

BBA 75033

CHARACTERIZATION OF THE PLASMA MEMBRANE OF *MYCOPLASMA LAIDLAWII*. II. MODES OF AGGREGATION OF SOLUBILIZED MEMBRANE COMPONENTS

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(Received September 27th, 1966)

SUMMARY

The process of assembly of membrane components from *Mycoplasma laidlawii*, previously solubilized by detergent (sodium dodecyl sulfate), is investigated under conditions of dialysis to remove the detergent and allow the introduction of different levels of Mg^{2+} . Material aggregated under these conditions is investigated by electron microscopy, density-gradient centrifugation, and analytical ultracentrifugation.

In all cases, removal of detergent leads to the formation of lipoprotein material, but large lipoprotein aggregates are only obtained in the presence of Mg^{2+} . Different morphologies and density-gradient profiles are seen for this aggregated material, depending on the initial detergent concentration and on Mg^{2+} concentration. Only under a limited set of conditions does the majority of this material closely resemble the original membrane.

INTRODUCTION

In studying the macromolecular organization of biologically ordered structures, it is often useful to disaggregate the structures into their component parts, to study the properties of these parts, and to investigate the modes of reassembly of these components under suitable conditions. Such a study of reassembly *in vitro* may yield information on the *in vivo* assembly process. Our purpose in this investigation has been to gain such information on cell membrane structure and synthesis, using macromolecular structural elements derived from completely dissolved plasma membrane.

In earlier work we described the formation of membrane-like material from detergent-solubilized membrane components of *M. laidlawii*¹. Subsequent studies² have shown that the state of this solubilized material is a function of the concentration of detergent (sodium dodecyl sulfate). In addition we have found that the nature of

* Part of this material will be submitted in partial fulfillment of the requirements for the Ph. D. degree at Yale University.

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the products formed depends on the Mg^{2+} concentration during the process of re-aggregation. The basic protocol of the experiments described below consists of dissolving purified membrane preparations with sodium dodecyl sulfate and reaggregating this material under a variety of conditions. The reaggregated material is examined by thin-section electron microscopy, isopycnic density-gradient analysis, and analytical ultracentrifugation.

MATERIALS AND METHODS

Membrane isolation

Growth of *M. laidlawii* B cells, introduction of [^{14}C]lipid label, and procedures for isolating pure membranes are described in the preceding paper².

Solubilization and reaggregation procedures

On the basis of studies described earlier², sodium dodecyl sulfate solutions were selected for given protein concentrations corresponding to (i) concentrations just sufficient to completely disaggregate membranes into soluble components (low sodium dodecyl sulfate) and (ii) concentrations 3 to 5 times this amount providing considerable excess of detergent (high sodium dodecyl sulfate). After adding sodium dodecyl sulfate to membrane suspensions and standing 1 h at room temperature with intermittent stirring, samples were centrifuged for 30 min at $37\,000 \times g$ (4°) and any residual pelleted material was discarded. The resulting yellow supernatants were placed in $\frac{1}{4}$ " dialysis tubing previously soaked for several hours in a 1:20 dilution of β -buffer (β -buffer contains: NaCl, 0.156 M; Tris, 0.05 M; 2-mercaptoethanol, 0.01 M; in deionized water, adjusted to pH 7.4 with HCl). Samples were dialyzed for 48 h at 4° against 2-3 changes of 2-l vol. of 1:20 β -buffer containing 0, 0.02 or 0.05 M $MgCl_2$. The fraction of dialyzed material which then pelleted upon centrifugation at $37\,000 \times g$ for 30 min (4°) was defined as aggregated material. Details of sample composition and analysis are discussed below.

Material dialyzed without magnesium

Two 5-ml samples containing 9 mg membrane protein were prepared, one 10 mM in sodium dodecyl sulfate (low), the other 50 mM in sodium dodecyl sulfate (high). After dialysis against 1:20 β -buffer in the absence of Mg^{2+} , the $37\,000 \times g$ supernatant was characterized as follows: (1) Analytical ultracentrifugation: 0.8-ml supernatant samples were centrifuged at 50 740 rev./min in a Spinco Model E analytical ultracentrifuge at 20° and examined using schlieren optics. Sedimentation coefficients were determined from microcomparator measurements. (2) Density-gradient centrifugation: 5-ml linear 20-45% (w/w) sucrose density gradients were formed using sucrose solutions made up in 1:20 β -buffer. 0.4-ml supernatant samples of the material described above were layered on the gradients and centrifuged at 40 000 rev./min for 30 h at 25° in an SW 50 rotor and Spinco Model L2 ultracentrifuge. 10-drop gradient fractions were bottom-collected, diluted to 1.7 ml with deionized water, and samples of 1 ml assayed for protein and 0.5 ml for radioactivity.

Material dialyzed with magnesium

Four 3-ml samples containing 2.6 mg protein in 1:20 β -buffer were prepared, two 6 mM in sodium dodecyl sulfate (low) and two 18 mM in sodium dodecyl sulfate

(high). One sample of each pair was dialyzed against 1:20 β -buffer containing 0.02 M and 0.05 M MgCl_2 , respectively. After dialysis as described above, sample volumes were brought to 5 ml with β -buffer, each sample divided into two 2.5-ml aliquots, and all samples centrifuged at $37\,000 \times g$ for 30 min (4°). One set of pellets representing half the material was set aside for electron microscopy; the other set was resuspended in 1:20 β -buffer and samples used for density-gradient analysis.

Isopycnic density-gradient analysis

Linear 4.6-ml 20–50% (w/w) sucrose gradients were constructed from sucrose solutions in 1:20 β -buffer. 0.7 ml of resuspended reaggregated material from each sample described above was layered on the gradients and centrifuged in an SW 50 rotor at 39 000 rev./min for 4 h (20°) in a Spinco Model L2 ultracentrifuge. Gradients were unloaded in 10-drop fractions, diluted to 2 ml, and 1 ml assayed for protein and radioactive lipid, respectively.

