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CHARACTERIZATION OF THE PLASMA MEMBRANE OF *MYCOPLASMA LAIDLAWII*. I. SODIUM DODECYL SULFATE SOLUBILIZATION

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SUMMARY

The preparation and properties of highly purified plasma membranes from *Mycoplasma laidlawii* B are described. The process of sodium dodecyl sulfate solubilization of these membranes is followed using 4 techniques: (1) measurement of the drop in turbidity, (2) release of membrane protein into non-sedimentable material, (3) schlieren patterns observed in analytical ultracentrifugation, and (4) distribution of protein and lipid in density-gradient sedimentation.

It is concluded that detergent solubilization of this membrane proceeds through a continuum of states with no well defined intermediates to the formation of separate lipid– and protein–detergent complexes. The appearance of single schlieren peaks in solubilized membrane preparations is considered to be an inadequate criterion for assessing the homogeneity of such a complex system. A previous conclusion that detergent solubilization of this membrane produced lipoprotein subunits is not consistent with these results.

INTRODUCTION

Investigation of the structural organization of lipids and proteins in plasma membranes has been hampered by the lack of suitable means of disrupting these predominantly lipophilic structures so that the component macromolecules can be studied in an aqueous environment. In this paper we have extended our studies[1] on the process of solubilization of a purified plasma membrane by a detergent. Some of the aggregation properties of the solubilized membrane components will be considered in the following report[2].

The plasma membrane of Mycoplasma was chosen as the object of study for a number of reasons. These organisms do not possess a cell wall; therefore, the prepara-

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tion of a membrane fraction free of other coat material is simplified. Further, the Mycoplasma appear to lack internal membranes so that isolates are not mixtures of membrane types. Owing to the small size of these cells, the membrane constitutes an unusually large portion (up to 40%) of the total dry mass, and membrane yields in preparative procedures are consequently high. Since many Mycoplasma are free-living organisms, their plasma membranes fulfil all membrane requirements of the organism including ion transport and a number of enzymatic activities. Moreover, the cells possess a general simplicity indicated by the small size of the genome as determined from DNA content[3].

Among the Mycoplasma, *Mycoplasma laidlawii* B was chosen for these experiments because of its high osmotic fragility[4]. On the basis of our past experiments sodium dodecyl sulfate was selected as the detergent for use in these studies.

MATERIALS AND METHODS

Organism and growth conditions

M. laidlawii B was grown as described by RAZIN, MOROWITZ AND TERRY[1] except that each liter of medium was supplemented with 10 μC [1-^{14}C]oleic acid (New England Nuclear Corp.; specific activity 8 mC/mmole in 0.2 ml of ethanol). Virtually all of this fatty acid is incorporated into the phospholipid fraction of the membranes[5].

Isolation of membranes

The membranes were isolated using the procedure of RAZIN, MOROWITZ AND TERRY[1]. For further purification the membranes were layered on a step-gradient composed of an upper layer of 25 ml 35% sucrose and a lower layer of 25 ml 50% sucrose and centrifuged at 25 000 rev./min for 3 h in the SW 25 2 rotor in a model L2 ultracentrifuge. (All sucrose percentages reported in this paper are weight to weight.) The membranes, which banded at the interface, were then removed with a Pasteur pipette and suspended in a 1:20 dilution of β-buffer (β-buffer is: NaCl, 0.156 M; Tris, 0.05 M; 2-mercaptoethanol, 0.01 M; in deionized water, adjusted to pH 7.4 with HCl). The membrane suspension was centrifuged at 37 000 $\times$ g for 30 min. This washing procedure was repeated 4 times, and the final pellet was resuspended in 1:20 β-buffer and stored at 4°.

Measurement of membrane solubilization by sodium dodecyl sulfate

The degree of membrane solubilization by sodium dodecyl sulfate was measured in 2 ways: (1) the decrease in optical density at 530 mμ of a 3-ml sample containing 3 mg membrane protein (25°, pH 7.5) was measured as aliquots of 0.2 M sodium dodecyl sulfate were added. (In general the protein concentration is used to characterize the concentration of the membrane preparation.) In every case 20 min were allowed after the addition of detergent for the reaction to proceed. (2) 0.1-ml aliquots of various concentrations of sodium dodecyl sulfate were added to 0.9-ml samples of membrane to final protein concentrations of 0.1, 0.5, and 1.0 mg/ml. The material was allowed to stand for 2 h at 25° and then centrifuged at 37 000 $\times$ g for 30 min. The protein in the supernatant was measured using the Folin reaction[6] with egg white lysozyme (2$\times$ crystallized; Worthington Biochemical Corp.) as a standard.

