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CHARACTERIZATION OF THE PLASMA MEMBRANE OF *MYCOPLASMA LAIDLAWII*

IV. STRUCTURE AND COMPOSITION OF MEMBRANE AND AGGREGATED COMPONENTS*

DONALD M. ENGELMAN** AND HAROLD J. MOROWITZ

Department of Molecular Biophysics, Yale University, New Haven, Conn. (U.S.A.)

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SUMMARY

Mycoplasma laidlawii membranes and various preparations of sodium dodecyl sulfate-dissolved and subsequently aggregated structures are compared with respect to amino acid composition, hexosamine composition, appearance in electron microscopy, protein to lipid ratio, buoyant density and magnesium to protein ratio. The various aggregates are virtually indistinguishable by these criteria with the exception of the microscopic appearance of the small lipoprotein aggregates and the Mg^{2+}-induced structures derived from these aggregates.

INTRODUCTION

In previous publications[1,2] the aggregation of lipid and protein membrane components which had been separated by exposure of purified membranes to detergent has been described. Removal of the detergent by dialysis against Mg^{2+}-containing buffer leads to the formation of lipoprotein membranes similar in electron microscopic appearance to the original membrane. Dialysis against buffer in the absence of Mg^{2+} leads to the formation of small lipoprotein aggregates which can be assembled into small membrane-like structures upon introduction of Mg^{2+}. The objective of the present work is to establish more detailed characteristics of the original membrane isolate and to examine the relationship of the aggregated structures to the original membrane in terms of such characteristics.

MATERIALS AND METHODS

Growth of *Mycoplasma laidlawii* B cells, introduction of [^{14}C]lipid label and procedures for the isolation of purified membranes have been described[3]. In summary,

* Part of this material has been submitted in partial fulfillment of the requirements of the Ph.D. degree at Yale University.

** Present address: Cardiovascular Research Institute, University of California Medical Center, San Francisco, Calif., U.S.A.

cells are lysed by exposure to low ionic and osmotic strength buffer, the membranes are banded in a sucrose gradient, and the banded material is washed to remove the sucrose.

Preparation of aggregates

Three types of lipid–protein aggregates will be discussed. They are the reaggregated membrane[1], the small lipoprotein aggregate[2] and the reaggregated membrane prime[2].

Reaggregated membrane was prepared by dissolving suspensions of purified original membranes at a protein concentration of 3 mg/ml with sodium dodecyl sulfate (to a final concentration of 10 mM). The solubilized membrane was then dialyzed against 1:20 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) containing 20 mM $MgCl_2$ for 36 h at 4°. The aggregates formed were then washed by centrifugation and resuspension in 1:20 β-buffer without Mg^{2+}.

Small lipoprotein aggregates were prepared as described previously[2] by dialysis of the solubilized membranes against 1:20 β-buffer.

M_r' was prepared from the small lipoprotein aggregates by dialysis against 1:20 β-buffer containing 20 mM $MgCl_2$ as described previously[2].

Both the reaggregated membrane and the M_r' were twice washed by centrifugation at 100000 $\times$ g for 15 min and resuspended in 1:20 β-buffer.

Analytical procedures

Protein was assayed by the Folin phenol method of LOWRY *et al.*[4] using egg white lysozyme (2 $\times$ crystallized, Worthington Biochemical Corp.) as a standard. Radioactive samples were dried on planchets and counted on a Beckman low-beta thin-window counter. Buoyant densities were determined by the method of TERRY, ENGELMAN AND MOROWITZ[1]. Mg^{2+} was determined by the atomic absorption method previously described[2].

Amino acid analysis

Samples containing 2–5 mg membrane were lyophilized (for amino acid analysis) in new, washed test tubes, resuspended in 3 ml constant boiling 6 M HCl, and sealed under vacuum. Hydrolysis was effected by heating for 22 h at 110°. Samples were then dried in a rotary evaporator to remove HCl, and resuspended in 1 ml distilled water for loading onto the analyzer.

Aliquots (0.5 ml) of the hydrolyzed protein were loaded onto the long and short columns of an automatic recording amino acid analyzer[5]. The resultant peak areas were compared with recent calibrations to obtain concentrations.