Electron microscopy

Pellets set aside for electron microscopy were left attached to centrifuge tubes during fixation and throughout all subsequent manipulations until dehydration in 90% ethanol. Initial fixation was carried out for 2 h at room temperature in 5 ml of a solution containing 9% formalin, 0.25 M sucrose, 0.05 M CaCl_2 , and 0.05 M veronal-acetate buffer adjusted to pH 7.2 with HCl (W. STOECKENIUS, personal communication). After rinsing in veronal-acetate buffer, the pellets were post-fixed overnight at 4° in 5 ml of veronal-acetate-buffered osmium tetroxide and then stained for 4 h with uranyl acetate solution, following the procedures of RYTER AND KELLENBERGER³. After rinsing with buffer, the pellets were dehydrated through a graded ethanol series. At 90% ethanol, the pellets were loosened from the centrifuge tubes and cut into small blocks about 1 mm³. After final dehydration in propylene oxide, the samples were infiltrated and embedded with Araldite 502 (ref. 4). 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.

Protein and radioactivity assays

Protein was assayed by the Folin phenol method of LOWRY *et al.*⁵, using egg white lysozyme ($2 \times$ crystallized, Worthington Biochemical Corp.) as standard. Radioactive samples were dried on planchets and counted on a Beckman low-beta thin-window proportional counter.

Density measure of gradient fractions

Gradients formed as described above, but with buffer in place of sample, were centrifuged concurrently. Undiluted 10-drop fractions were assayed directly for refractive index in a Bausch and Lomb Abbe-3L refractometer, and fraction densities calculated from refractive indices.

Reaggregation in the presence of ³⁵S-labeled sodium dodecyl sulfate

³⁵S-labeled sodium dodecyl sulfate (New England Nuclear Corp.; specific activity 8 mC/mmol) was dissolved in deionized water in the presence of unlabeled

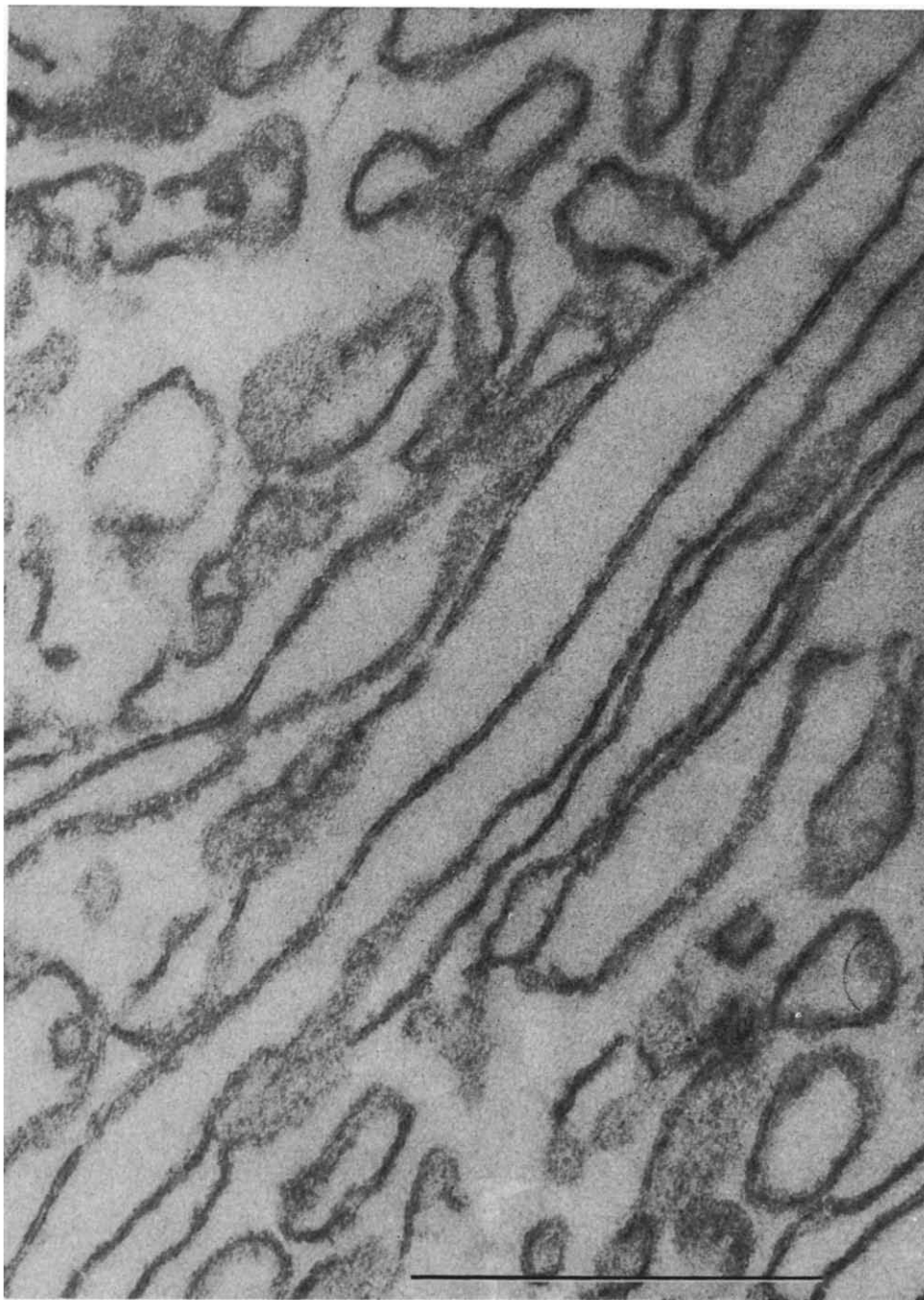


Fig. 1. Thin section of purified plasma membrane from *M. laidlawii*. Scale marker represents 0.5μ . $\times 112,000$.

sodium dodecyl sulfate to an activity appropriate for counting. Two membrane samples of 3 ml containing 1 mg protein were made 3 mM (low) and 18 mM (high) in sodium dodecyl [^{35}S]sulfate. After standing for 1 h the samples were centrifuged at $37\,000 \times g$ for 30 min to remove residual sedimentable material and dialyzed against 1:20 β -buffer with 0.02 M MgCl_2 as described above. Samples of each 3 l buffer solution were dried on planchets and counted for radioactivity after removal of the dialysis sample. Samples of the $37\,000 \times g$ pellet and supernatant after dialysis were also counted, and the distribution of sodium dodecyl [^{35}S]sulfate in these 4 fractions computed.

