasing amounts of SLS were added to 3 ml of membrane suspensions containing the following amounts of membrane protein: A = 1,080 μg; B = 540 μg; C = 270 μg; D = 108 μg. Optical density was measured at 400 mμ following each addition of SLS.

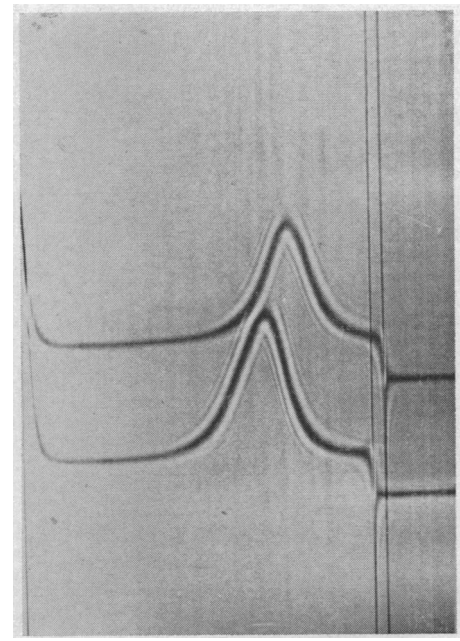

FIG. 2.—Sedimentation patterns. Membranes and reaggregated membrane material were dissolved by sodium lauryl sulfate and centrifuged in a Spinco model E analytical ultracentrifuge at 44,770 rpm at 20°. Upper schlieren peak = dissolved membranes, uncorrected sedimentation constant 3.3S. Lower schlieren peak = dissolved reaggregated material, uncorrected sedimentation constant 3.5S. Both patterns were obtained in the same centrifuge run.

membrane suspension was too small to cause complete dissolution, several fast-moving peaks appeared in addition to the main peak. Similar sedimentation patterns were observed with membrane preparations dissolved by Lubrol W and sodium deoxycholate.

Reaggregation of membrane subunits: Prolonged dialysis (1–3 days) of the SLS-dissolved membranes against 1:20 β-buffer containing 0.01 M Mg^{++} caused the appearance of turbidity in the dialysis bag. Centrifugation of the dialysis bag contents at 37,000 g for 2–4 hr yielded a translucent yellow sediment and a colorless supernatant. Reaggregation of membrane material did not occur when magnesium was not present in the dialyzing fluid. However, 0.01 M Ca^{++} or 0.001 M spermine were as effective as Mg^{++} in reaggregation of membrane material. The reaggregated material could be dissolved completely by the same amount of SLS required for dissolution of the original membranes. Ultracentrifugal analysis of the dissolved material showed a schlieren peak similar to that observed with dissolved original membranes (Fig. 2).

Electron microscopy: Thin sections of whole cells showed them to be bounded by a unit membrane approximately 80 Å in thickness (Fig. 3). Purified membrane preparations were made up predominantly of structures showing the same triple-