Cysteine was determined as cysteic acid in a separate analysis of protein oxidized with performic acid by the method of HIRS[6]. Methionine was taken to be the sum of the methionine and methionine sulfone peaks. The corrections used for destruction during hydrolysis are those of MOORE AND STEIN[5]; they are 5 % for threonine and tyrosine and 10 % for serine.

Several attempts were made to determine the content of tryptophan using the spectrophotometric techniques of GOODWIN AND MORTON[7]. Unfortunately, interference of the carotenoid absorption with the protein spectrum persisted after various

extraction procedures and no satisfactory value for the tryptophan content was obtained.

In addition to the normal amino acid peaks, three peaks not usually found in protein hydrolysates appeared that were not readily identified. The first appeared as a very small peak between cysteic acid and aspartic acid and appeared to absorb more at 440 mμ than at 570 mμ when stained with ninhydrin. The second and third unknown peaks followed phenylalanine. Much larger than the first unknown peak and rather broad, these peaks absorbed substantially more at 570 mμ than at 440 mμ.

From the inspection of the results of ZACHARIUS AND TALLEY[8] the compounds responsible for the unknown peaks were tentatively identified as levulinic acid (between cysteic acid and aspartic acid), glucosamine (following phenylalanine), and galactosamine (following glucosamine). The analyzer was then calibrated with these compounds (Sigma Biochemicals) and the resultant peaks were found to be indistinguishable from those seen in the membrane analysis. The presence of levulinic acid is consistent with the presence of the amino sugars and the presence of phosphatidyl glucose[9] as it is a product of acid hydrolysis of sugars.

Density gradient centrifugation

Two types of linear sucrose gradients were used: (1) 5 ml in 20–50 % (w/w) linear sucrose gradients were formed using sucrose dissolved in 1:20 β-buffer and 10 mM in sodium dodecyl sulfate. Samples of the original membrane, reaggregated membrane, and small lipoprotein aggregate were layered into the gradients and the gradients were centrifuged for 24 h at 35000 rev./min in the SW 50 rotor of Spinco model L-2 ultracentrifuge at 22°; (2) 5 ml linear 25–50 % sucrose gradients were formed from sucrose dissolved in 1:20 β-buffer. Samples of the original membrane, the reaggregated membrane and the M_r' were layered onto the gradients and centrifuged for 42 h at 22° and 50000 rev./min.

In all cases fractions were bottom collected and analysed for protein (Folin reaction) and lipid (^{14}C counts).

Electron microscopy

The microscopic techniques used were those of TERRY, ENGELMAN AND MOROWITZ[1]. In summary, samples were centrifuged to form pellets, fixed with formaldehyde and osmium tetroxide, stained with uranyl acetate, embedded in araldite, sectioned, post-stained with uranyl acetate and lead citrate, and examined in a Philips E.M. 200 electron microscope.

Properties of the original membrane

The membrane suspension obtained by the isolation procedures described previously[3] is turbid and yellow in color. The color is presumed to be due to the presence of a carotenoid (absorption maxima at 477, 447 and 421 mμ). Centrifugation of a sample of the membranes to equilibrium in a 25–50 % sucrose gradient produces a single band with a peak buoyant density of 1.18 ± 0.005 g/cm^3. Analysis of the fractionated gradient for protein and lipid content reveals a single peak in which the protein and lipid curves coincide (Fig. 1A).

Analysis of the amino acid composition of the membrane isolate was undertaken with two objectives. First, it was thought that the class of proteins bound to the mem-

brane might have a high content of amino acids which would contribute to hydrophobic interactions with the lipids. Second, a comparison of the protein moiety of the original membrane with that of the reaggregated material to be described below was desired. Additionally, other unusual compositional features might be revealed using such a procedure.

It was observed that upon lipid extraction with chloroform–methanol (2:1, by vol.) approx. 10% of the protein was extracted into the lipid phase. Application of the FOLCH test[10] indicated the possible existence of a proteolipid. Since an analysis of the total protein was desired, it was decided to use unextracted membranes for hydrolysis.

The average amino acid composition obtained from two independent analyses is shown in Table I. A high content of alanine, glycine, valine, leucine, isoleucine, phenylalanine, and tyrosine is seen, but the content is not so high as to suggest that the insoluble character of the protein in aqueous solutions can be explained solely by the amino acid composition.