RESULTS

The initial membrane preparation² gave a characteristic translucent yellow pellet upon centrifugation, and banded in a sucrose gradient at an equilibrium density of about 1.18 g/cm³. Thin-sectioned electron micrographs of this material (Fig. 1) display uniform fields of triple-layered 'unit' membrane⁶ structure 75–90 Å thick.

The choice of conditions of solubilization and reaggregation, together with a gross description of the pellets of reaggregated material, are given in Table I. These

TABLE I

APPEARANCE OF PELLETS OF AGGREGATED MATERIAL OBSERVED AT DIFFERENT SODIUM DODECYL SULFATE AND MAGNESIUM CONCENTRATIONS

Sodium dodecyl sulfate concentration	<i>Mg</i> ²⁺ concentration		
	0	0.02 M	0.05 M
Low	(1) no pellet	(3) translucent yellow pellet	(5) two-layered pellet; opaque lower core overlaid with translucent yellow material
High	(2) no pellet	(4) two-layered pellet; opaque lower core overlaid with translucent yellow material	(6) opaque pellet, faintly yellow

conditions comprise a grid of 6 fields representing 3 different Mg^{2+} concentrations and 2 different ranges of sodium dodecyl sulfate concentration; detailed analytical results for each field are discussed below.

(1) Low sodium dodecyl sulfate, no Mg^{2+} . No significant quantity of material was pelleted at $37\,000 \times g$ after dialysis. The supernatant material gave a single schlieren peak on analytical ultracentrifugation characterized by a sedimentation coefficient ($s_{20,w}$) of 4.0 S.

When the sample was centrifuged for 30 h in a density gradient, the velocity distribution of material in Fig. 2a was observed. Lipid and protein seem to be bound in a lipoprotein band, and some unbound protein is also visible above this band.

(2) High sodium dodecyl sulfate, no Mg^{2+} . This material resembled sample (1) above in giving no appreciable quantity of precipitable material and in exhibiting a single schlieren peak with $s_{20,w}$ of 4.1 S.

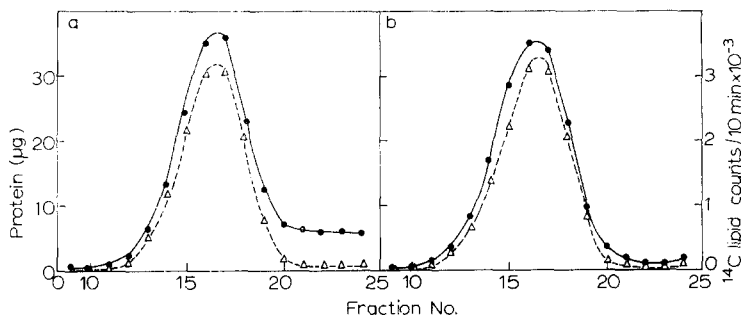


Fig. 2. Distribution of protein and lipid components in velocity sedimentation in a density gradient. a, Low sodium dodecyl sulfate, no Mg^{2+} dialyzed material. b, High sodium dodecyl sulfate, no Mg^{2+} dialyzed material. Gradients were centrifuged for 30 h at 40 000 rev./min in an SW 50 rotor. ●—●, protein; \triangle — \triangle , radioactive lipid. In each case the top of the gradient is on the right.

Density-gradient analysis of this material produced the distribution seen in Fig. 2b. A single lipoprotein band is seen with little indication of the presence of any other material; in particular, the unbound protein seen in Fig. 2a is absent.

(3) Low sodium dodecyl sulfate, 0.02 M Mg^{2+} . Reaggregation under this set of conditions has been repeated several times with sodium dodecyl sulfate concentrations

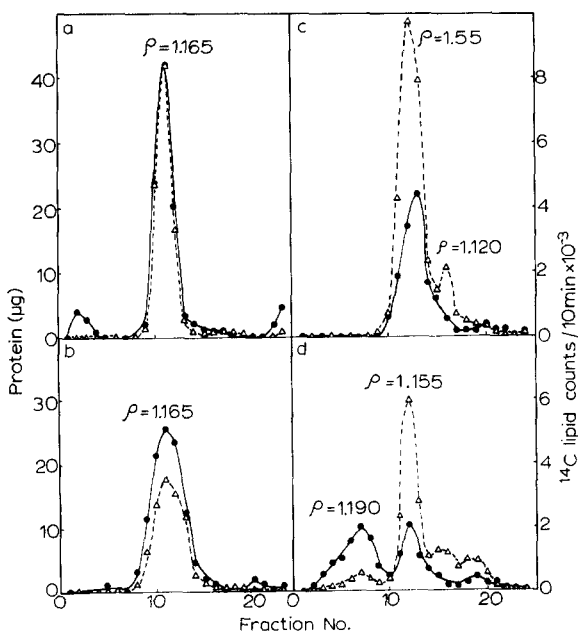


Fig. 3. Equilibrium density-gradient distribution of protein (●—●) and $[^{14}\text{C}]$ lipid (\triangle — \triangle) in different samples of reaggregated membrane components: a, Low sodium dodecyl sulfate, 0.02 M Mg^{2+} . b, Low sodium dodecyl sulfate, 0.05 M Mg^{2+} . c, High sodium dodecyl sulfate, 0.02 M Mg^{2+} . d, High sodium dodecyl sulfate, 0.05 M Mg^{2+} . All samples spun for 4 h at 39 000 rev./min (20°) in an SW 50 rotor. Density ($\rho \pm 0.005 \text{ g/cm}^3$) of peak fraction (determined from refractive index) is indicated above each peak. In each case the top of the gradient is on the right.

in the range 4–7 mM, for protein concentrations in the range 0.5–1.0 mg/ml. Equilibrium density-gradient banding of these samples has always produced a single band with a peak density of 1.16–1.18 g/cm³ containing both lipid and protein (Fig. 3a). A thin-section electron micrograph of a specimen typical of most preparations obtained under these conditions is seen in Fig. 4; triple-layered structures 75–90 Å wide, closely resembling the original membrane, are seen both in closed vesicles and in long linear arrays. The vesicles seem fairly uniform in size; most have circumferences in the range 0.3–0.5 μ , in contrast to the original membrane preparation which exhibits a range of 0.2–2.0 μ .