Analytical ultracentrifugation

Samples at 2 protein concentrations (3.45 mg/ml and 1.65 mg/ml) were solubilized with different amounts of sodium dodecyl sulfate. The material was placed in a 4°-sector cell and centrifuged at either 44 770 or 50 740 rev./min and 20° in the Spinco Model E analytical ultracentrifuge. Sedimentation coefficients were determined from microcomparator measurements of the schlieren patterns.

Electron microscopy

(1) Preparation for thin sectioning: a small volume of stock membrane solution containing about 2 mg membrane was centrifuged into a tightly packed yellow pellet at 37 000 × *g* for 30 min at 4°; the latter was left attached to the centrifuge tube in all subsequent manipulations until dehydration in 90% ethanol. Fixation, dehydration and embedding were carried out as described in the following paper[2]. Sections showing gold or silver interference colors were cut on an LKB ultratome, picked up on uncoated 1000-mesh Cu grids, and examined in a Philips EM 200 electron microscope after staining for 15 min with saturated uranyl acetate in 50% ethanol.

(2) Negative staining: small droplets of diluted stock membrane solution mixed with 2% phosphotungstic acid which had been adjusted to pH 7.0 with NaOH were placed on the surface of 400-mesh Cu grids previously coated with Formvar films and a shadow-cast layer of carbon. Excess liquid was removed with filter paper and the grids examined in a Philips EM 200 electron microscope.

(3) Shadowing: small droplets of diluted stock membrane solution were placed on the surface of Formvar-coated 200-mesh stainless-steel grids. After drying, the grids were shadowed with Pt–Pd, then examined in an RCA EMU 3B electron microscope.

Density-gradient centrifugation

Three types of linear density gradients were used: (1) 5 ml 20–45% sucrose gradients were formed in which the sodium dodecyl sulfate concentrations were uniform at 0.0, 0.5, 1.2, 5.0, 10.0, or 30.0 mM. The sucrose solutions were made with 1:20 β-buffer. Six 0.4-ml samples containing 1.8 mg protein/ml and 0.0, 1.7, 2.8, 7.0, 12.0, or 32.2 mM sodium dodecyl sulfate respectively were layered onto the gradients. The gradients were centrifuged at 40 000 rev./min in an SW 50 rotor in the Spinco Model L2 ultracentrifuge for 30 h at 25°. Fractions were collected, diluted, and assayed for protein (Folin reaction) and lipid (counts of aliquots dried on planchets). (2) A three-layered 'sandwich' gradient was constructed. The lower layer was a 2.5-ml linear gradient from a density of 1.25 g/cm^3 (obtained by making a 35% sucrose solution with 90% deuterium oxide (^2H_2O)) to a density of 1.19 g/cm^3 (obtained by making a 24% sucrose solution with 90% ^2H_2O). The middle layer consisted of 0.3 ml of a membrane pellet resuspended in 90% ^2H_2O to a final density of approx. 1.17 g/cm^3 and a protein concentration of approx. 4 mg/ml. The upper layer was a 2.5-ml linear gradient from a density of 1.15 g/cm^3 (obtained by making a 15% sucrose solution with 90% ^2H_2O) to a density of 1.10 g/cm^3 (obtained by making a 3% sucrose solution with 90% ^2H_2O). The entire gradient including the sample contained 9.0 mM sodium dodecyl sulfate. The gradient was centrifuged for 48 h at 50 000 rev./min in the SW 50 rotor as described above. Fractions were collected and analyzed for radioactivity and protein content. (3) 5-ml linear density gradients were formed from 50% to 75%