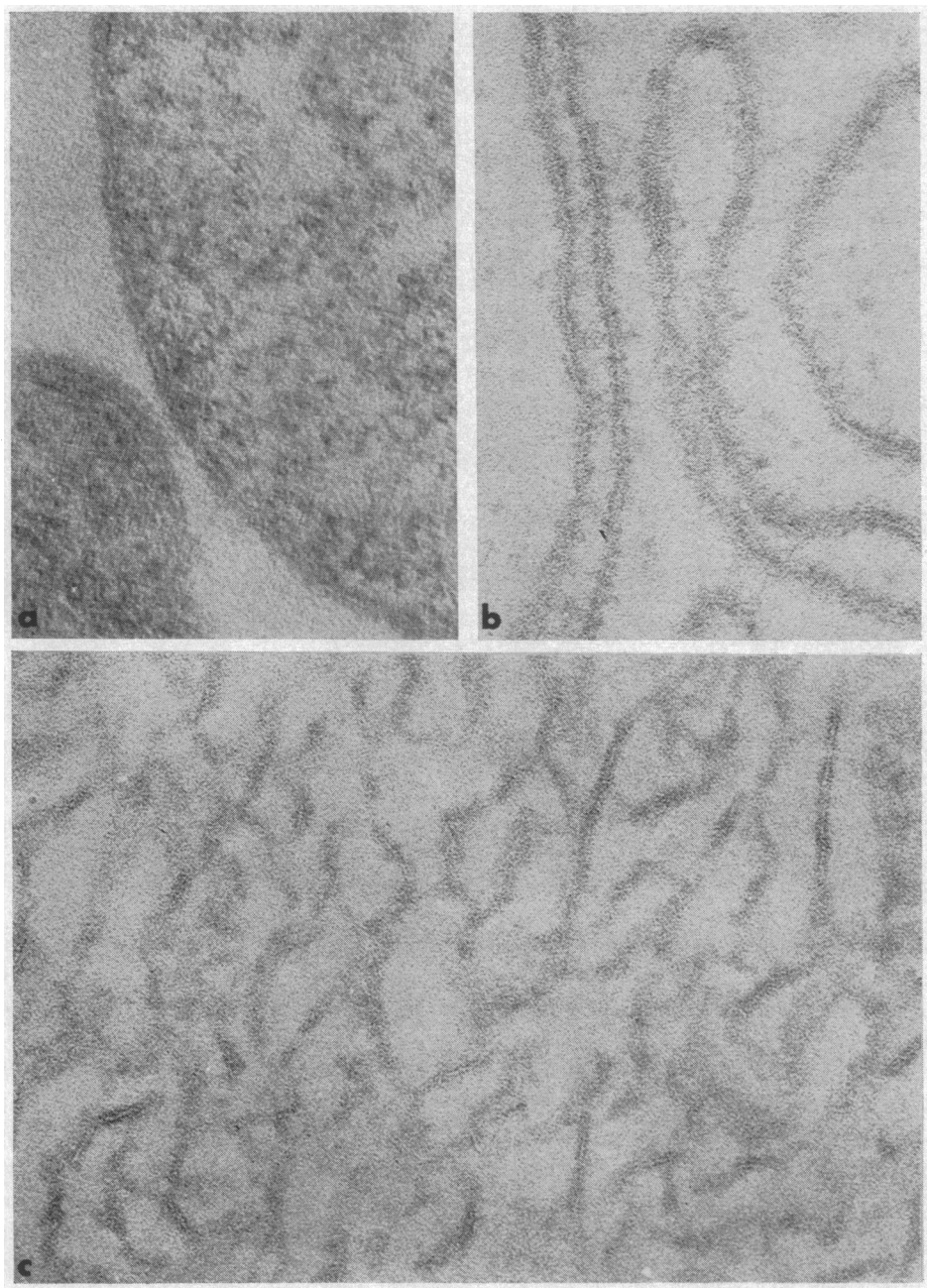

FIG. 3.—Electron micrographs of thin sections of: (*a*) cells of *Mycoplasma laidlawii;* (*b*) isolated membranes; (*c*) reaggregated membrane material. Picture magnification × 163,000.

layered "unit membrane" pattern. Thin sections of reaggregated membrane material also showed the same pattern, but the membranes were approximately 70 Å thick (Fig. 3).

Chemical analysis of membranes and reaggregated membrane material: The data

TABLE 1

CHEMICAL COMPOSITION AND DENSITY OF *Mycoplasma laidlawii* MEMBRANES AND REAGGREGATED MEMBRANE MATERIAL

	% Dry Weight							
	Lipid			Protein	Carbohydrate	Nucleic Acids		Density
	Total	Acetone-insoluble (phospholipids)	Cholesterol			RNA	DNA	
Membranes	37.9	22.7	0.82	53.1	1.6	0.55	0.15	1.17–1.19
Reaggregated material	65.6	40.1	0.80	29.5	0.9	<0.03	<0.06	1.165

Each result represents the average of 3–6 determinations performed on different batches of membranes and reaggregated material.

presented in Table 1 show that isolated *M. laidlawii* membranes were composed almost entirely of protein and lipid. A small amount of carbohydrate and traces of nucleic acids were also present. The reaggregated material consisted essentially of lipid and protein, and was practically free of nucleic acids. The main difference in chemical composition between the original membranes and reaggregated membrane material was the much higher percentage of lipid in the latter. The lipid of the reaggregated material resembled the lipid of the original membranes in composition, being composed mainly of acetone-insoluble phospholipids. This lipid contained virtually all the carotenoids of the original membranes. Carotenoids could not be detected in the supernatant fluid obtained after sedimentation of the reaggregated material. Most of the membrane cholesterol was also recovered in the reaggregated material. The higher lipid content of the reaggregated material was also indicated by its lower density (Table 1). This material showed considerably more density homogeneity than the original membrane preparations.

Enzymic activity of the reaggregated membrane material: $NADH_2$ oxidase has been shown to be localized almost exclusively in the membranes of *M. laidlawii*.[6] Preliminary experiments have shown that $NADH_2$ oxidase activity of the membranes was not affected at a SLS concentration sufficient for complete dissolution of the membranes. The results presented in Table 2 show that $NADH_2$ oxidase activity was retained to a large extent in the reaggregated membrane material.