The appearance of another sample which had the lowest density (1.16 g/cm³) observed for material reaggregated under these conditions is shown in Fig. 5. Many small rounded vesicles with circumferences generally around 0.3 μ are visible; in many cases these appear to be made up of 2 or more concentric layers of alternating dark and light bands with a periodicity of about 40–50 Å. Triple-layered structures are also present; some are 75–90 Å wide, while others are considerably thicker, 160–190 Å.

(4) Low sodium dodecyl sulfate, 0.05 M Mg²⁺. The appearance of the reaggregated pellet (noted in Table I) suggested the presence of more than 1 type of component in this sample. In the density gradient after equilibrium banding 3 bands were visible, 2 faint bands surrounding a central dense band. The gradient profile (Fig. 3b) fails to resolve these bands into separate peaks, but comparison with the gradient profile displayed in Fig. 3a, run under identical conditions, suggests that the wide band width observed here is due to a broad range of density variation in the sample (1.15–1.18 g/cm³) rather than to diffusion.

Fig. 6 shows the electron microscopic appearance of this fraction. Small dense globules, ranging from 0.06–0.3 μ in diameter, are prominent and in many cases show concentric striations of alternating dark and light bands with a periodicity of 40–50 Å. Also present are triple-layered structures, some about 75–90 Å wide, others about 160–190 Å.

(5) High sodium dodecyl sulfate, 0.02 M Mg²⁺. Fortunately, the 2 layers comprising the reaggregated pellet of this sample separated during dehydration procedures, and it was possible to embed and examine them separately. The outer pellet layer, containing the major amount of sample material, showed numerous thin string-like structures (Fig. 7), parts of which clearly exhibited triple-layered structure about 75–90 Å wide; no vesicles were seen. Micrographs of the inner pellet, which contained only a small part of the sample material, appear in Fig. 8; tightly packed globular structures ranging in diameter from 0.25–1.25 μ are seen throughout the field. Numerous concentric lamellar striations of alternating dark and light bands with 40–50 Å periodicity are visible in suitable sections of these globules.

Gradient analysis of this sample (Fig. 3c) revealed a large band with a peak density of about 1.15 g/cm³ which contained both lipid and protein, as well as a small upper band of density 1.12 g/cm³ which consisted primarily of lipid with a small shoulder of protein. It seems reasonable to identify the major, denser band with the detached outer pellet described above (Fig. 7), and the smaller, less dense band with the smaller inner core of the pellet (Fig. 8).

(6) High sodium dodecyl sulfate, 0.05 M Mg²⁺. This sample, comprising an opaque, tightly packed pellet, was extremely heterogeneous both in morphological

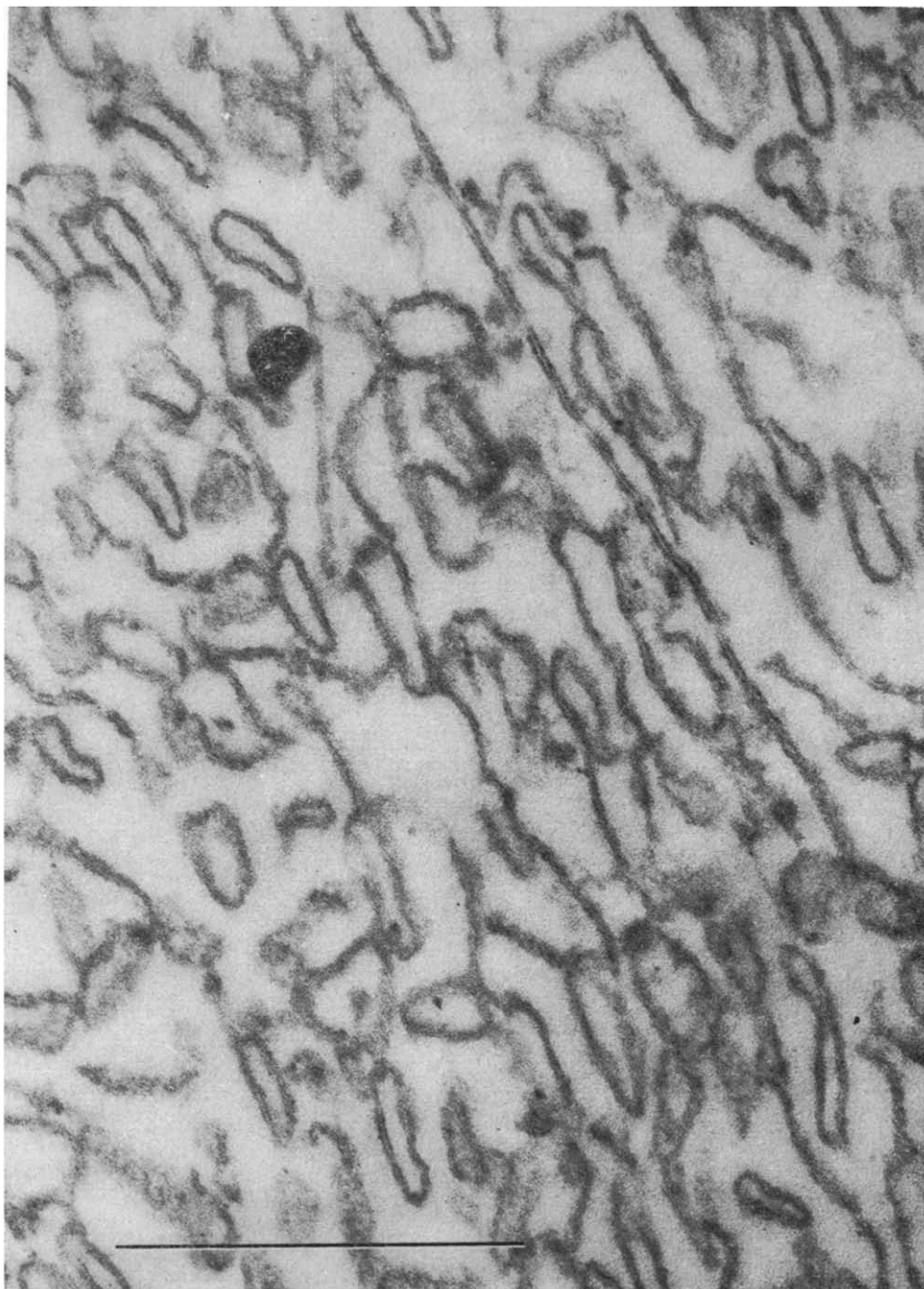


Fig. 4. Solubilized membrane reaggregated under conditions of low sodium dodecyl sulfate, 0.02 M Mg^{2+} . Scale marker represents 0.5 μ . $\times 112\ 000$.