Three additional peaks were observed in the analysis. They have been tentatively identified as levulinic acid, glucosamine and galactosamine on the basis of calibration of the column with the pure compounds. The hexosamines do not derive from the lipid component of the membrane, since ninhydrin-positive compounds are known to be absent from the lipids[9]. The amounts observed are listed in Table I. Since it is to be expected that the sugars would be considerably degraded during the hydrolysis, these values are probably low.

It appeared curious that such a large hexosamine content was not revealed in

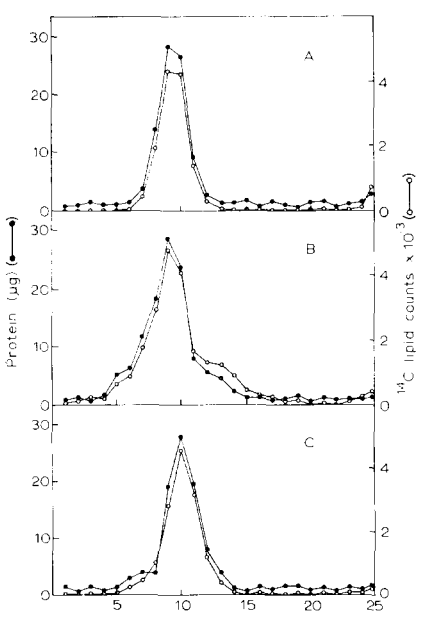

Fig. 1. Equilibrium distribution of original membrane (A), reaggregated membrane prime (B) and reaggregated membrane (C) in 25–50% sucrose gradients. Fractions were analyzed for protein (●—●) and lipid (○—○) following centrifugation for 24 h at 35000 rev./min. The tops of the gradients are on the right.

TABLE I

AMINO ACID ANALYSES OF ORIGINAL MEMBRANE, REAGGREGATED MEMBRANE, SMALL LIPOPROTEIN AGGREGATE, AND REAGGREGATED MEMBRANE PRIME PREPARATIONS

Corrections for losses in hydrolysis were taken as 5% for threonine and tyrosine and 10% for serine. The amino sugar content was divided by the normalizing factor used in each analysis for conversion to mole%. "Trace" implies less than 0.25 mole%.

Amino acid	*Original membrane (mole %)*	*Reaggregated membrane (mole %)*	*Lipoprotein particle (mole %)*	*Reaggregated membrane prime (mole %)*
Lys	6.37	6.17	6.91	6.40
His	1.46	1.40	1.41	1.63
Arg	2.95	2.96	2.96	2.59
Cys	0.19	trace	trace	trace
Asp	11.43	10.07	11.80	11.66
Met	2.33	2.53	2.23	2.20
Thr	6.77	6.97	6.70	7.42
Ser	6.40	6.87	6.97	8.33
Glu	8.23	7.57	7.91	7.16
Pro	3.60	3.88	4.17	3.51
Gly	6.95	7.65	7.24	7.04
Ala	8.23	8.33	8.39	8.38
Val	7.55	7.28	6.98	7.02
Ile	7.39	7.56	6.85	6.72
Leu	9.79	10.13	9.69	9.84
Tyr	4.81	4.81	4.55	4.73
Phe	5.40	5.82	5.22	5.37
GlcN	1.73	2.18	1.59	1.80
GalN	5.75	7.52	5.54	5.36

the analysis of *M. laidlawii* membranes by RAZIN, MOROWITZ AND TERRY[11] using the method of DUBOIS *et al.*[12]. Since no hexosamines were considered in the otherwise thorough exploration of the technique by DUBOIS *et al.* a possible explanation is that they are not revealed by the phenol sulfuric acid reagent. To investigate this possibility, an experiment was conducted in which the method was applied to glucose, glucosamine and galactosamine solutions at a series of concentrations of the sugars. Virtually no reaction with the amino sugars occurs over a concentration range in which the full range of the color reaction is seen with glucose. The failure of the analysis of RAZIN, MOROWITZ AND TERRY to reveal the amino sugar content of the membranes is therefore explained. This result suggests that the compositional data of these authors must be modified to represent the rather large hexosamine content of the membrane.

The appearance of the isolated membranes is that of three-layered unit membranes free from large amounts of extraneous material and having no obvious substructural divisions (Fig. 2).