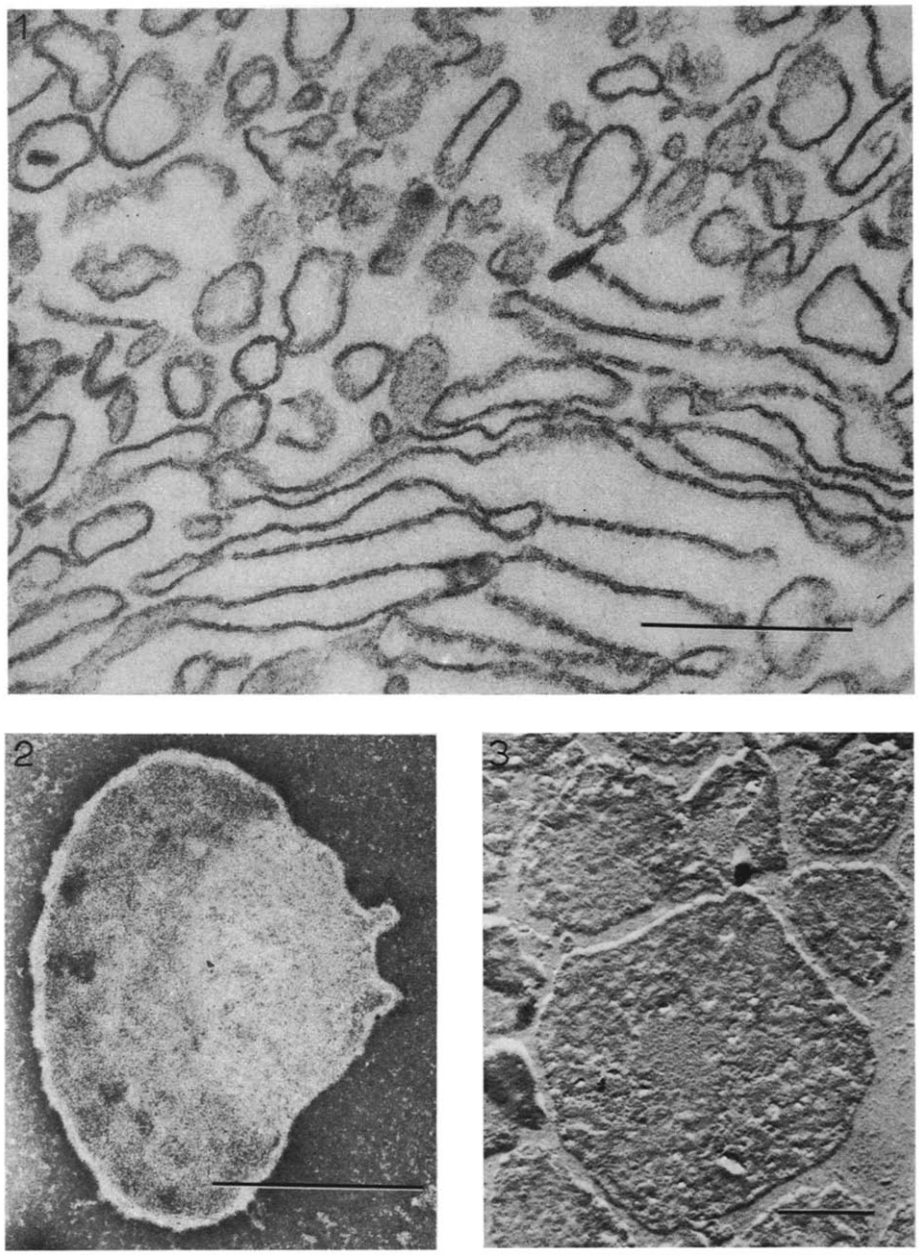

Fig. 1. Purified *M. laidlawii* plasma membrane; thin section through membrane pellet, × 61 600.

Fig. 2. Negatively stained membrane 'ghost', × 61 600.

Fig. 3. Shadowed membrane preparation, × 28 000. All scale markers represent 0.5 μ.

^2H_2O, containing 1:20 β-buffer and 10 mM sodium dodecyl sulfate. 0.3-ml samples of a membrane preparation (which had not been exposed to sucrose) at a concentration of 3.0 mg protein/ml and 10 mM sodium dodecyl sulfate were layered onto the gradients and centrifuged for 8 h at 50 000 rev./min as described above. Fractions were collected and analyzed for radioactivity and protein content.