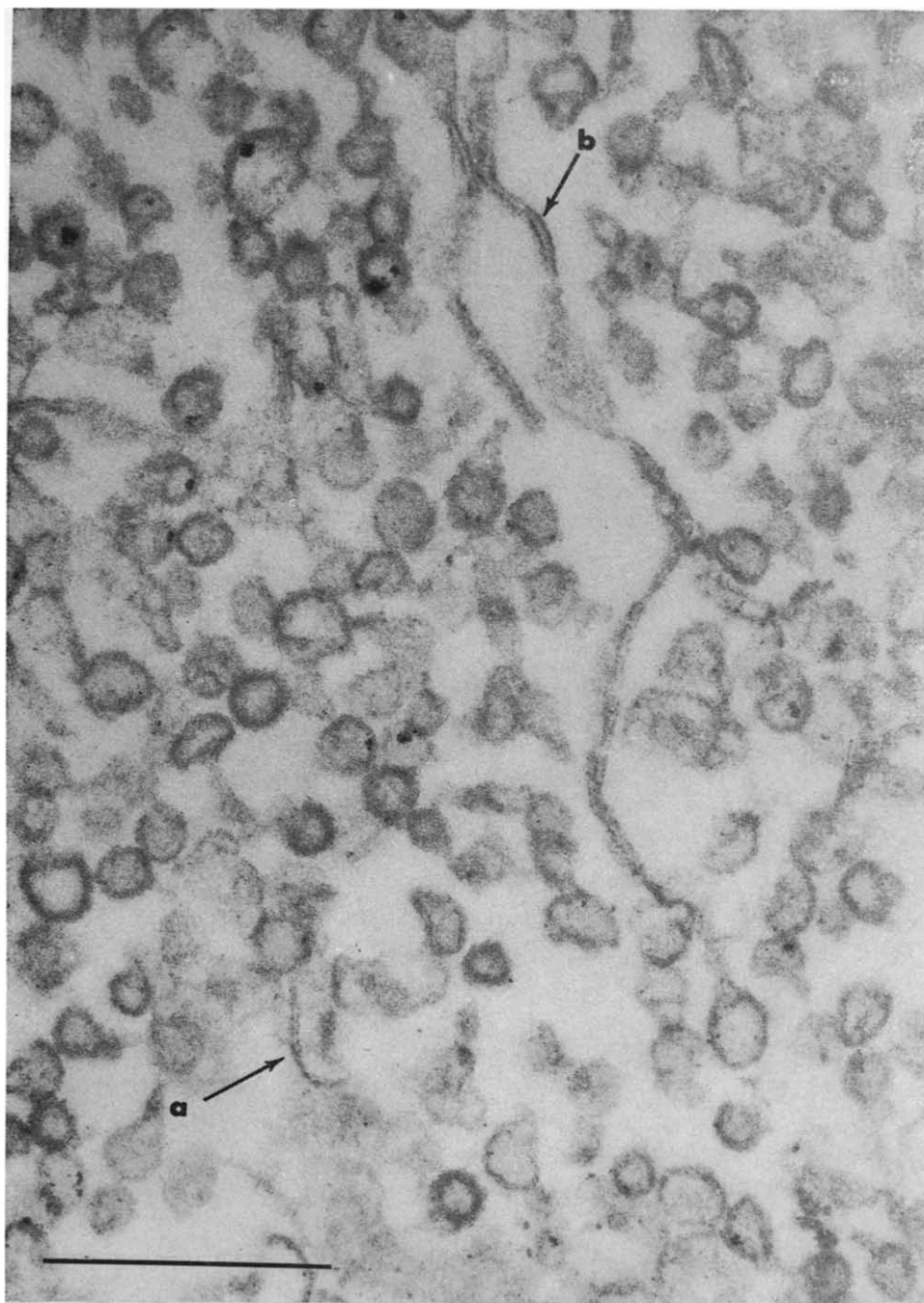


Fig. 5. Solubilized membrane reaggreated under conditions of low sodium dodecyl sulfate, 0.02 M Mg^{2+} . In addition to many vesicles, note 75–90 Å thick triple-layered structures (arrow a) and 160–190 Å thick structures (arrow b). Scale marker represents 0.5 μ . $\times 78\ 500$.