Centrifugation of a sample of the original membrane solubilized with 10 mM sodium dodecyl sulfate on a sucrose gradient containing 10 mM sodium dodecyl sulfate results in the profile shown in Fig. 3C. The separation of lipid and protein peaks is thought to indicate that the lipid and protein are separate or weakly interacting in the detergent-solubilized state[3].

Properties of aggregates formed from solubilized membrane components

Removal of the detergent from samples of the original membrane preparation leads to the formation of lipid–protein aggregates[1,2]. Three aggregates are obtained

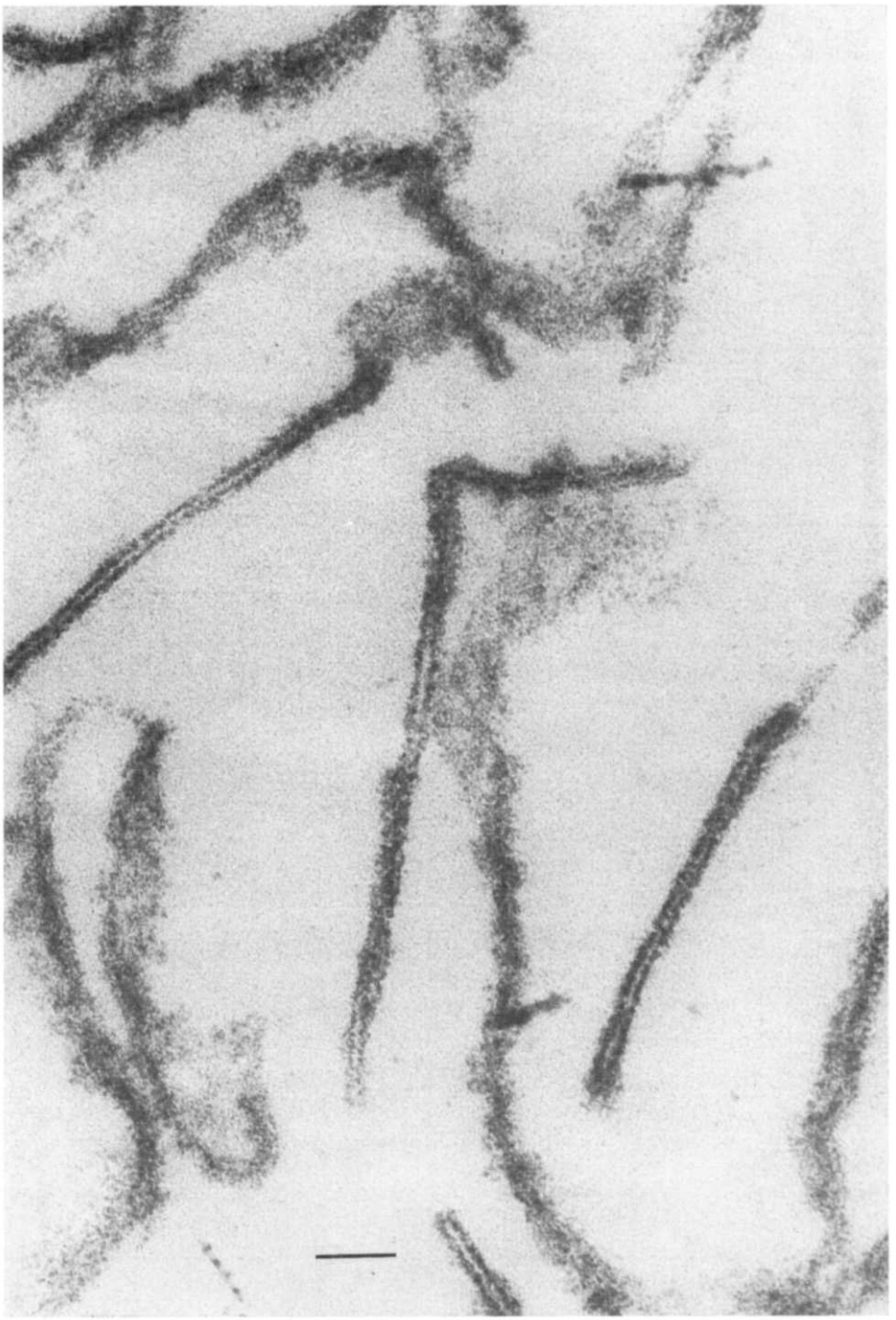

Fig. 2. Original membrane preparation. A pellet of material was fixed with formaldehyde, post-fixed with OsO_4, and stained with uranyl acetate and lead citrate. Clearly delineated regions of unit membrane structure are seen. No clear substructure (*e.g.* cross bridges between the dense layers) is evident. Magnification is 210000 ×. The scale marker represents 500 Å.