Discussion.—Lysis of membranes by detergents has been reported for a number of biological entities. Depending on the structure of the membrane, the products of lysis may be either uniform subunits or more complex structures. In the case of *Mycoplasma laidlawii* the major fraction of the membrane appears to be made up of centrifugally homogeneous lipid-protein subunits. The sedimenting particles probably consist of these subunits complexed with molecules of SLS. The existence of a

TABLE 2

$NADH_2$ OXIDASE ACTIVITY OF *Mycoplasma laidlawii* MEMBRANES AND REAGGREGATED MEMBRANE MATERIAL

	$NADH_2$ oxidase activity*
Membranes	2.2
Reaggregated material	1.6
Supernatant fluid†	0.05

* Reaction mixtures consisted of 2.9 ml of β-buffer containing 3.6 μmoles $NADH_2$ and 0.1 ml of tested material containing 0.096–0.875 mg protein. Temperature of incubation was 30°. Data are expressed as decrease in optical density at 340 mμ per min per mg protein.

† The supernatant fluid obtained after sedimentation of the reaggregated membrane material.

single rather symmetrical schlieren peak indicates that the sedimenting units are similar in molecular weight and partial specific volume.

While our work was in progress, Gent *et al.*[16] showed that myelin is dissolved by detergents to give uniform lipid-protein subunits. Preliminary experiments in our laboratory indicate that subunits can be obtained by dissolution of bacterial protoplast membranes and erythrocyte membranes by detergents. Hence, the disaggregation of biological membranes by detergents into uniform lipid-protein subunits seems to be a general phenomenon. This accords with recently reported morphological evidence showing subunit architecture in membranes of various microorganisms.[17-20]

The finding that the membrane subunits reaggregate in the presence of di- or multivalent cations when the SLS is removed opened the way for the purification and characterization of the subunits. The preliminary chemical analysis and density determinations (Table 1) demonstrate that the reaggregated membrane material shown in Figure 3*c* is indeed composed of lipid and protein.

The amount of carotenoids and cholesterol in *M. laidlawii* membranes can be considerably varied as a result of growth conditions.[21] Hence, some caution must be observed in considering the uniformity of the lipid portion of the subunits. The enzymatic activity of the reaggregated material demonstrates that the protein moiety of the subunits does not fulfill solely a structural role.

The fact that both SLS removal and the presence of di- or multivalent cations are required for reaggregation of membrane subunits indicates that both apolar and ionic bonds take part in this process. If the reformed material is indeed identical with the original membrane backbone, as is suggested by the electron micrographs, then it seems likely that simple physicochemical forces suffice for the assembly of membrane material. That such forces can act to cause spontaneous aggregation of lipid systems into membranelike structures is demonstrated by the work of Stoeckenius[22] and Lucy and Glauert.[23]

The demonstration of the effectiveness of di- and multivalent cations in assembly of *Mycoplasma* membrane subunits provides an explanation for the pronounced protective effect of the cations against osmotic lysis of *Mycoplasma* cells[3] and other osmotically fragile organisms.[24]

Summary.—Cell membranes of the pleuropneumonialike organism *Mycoplasma laidlawii* were dissolved by sodium lauryl sulfate. The dissolved material consisted of ultracentrifugally homogenous subunits having a sedimentation constant of 3.3 Svedberg units. These subunits were composed of lipid and protein and could be reaggregated to form membranelike structures in the presence of di- or multivalent cations.

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*NUCLEAR MAGNETIC RESONANCE EVIDENCE FOR COMPLEXING OF SODIUM IONS IN MUSCLE**

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Most investigators have assumed that the Na^+ of the cell is largely in free solution in intracellular water. The opposite conclusion was drawn from the application of a kinetic theory proposed by Cope[1, 2] to van der Kloot's data on Na^+ leakage from muscle.[3] On other grounds, Troshin[4] and Ling[5, 6] previously had deduced that a large fraction of intracellular Na^+ existed in a complexed state. To test the prediction of Cope's kinetic theory, and because a knowledge of the extent of complexing of Na^+ in the cell is of general importance for the derivation of theories of ion transport and nerve conduction, a new experimental approach to cellular Na^+ complexing was sought. Nuclear magnetic resonance (NMR) analysis of muscle Na^+ proved to have the required sensitivity, specificity, and clarity of interpretation.

A large bullfrog (*Rana catesbeiana*) was killed by decapitation, the muscle was cut off in fairly large pieces from the upper portion of the hind leg, and was blotted to remove blood and extracellular fluid insofar as possible. Pieces of muscle then were packed tightly up to an 8-ml mark in a Pyrex test tube that had been lined with