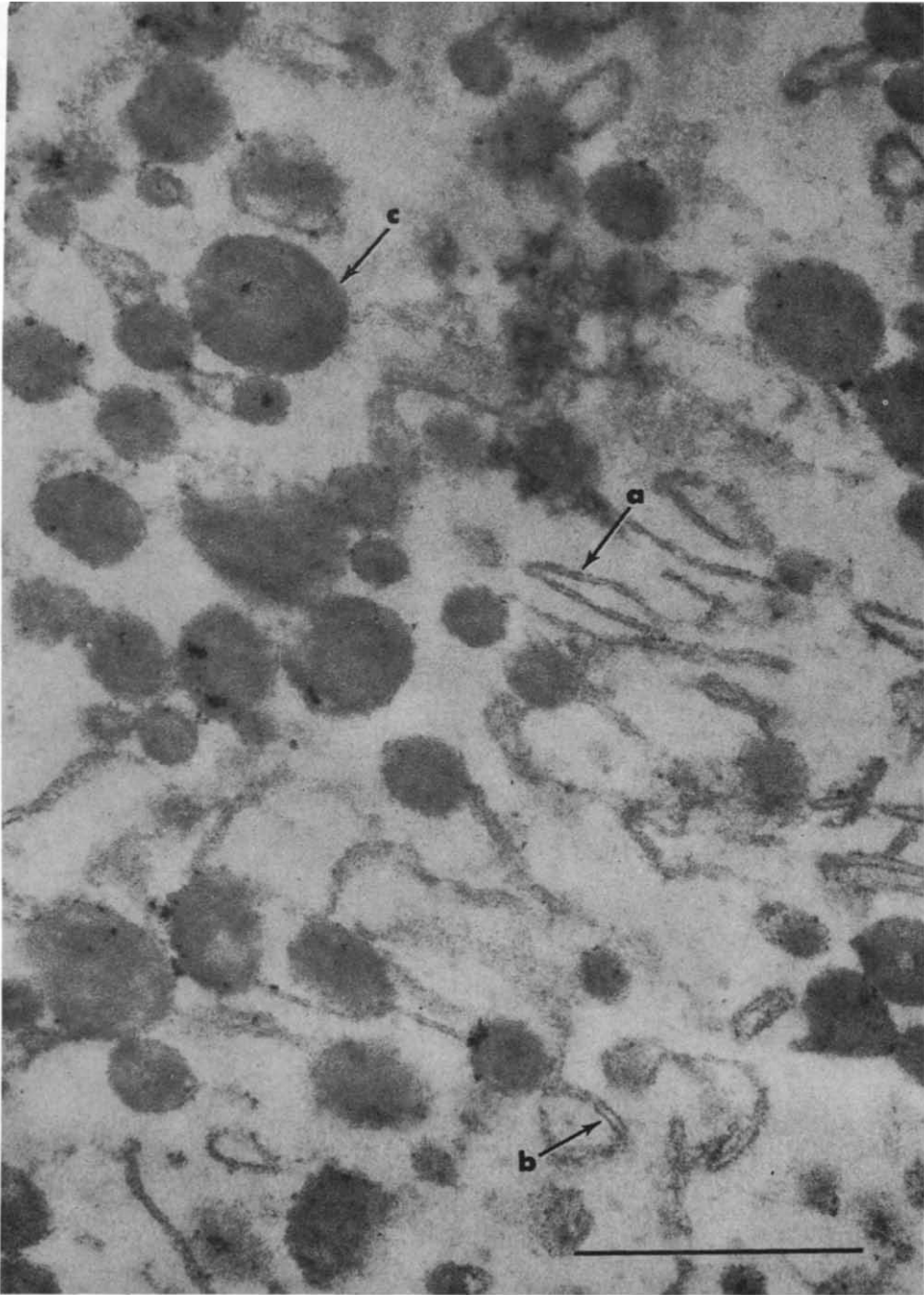


Fig. 6. Solubilized membrane reagggregated under conditions of low sodium dodecyl sulfate, 0.05 M Mg^{2+} . Typical structures are: 80 \AA wide triple-layered structure (arrow a), 170 \AA wide structure (arrow b), and globule (arrow c) containing lamellar striations with $40\text{--}50 \text{ \AA}$ periodicity. Scale marker represents 0.5μ . $\times 78\,500$.

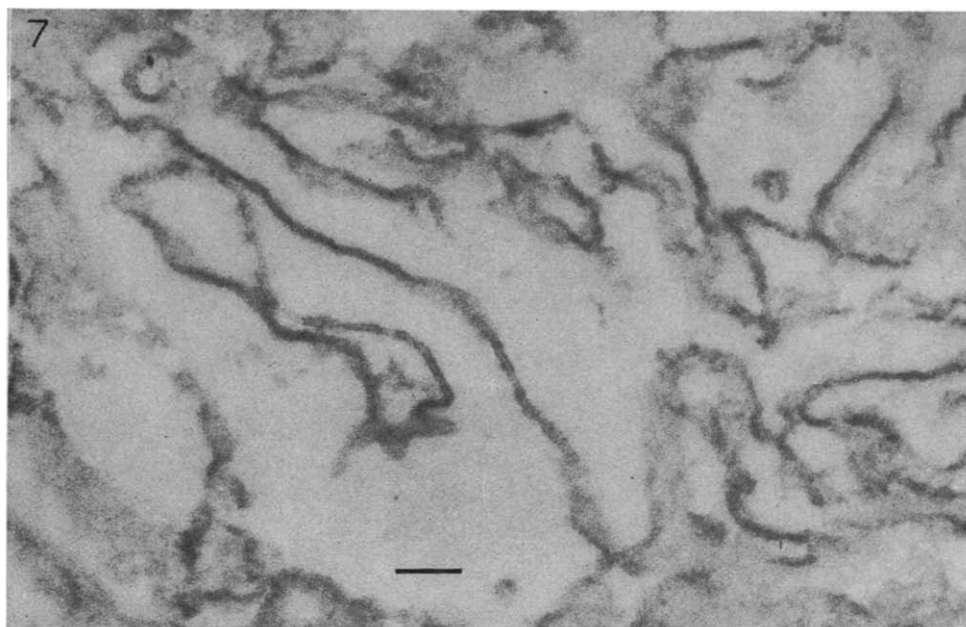


Fig. 7. Solubilized membrane reaggreated under conditions of high sodium dodecyl sulfate, 0.02 M Mg^{2+} . This outer layer of pelleted material became detached from inner layer shown in Fig. 8. Scale marker indicates 0.1 μ . $\times 82\ 800$.

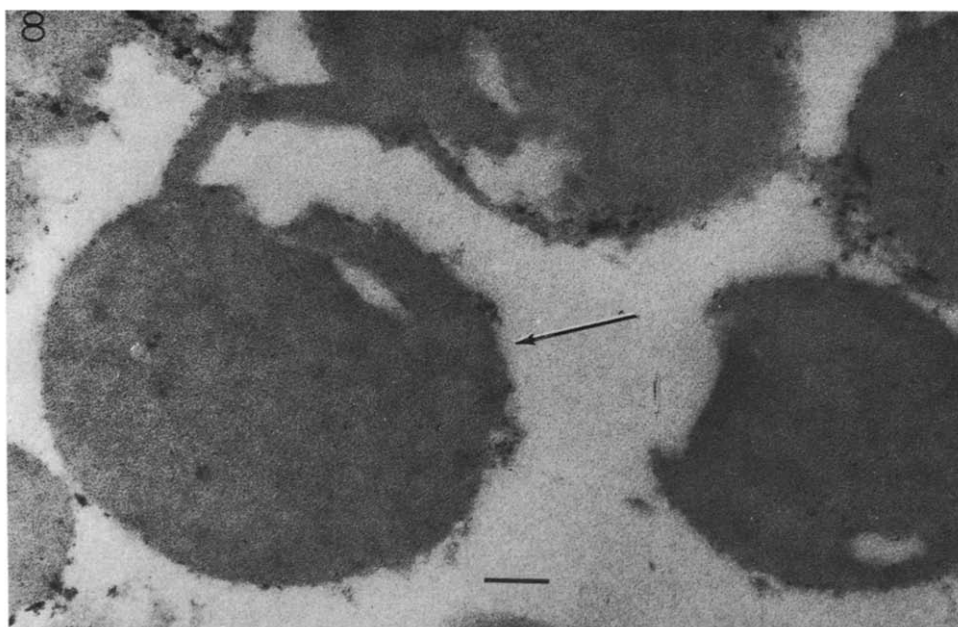


Fig. 8. Solubilized membrane reaggreated under conditions of high sodium dodecyl sulfate, 0.02 M Mg^{2+} . This inner layer of pelleted material was much smaller in amount than that shown in Fig. 7. Arrow indicates globule in which lamellar striations are prominent. Scale marker indicates 0.1 μ . $\times 82\ 800$.