by different dialysis procedures: reaggregated membrane, by dialysis of solubilized membrane against 1:20 β-buffer containing 20 mM Mg^{2+} (ref. 1); small lipoprotein aggregates, by dialysis of solubilized membrane against 1:20 β-buffer[2], and reaggregated membrane prime, by dialysis of the small lipoprotein aggregates against 1:20 β-buffer containing 20 mM magnesium[2]. The following experiments compare properties of the aggregates with those of the original membrane.

Protein (Folin reacting material) and lipid ([^{14}C]oleic acid label) contents of the original membrane and the various aggregates were determined. A ratio of protein to lipid (Folin/counts) was calculated and the ratio was normalized to unity for the original membrane. The ratios obtained in 4 experiments were averaged to give the values shown in Table II. The ratio is quite similar in the different preparations.

Samples of the original membrane, reaggregated membrane, and reaggregated membrane prime were centrifuged to approximate equilibrium in sucrose gradients,

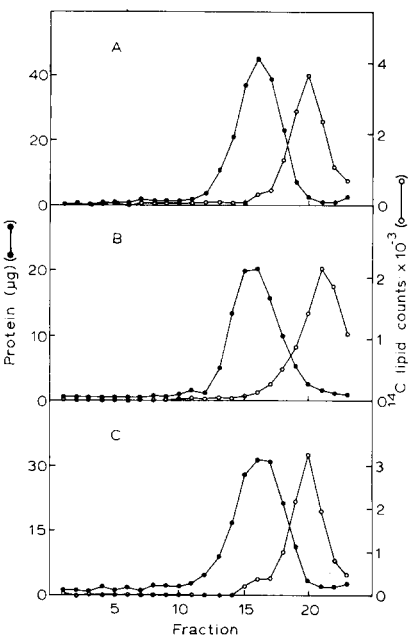

Fig. 3. Sodium dodecyl sulfate sedimentation of the small lipoprotein aggregates (A), reaggregated membrane (B) and original membrane (C). Gradients from 20–50% sucrose containing 10 mM sodium dodecyl sulfate were centrifuged for 42 h at 50000 rev./min. Fractions were analyzed for protein (●—●) and lipid (○—○).

TABLE II

COMPARISON OF DIFFERENT MEMBRANE PREPARATIONS WITH RESPECT TO COMPOSITION AND DENSITY

	Protein to lipid ratio (arbitrary units)	*Buoyant density (g/cm^3)*	*mg Mg/mg protein (× 10^{-3})*
Original membrane	1.0	1.18 ± 0.005	1.30
Reaggregated membrane	0.97	1.175 ± 0.005	1.32
Small lipoprotein aggregates	1.05	not determined	0.62
Reaggregated membrane prime	1.02	1.18 ± 0.005	1.40