Electrophoresis

Samples consisting of fractions from one of the 50–75% ^2H_2O gradients were layered onto spacer gels above 7% polyacrylamide separating gels[7]. Electrophoresis was conducted at 5 mA per gel and the gels were stained and fixed for 2 h with Amido Black and 7.5% acetic acid. They were then destained at 12.5 mA per gel and examined.

RESULTS

Characteristics of isolated membranes

The membrane suspension obtained by the isolation procedures described above is turbid and yellow in color due to the presence of a carotenoid (absorption maxima at 477, 447, and 421 mμ). When a small volume of the suspension is centrifuged at 37 000 $\times$ g for 30 min, a translucent yellow pellet is obtained. Most of the membrane preparations have a density of 1.18$\pm$0.01 g/cm^3 as determined from equilibrium banding in a sucrose density gradient. Electron microscopic examination of thin sections of the preparation shows a unit membrane structure with no extraneous coats (Fig. 1). Negatively stained or shadowed preparations reveal what appear to be collapsed envelopes of a size commensurate with that of the original cells (Figs. 2 and 3). Upon filtration of a suspension of the membranes more than 80% of the material passes through a Millipore filter with 0.45 μ pore diameter, but less than 20% passes

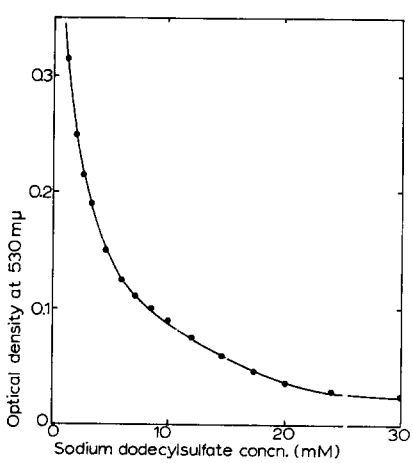

Fig. 4. Optical density of membrane suspension *versus* concentration of sodium dodecyl sulfate. Aliquots of 0.2 M sodium dodecyl sulfate were added to 3 ml of membrane at 1.0 mg protein/ml. Twenty min after the addition of sodium dodecyl sulfate at pH 7.5 and 25°, the optical density was read at 530 mμ against a distilled water blank. Each point is corrected for dilution by the sodium dodecyl sulfate solution. The optical density of the point at zero sodium dodecyl sulfate was 1.25.

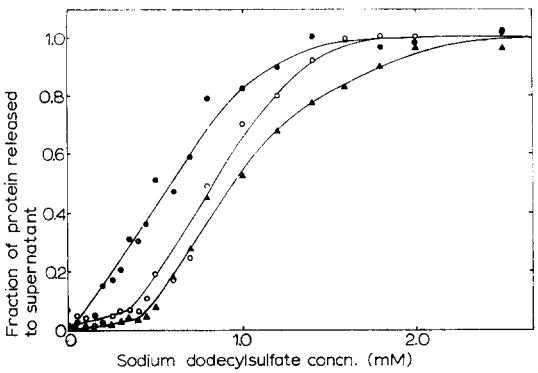

Fig. 5. Protein solubilized *versus* sodium dodecyl sulfate concentration. The fraction of membrane protein in the 37 000 × *g* supernatant of membrane samples at 3 different membrane concentrations treated with various amounts of sodium dodecyl sulfate (●—●, 0.1 mg protein/ml; ○—○, 0.5 mg protein/ml; ▲—▲, 1.0 mg protein/ml).

through a filter with a pore diameter of 0.22 μ. These observations indicate that the preparation consists mainly of individual cell ghosts.

Solubilization by sodium dodecyl sulfate

The process of solubilization of the membrane by sodium dodecyl sulfate has been followed using 4 techniques: (1) measurement of the drop in turbidity, (2) amount of protein in the supernatant fraction following centrifugation, (3) the schlieren pattern observed in analytical ultracentrifugation, and (4) the distribution of material in various density gradients.