appearance and in its gradient profile. Fig. 3d illustrates the distribution of protein and lipid after density-gradient banding (4 bands were seen in the gradient prior to unloading). A protein-rich broad band is seen with a density range of 1.21–1.17 g/cm³, while a large band containing lipid and protein with peak density of 1.16 g/cm³ occupies the center of the gradient; 2 small bands are also visible toward the top of the gradient.

Electron micrographs through different regions of the pellet showed many different structures, in contrast to other samples in which different sections showed comparable results. Fig. 9 shows one such field, containing both large and small globules of somewhat amorphous structure as well as a few string-like structures which occasionally show some triple-layered character. Other sections showed massive globules as large as 10 μ in diameter. In some sections it was possible to distinguish concentric lamellar striations with a periodicity of 40–50 Å within the body of such globules.

Solubilization and reaggregation with ³⁵S-labeled sodium dodecyl sulfate

In order to examine the possibility that significant amounts of sodium dodecyl sulfate might be incorporated into the reaggregated structures described above, solu-

TABLE II

PERCENTAGE DISTRIBUTION OF ³⁵S-LABELED SODIUM DODECYL SULFATE IN DIFFERENT FRACTIONS OF LOW AND HIGH SODIUM DODECYL SULFATE-SOLUBILIZED MEMBRANE REAGGREGATED IN 1:20 β -BUFFER CONTAINING 0.02 M MgCl₂

Fraction	Sodium dodecyl sulfate recovered in different fractions	
	Sodium dodecyl [³⁵ S]sulfate concentration	Low sodium dodecyl sulfate (%) High sodium dodecyl sulfate (%)
1st dialysis solution		93 94
2nd dialysis solution		3 3
Supernatant of dialyzed sample		3 2
Pellet of dialyzed sample (reaggregated material)		0.2 0.2

tions containing sodium dodecyl sulfate labeled with ³⁵S were used to solubilize unlabeled membrane and the samples (low and high sodium dodecyl sulfate) reaggregated in buffer containing 0.02 M Mg²⁺. The distribution of sodium dodecyl sulfate in the 2 dialysis washes and in the reaggregated material are summarized in Table II. In both cases almost all detergent is released into the first dialysis buffer; extremely little (less than 1% by weight) is incorporated into the reaggregated material.

DISCUSSION

The results of this study indicate that the same solubilized membrane components can interact in a wide variety of ways, depending in part on the initial conditions of solubilization and in part on the ionic environment to which they are subsequently subjected. Sodium dodecyl sulfate has been shown to interact with membranes in such a way as to separate lipid and protein components in the process of disrupting mem-