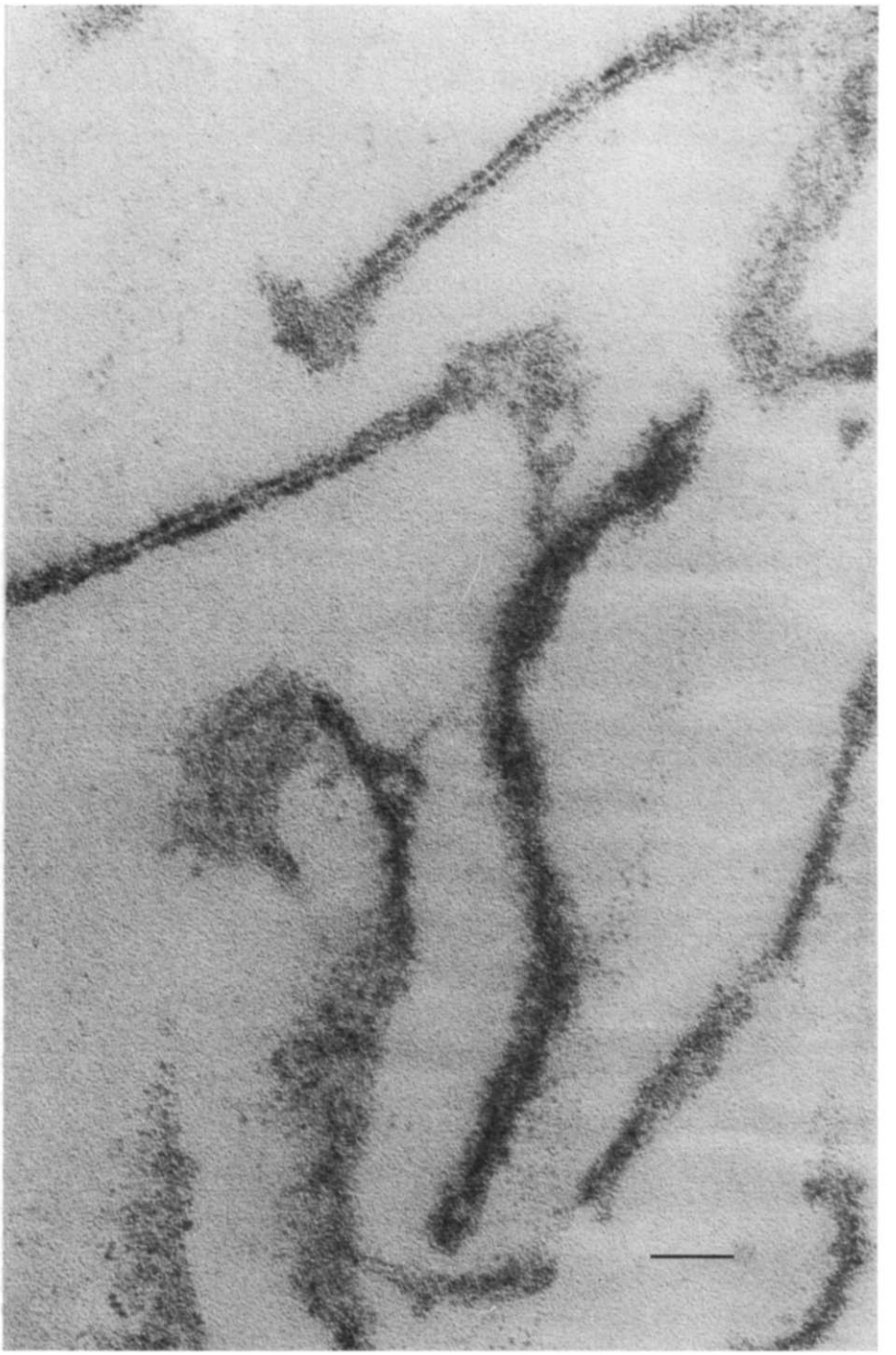

Fig. 4. Reaggregated membrane. A pellet of material was fixed with formaldehyde, post-fixed with OsO_4, and stained with uranyl acetate and lead citrate. Clearly delineated regions of unit membrane are seen. The magnification is 210000 ×. The scale marker indicates 500 Å.

and the profiles obtained from analysis of the fractions for protein and lipid are shown in Fig. 1. The buoyant densities represented by the peak fractions are presented in Table II. The profiles show the preparations to be similar with some heterogeneity evident in the M_r'.

The reaggregated membrane has been shown to have an appearance very similar to that of the original membrane[1]. A view of a thin section of this material is shown in Fig. 4. The appearance of the reaggregated membrane prime is somewhat different, but is consistent with the appearance of small pieces of membrane[2].

The amino acid compositions of the original membrane and the three aggregates are shown in Table I. Comparison shows that the compositions are quite similar, supporting the view that no major class of protein is excluded in the various aggregation reactions. The amino sugars are seen to appear in the aggregated materials and the amounts, while not precisely the same, are comparable. Since the sugars are soluble in water as free molecules, they must be bound to other molecules being bound in turn by the reaggregated structure; otherwise there is no reason to expect them to appear in the reaggregates. Since the lipid fraction is known to lack ninhydrin-positive material[9], it is likely that the sugar is in some way bound to the protein.