(1) Turbidity change: incremental amounts of sodium dodecyl sulfate were added to a membrane sample at pH 7.5 and 25°. The optical density was read at 530 mμ against a distilled water blank (Fig. 4). A rapid drop in turbidity was observed to approx. 10% of the initial value, after which a slow decline appeared. No definite plateau was seen.

(2) Release of protein: the fraction of protein found in the supernatant fraction after centrifugation for 30 min at 37 000 × *g* is shown in Fig. 5. The solubilization of the membrane by sodium dodecyl sulfate is followed at 3 protein concentrations, and 3 similar curves displaced from one another are observed.

(3) Schlieren sedimentation patterns: upon sedimentation in the model E analytical ultracentrifuge, the dissolved membrane material has consistently given a single schlieren peak (similar single peaks have been observed in solubilized membrane preparations from *Escherichia coli*, *Micrococcus lysodeikticus*, rat-heart mitochondria, and rat peripheral nerve myelin). The behavior of the sedimentation coefficient characterizing this peak has been followed as a function of sodium dodecyl sulfate concentration (Fig. 6). After an initial rapid drop, a steady decline is seen with no indication of a plateau. Only at low concentrations of sodium dodecyl sulfate do the $s_{20,w}$ values for the 2 different membrane concentrations differ significantly.

The sedimentation coefficients of the peaks for 3 concentrations of membrane, 1.94, 1.43, and 0.96 mg protein/ml, were measured at a single concentration of detergent (10 mM) sufficient to produce complete solubilization as determined from Fig. 5,

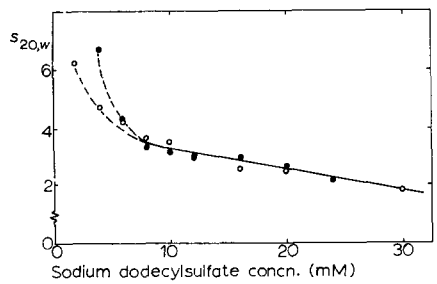

Fig. 6. Sedimentation coefficients ($s_{20,w}$) of different membrane samples solubilized with various sodium dodecyl sulfate concentrations, then centrifuged at 44 770 or 50 740 rev./min (20°) in a Model E ultracentrifuge. ●—●, 3.45 mg protein/ml; ○—○, 1.65 mg protein/ml.

and found to be 3.0, 3.0, and 2.9 S respectively. This result supports the idea that, at the concentrations of sodium dodecyl sulfate used in the sedimentation experiments, the amount of bound sodium dodecyl sulfate did not appreciably alter the solution concentration.

(4) Density-gradient centrifugation: linear sucrose density gradients were employed to follow the behavior of the lipid and protein components at various concen-