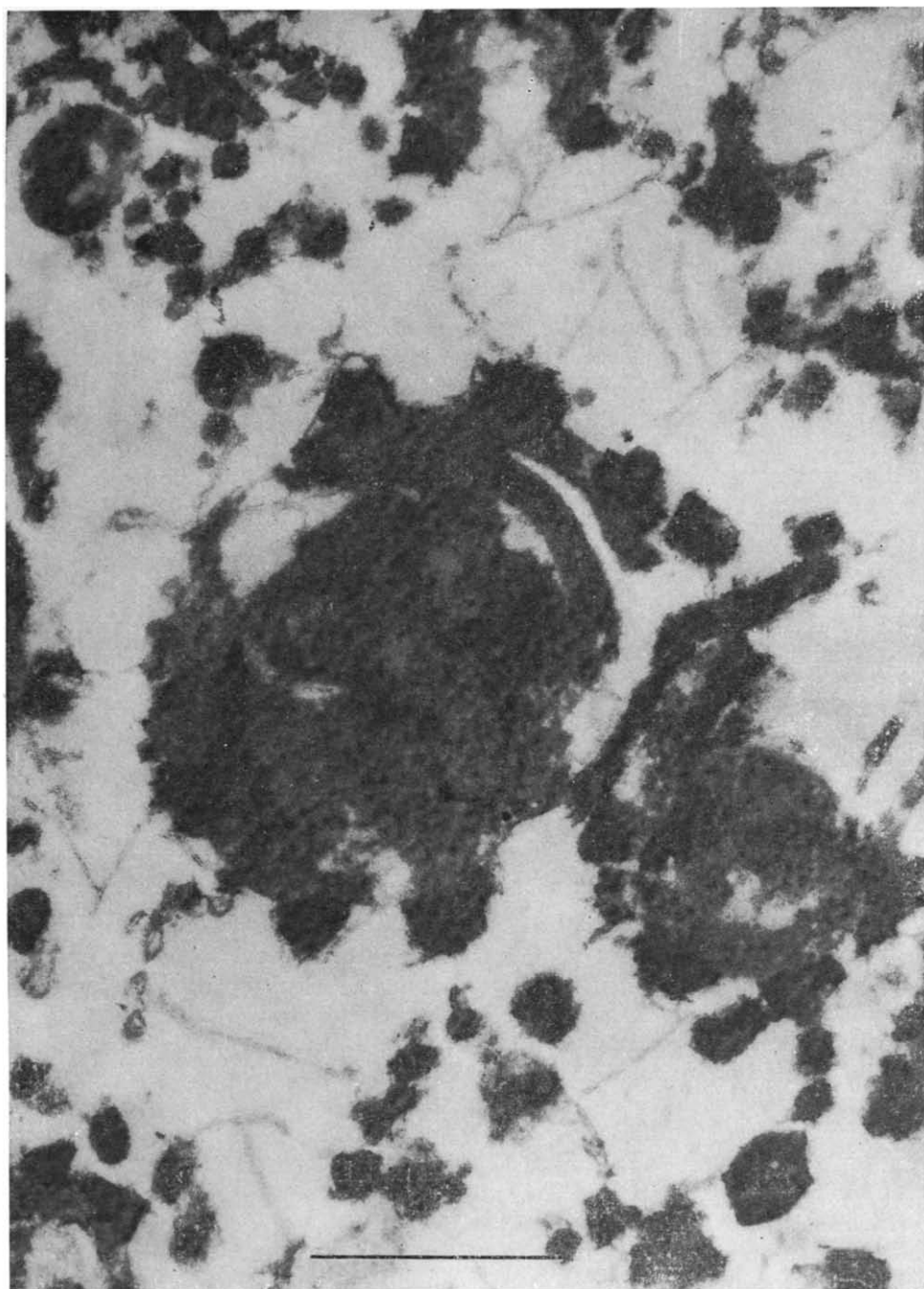


Fig. 9. Solubilized membrane reaggreated under conditions of high sodium dodecyl sulfate, 0.05 M Mg^{2+} . Scale marker indicates 1 μ . $\times 34\ 400$.

brane structure². In all cases in which the detergent is removed by dialysis, these lipid and protein elements interact to produce lipoprotein structures. Whether these structures are aggregated into large sedimentable material or remain as soluble components in the supernatant depends on whether or not Mg^{2+} is introduced into the dialysis solution. There is some evidence that other divalent or multivalent cations can serve the same purpose¹.

In the absence of Mg^{2+} , dialysis of sodium dodecyl sulfate-solubilized membrane results in small lipoprotein particles with an $s_{20,w}$ of approx. 4 S, little difference is seen between samples prepared with low or high concentrations of detergent (Fig. 2a, b). The detailed composition of these particles is currently under investigation. It is important to consider this result in evaluating the composition of detergent-solubilized membrane products. GENT *et al.*⁷, in a study of lysolecithin solubilization of myelin, dialyzed their solubilized material in a buffer lacking divalent cations in preparing the sample for electrophoresis. The single electrophoretic peak observed for this dialyzed material might well reflect a reassociation of lipid and protein which had been separated during the process of detergent solubilization. The existence of such reassociated lipoprotein particles would not constitute satisfactory evidence for a lipid-protein subunit in the original material.

In the presence of Mg^{2+} , we observe a variety of structures assembled from the solubilized membrane components (Figs. 4-9). These structures fall predominantly into 2 classes: linear triple-layered structures, 75-90 Å or occasionally 160-190 Å thick; and densely packed globules of different sizes, on some of which can be seen concentric lamellar striations of alternating dark and light bands with a periodicity of 40-50 Å. Several factors may play a role in causing the appearance of the different observed structures. Different concentrations of sodium dodecyl sulfate may lead to different degrees of denaturation of protein or dispersion of lipid², and so affect the manner in which these components can subsequently interact. Alternate types of interaction or aggregation of the lipid and protein components may be favored by different levels of Mg^{2+} concentration.

Thin sections of the original membrane preparation (Fig. 1) exhibit triple-layered 'unit' membrane structure, 75-90 Å wide, which is in accord with the characteristic thin-section electron microscopic appearance of fixed, dehydrated, and embedded plasma membrane⁸. Among the structures observed after reaggregation under the experimental conditions used in this study, those shown in Fig. 4 exhibit the closest correspondence with the original membrane preparation, although some triple-layered membrane-like pieces are seen in almost all preparations. The spatial extension of such reaggregated membrane-like material also seems to vary under different conditions; for example, Fig. 4 seems to contain primarily closed vesicles of fairly uniform size, while Fig. 7 shows more elongated, sheet-like structures. The important features of the preparation seen in Fig. 4 are the use of a relatively low concentration of sodium dodecyl sulfate to solubilize the membrane initially and the presence of 0.02 M Mg^{2+} in the dialysis buffer. Density-gradient equilibrium banding of this material also corresponds closely to results obtained with the original membrane preparation; a single, sharp band containing lipid and protein is seen (Fig. 3a), and the isopycnic density of this band corresponds closely to that of the similar band observed with control membrane (see ENGELMAN, TERRY AND MOROWITZ²). It seems unlikely that structures of significantly different lipid-protein composition would correspond so closely both

in equilibrium density and in morphological appearance with the original membrane; therefore, it seems plausible to regard these structures as having a lipid-protein composition and a structure similar to the membrane from which they were derived. The fact that such structures can be reassembled spontaneously under suitable conditions suggests the possibility that, in a living cell, the synthesis of appropriate proteins and the availability of suitable lipids may provide sufficient structure-determining information for the synthesis of new membrane. The results of these studies, however, can only be construed as indirect evidence for this view in so far as the presence of detergent in our experimental system constitutes a significant departure from the conditions of membrane synthesis *in vivo*. Furthermore, the lack of any suitable assay for selective permeability and transport properties in isolated membrane fractions has rendered it infeasible for us to demonstrate that the molecular arrangement of the re-aggregated membrane is identical to or different from that of the original membrane with respect to these functions.

The appearance in several samples of exceptionally wide triple-layered structures might be the result of a coalescence of 2 or more bimolecular lipid leaflets, coated on the outside with protein. The 40–50 Å wide concentric lamellar striations observed in the different sized globules present in many of the samples are suggestive of the myelin figures produced by dispersions of phospholipids in aqueous solutions. Thin-section electron micrographs of such phospholipid dispersions often reveal lamellar striations with a periodicity of about 40 Å (see ref. 8). However, these globules have been observed to band at isopycnic densities significantly in excess of those expected for pure phospholipid micelles (Fig. 3b–d). It is therefore probable that the globules contain some protein.

The wide variety of possible states of aggregation of solubilized membrane components indicates that only under fairly restricted conditions both of initial detergent concentration and of subsequent exposure to divalent cations do these components interact to produce material which resembles the original membrane in morphology and density. Although other modes of aggregation differ in these 2 respects, they all exhibit some degree of structural order.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. J. G. GALL for the use of his electron microscope facilities.

This work was supported by U.S. Atomic Energy Commission Grant AT 30-1-2687 and by Public Health Service Grant AM 05226.

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