The magnesium content of the aggregates was determined by atomic absorption spectroscopy and the values obtained are shown in Table II.

The solubilization of the reaggregated membrane and the small lipoprotein aggregate have been examined by sedimentation of the solubilized material in sucrose density gradients containing 10 mM sodium dodecyl sulfate. The profiles are shown in Figs. 3A and 3B. Incipient separation of the protein and lipid peaks similar to that seen for the original membrane is observed.

In order to examine the retention of aggregation characteristics by the membrane components as they are passed through the various aggregation procedures used, the following experiment was conducted. A sample of the original membrane was solubilized with 10 mM sodium dodecyl sulfate and dialyzed in the absence of magnesium to give small lipoprotein aggregates. These were then dialyzed against 20 mM Mg^{2+} to form reaggregated membrane prime. The reaggregated membrane was washed by repeated centrifugation and resuspension in 1:20 β-buffer, and then solubilized with 10 mM sodium dodecyl sulfate. The solubilized material was then dialyzed against 1:20 β-buffer containing 20 mM Mg^{2+}, presumably giving reaggregated membrane if the properties of the components had not been altered in manipulation. The final material was prepared for electron microscopy and is seen in Fig. 5. Vesicles showing regions of three-layered membrane structure are seen, and it is concluded that the material is similar to the reaggregated membrane.

DISCUSSION

The principal thrust of the experiments described in the preceding studies[1–3] is toward the understanding of the states of membrane components on exposure to and subsequent removal of detergent. For purposes of discussion, a scheme in which the interactions observed are interrelated is developed below.

The material previously presented[3] established that the addition of 10 mM sodium dodecyl sulfate (SDS) to a membrane isolate produces separation and solubili-