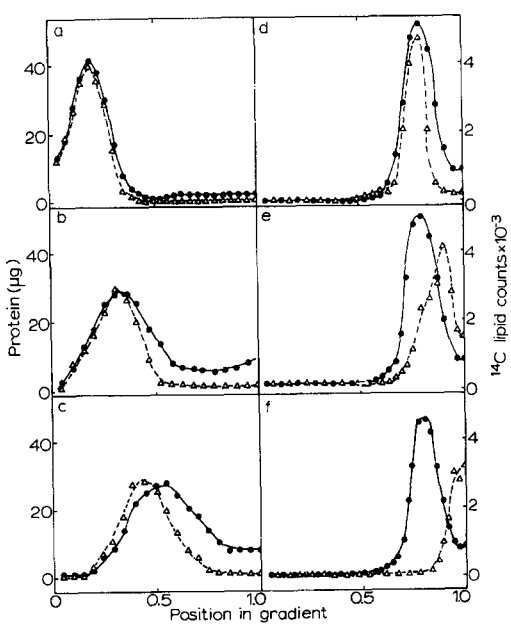

Fig. 7. Sucrose density gradients. Five ml 20–45% sucrose gradients (w/w) were made at various concentrations of sodium dodecyl sulfate. Samples containing 1.8 mg protein/ml were exposed to different levels of detergent and 0.4-ml vol. were layered on the gradients. The gradients were then centrifuged at 40 000 rev./min for 30 h in an SW 50 rotor and analyzed for protein (●—●) and lipid (△- - - -△). a, No sodium dodecyl sulfate in sample, no sodium dodecyl sulfate in gradient. b, 1.7 mM sodium dodecyl sulfate in sample, 0.5 mM sodium dodecyl sulfate in gradient. c, 2.8 mM in sample, 1.2 mM sodium dodecyl sulfate in gradient. d, 7.0 mM sodium dodecyl sulfate in sample, 5.0 mM sodium dodecyl sulfate in gradient. e, 12.0 mM sodium dodecyl sulfate in sample, 10.0 mM sodium dodecyl sulfate in gradient. f, 32.0 mM sodium dodecyl sulfate in sample, 30.0 mM sodium dodecyl sulfate in gradient. In all cases the top of the gradient is on the right.

trations of detergent. In each case the gradient concentration of sodium dodecyl sulfate was adjusted to the approximate level of unbound detergent in the sample determined from Fig. 5. The profiles resulting from centrifugation are shown in Fig. 7, a–f. As the particle size decreases in Fig. 7, a–c, a shift from equilibrium banding to velocity sedimentation occurs. An initial release of protein is indicated by the elevated plateau following the protein peak seen in Fig. 7, b and c. In Fig. 7d the particle size has decreased radically; in Fig. 7e there is an apparent separation of lipid and protein components which is observed to have progressed further in Fig. 7f. It is to be noted that the protein peaks in Fig. 7, d–f are in approximately the same position with respect to the gradient.

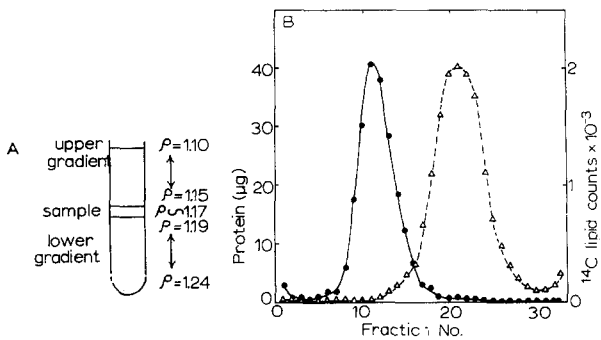

Fig. 8. 'Sandwich' gradient. A, A three-layered gradient was constructed as shown. The lower gradient was linear from a density of 1.19 to 1.24 g/cm³. The upper gradient was linear from 1.15 to 1.10 g/cm³. The sample was introduced at an approximate density of 1.17 g/cm³. The entire gradient contained 9 mM sodium dodecyl sulfate and 1:20 β-buffer. The densities were achieved by mixing various amounts of sucrose with 90% ^2H_2O. B, The gradient shown in (A) was centrifuged for 48 h at 50 000 rev./min in an SW 50 rotor at 25°. Fractions were collected and analyzed for protein (●—●) and lipid (△— — —△). The top of the gradient is on the right.

In order to establish the degree of separation of lipid and protein present in the 10 mM sodium dodecyl sulfate-treated membrane, a 'sandwich' gradient was formed in which the sample was introduced between an upper and a lower density gradient. The densities were adjusted so that lipoprotein particles of approximate density 1.18 g/cm³ would not sediment, particles of higher density would sediment downward, and particles of lower density would sediment upward. The gradient is shown diagramatically in Fig. 8A. The sucrose used in forming the gradient was dissolved in 90% ^2H_2O in order to achieve higher densities while maintaining relatively low viscosities. The profile resulting from centrifugation is seen in Fig. 8B. A high degree of separation si evident.

In order to examine the possibility that the separation of lipid and protein appearing in Figs. 7e and 8B might have been the result of exposure to the sucrose, a similar sample was run on a 50–75% ^2H_2O gradient. Furthermore, the character of the protein peak was explored. Two ^2H_2O gradients were formed, each with 10 mM sodium dodecyl sulfate. Samples brought to 10 mM sodium dodecyl sulfate were layered on, and the gradients were centrifuged. One gradient was analyzed for ^{14}C and protein as shown in Fig. 9, and separation of lipid and protein was observed. Corresponding fractions from the other gradient were used as samples for polyacryl-

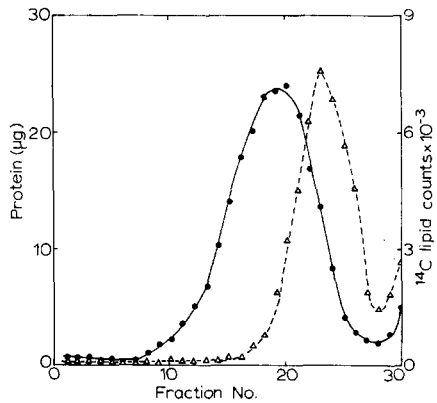

Fig. 9. ^2H_2O gradient. The 5-ml gradient was formed from 50% to 75% ^2H_2O with 1:20 β-buffer and 10 mM sodium dodecyl sulfate. A membrane sample containing 10 mM sodium dodecyl sulfate was layered on and the gradient was centrifuged at 50 000 rev./min for 8 h in an SW 50 rotor at 25°. Fractions were analyzed for protein (●—●) and labeled lipid (△— — —△). The top of the gradient is on the right.

amide-gel electrophoresis. The electrophoretic patterns on 7% gels showed certain bands to be predominant at the leading edge of the protein peak and others at the trailing edge, indicating that the peak is somewhat heterogeneous in protein composition.

DISCUSSION

The conception of what constitutes pure membrane is difficult since relatively weak interactions may stabilize associations of a wide variety of molecules, and it follows that any notion of the detailed composition of plasma membrane has a certain arbitrary character. In this study, the plasma membranes are considered to be purified on the basis of extensive washing procedures and isopycnic banding. Electron micrographs (Fig. 1) show that the preparation is free from large amounts of contaminating material. Furthermore, it is apparent from Fig. 7a that the preparation is confined to a relatively narrow density range. Owing to the large differences in density between the protein and lipid components the observed membrane density is primarily a function of the lipid–protein ratio. The fairly narrow density range observed (1.17–1.19 g/cm^3) suggests a roughly constant composition.

The process of solubilization of the membrane by sodium dodecyl sulfate appears to involve a continuum of states with no well defined configuration intermediate between the intact membrane and a condition in which the protein and lipid components exist as separate species. The absence of a plateau in both graphs of optical density and sedimentation coefficient *versus* sodium dodecyl sulfate concentration supports this idea (Figs. 4 and 6). The initial step in the detergent attack appears to be the release of protein as seen in Fig. 7, b and c. This is followed by a decrease in particle size indicated by the displacement of the curves in Fig. 7, c and d, and a dissociation of the lipid and protein shown in Fig. 8B. In the experiment shown in Fig. 7 the free lipid is prevented from sedimenting far into the gradient since the density of the gra-

dient begins at a point above that of the lipid. The breadth and displacement of the lipid peak in Fig. 8B indicate that the lipid molecules are in the form of lipid–sodium dodecyl sulfate aggregates or lipid micelles of variable size at 9 mM sodium dodecyl sulfate. As higher levels of detergent are encountered, the lipid particle size appears to decrease (Fig. 7, e and f). Little change in the protein curve is seen between Fig. 7, d and f, which supports the argument that the principal site of action of the detergent in this region is on the lipid.

In a previous publication[1] it was concluded that the primary breakdown product of the membrane on exposure to sodium dodecyl sulfate is a lipoprotein subunit of homogenous size. The main point of evidence on which this conclusion was based was that the material appeared as a single schlieren peak in the analytical ultracentrifuge. In the present study, well defined schlieren peaks were seen for material exposed to 7 and 12 mM sodium dodecyl sulfate, yet examination of the sucrose gradient profiles shows them to be composed of separate protein and lipid components. Such a result is reason to question the utility of the schlieren pattern as a criterion for the homogeneity of such complex systems. If the protein and radioactivity curves in Fig. 9 are added together, a single peak results; a similar coincidence may explain the appearance of the schlieren peak observed. Moreover, if there exist weak interactions between components of different sedimentation coefficients, it is possible that centrifugally heterogeneous material might give rise to a single schlieren peak[8].

The evidence from these studies is opposed to the idea that the membrane of *M. laidlawii* breaks down into homogeneous lipoprotein subunits under conditions of exposure to sodium dodecyl sulfate.

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