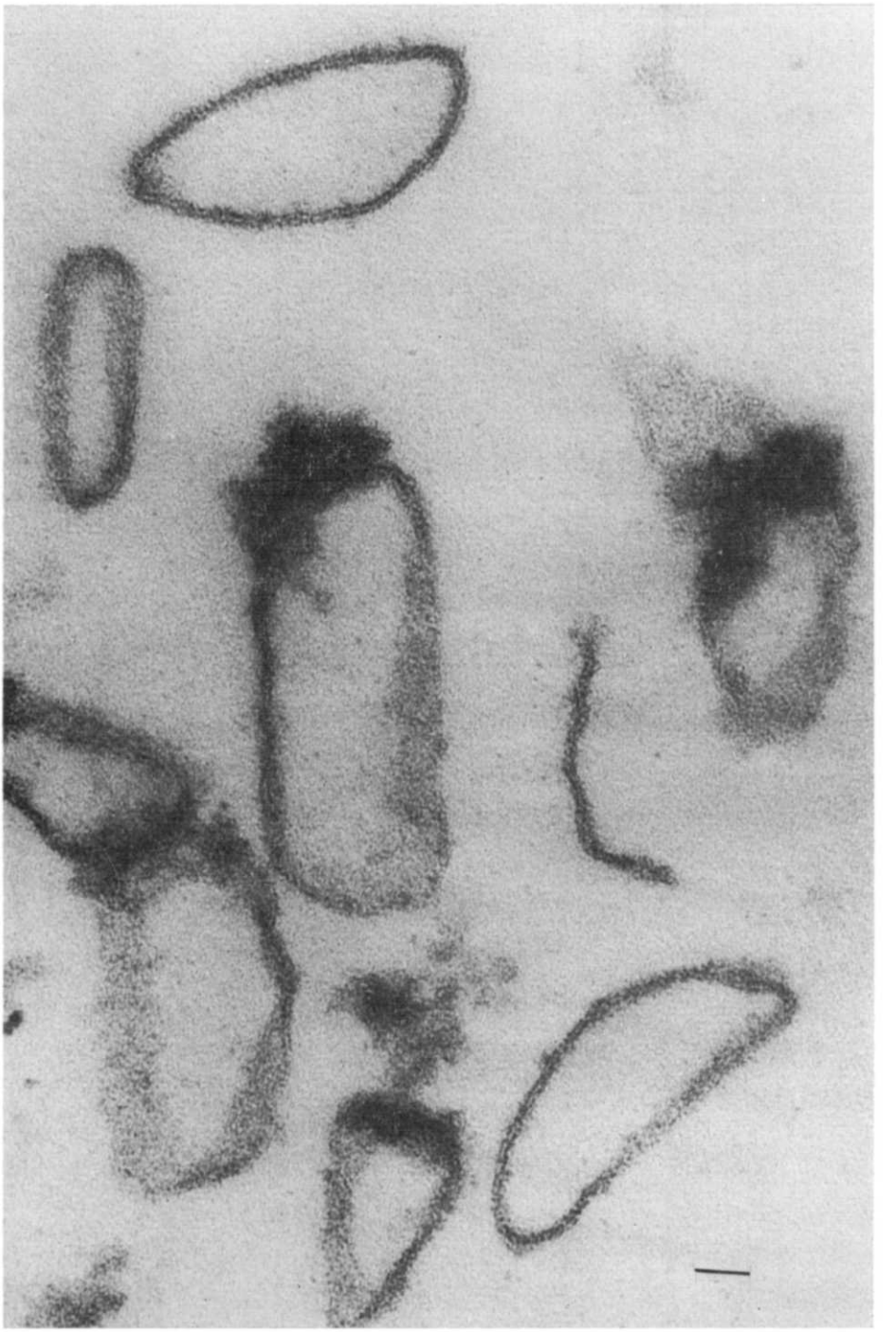

Fig. 5. Reaggregated membrane obtained from solubilized reaggregated membrane prime by dialysis against Mg^{2+}-containing buffer. A pellet of the material was fixed with formaldehyde, post-fixed with OsO_4, and stained with uranyl acetate and lead citrate. Most of the material present is in the form of vesicles showing regions of unit membrane structure. Some heterogeneity may be indicated by the densely staining regions seen to be associated with some of the vesicles The magnification is 140000 ×. The scale marker indicates 500 Å.

zation of its protein and lipid components. If the original membrane is represented by M_0 and the lipid and protein by L and P, the reaction can be written:

$$M_0 \xrightarrow{+\ SDS} L + P$$

In the paper by TERRY, ENGELMAN AND MOROWITZ the experimental evidence was interpreted to show that removal of the detergent by dialysis in the presence of Mg^{2+} produces aggregation of the lipid and protein into a membrane structure. If the reaggregated membrane is represented by M_r, the interaction is summarized:

$$L + P \xrightarrow[+\ Mg^{2+}]{-\ SDS} M_r$$

In the preceding papers[2,3] the effect of removing the detergent in the absence of magnesium was explored and the formation of a small aggregate was observed. If the lipoprotein aggregate is represented by LP, the interaction is

$$L + P \xrightarrow{-\ SDS} LP$$

Finally, the interaction of the small lipoprotein aggregates in the presence of Mg^{2+} to form a reaggregated structure which has several membrane-like properties was observed. If this second kind of reaggregated membrane is represented by M_r', the reaction is written

$$LP \xrightarrow{+\ Mg^{2+}} M_r'$$

These reactions can be assembled into the scheme shown in Fig. 6 where the dashed arrows indicate the apparent reversibility of the reaction shown in Fig. 3. It is apparent from the experiments in going from M_0 to L + P to LP to M_r' to L + P to M_r that the lipid and protein components involved in the various reactions are not radically altered in their properties with respect to aggregation and that the scheme proposed does not conceal extreme alterations of the properties of the components.

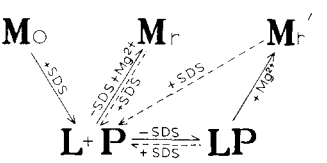

Fig. 6. Overall representation of the transitions among the various membrane derived materials. M_0, original membrane; M_r, reaggregated membrane; M_r', reaggregated membrane prime; L, lipid; P, protein; LP, lipoprotein aggregate; SDS, sodium dodecyl sulfate.

In summary M_0, M_r and M_r' may be compared with respect to the following properties. The three membrane preparations are seen to be rather similar in protein to lipid ratio, buoyant density, and magnesium content (Table II). The distributions of M_r and M_r' in sucrose density gradients centrifuged to bring the material to equilibrium are very much like that of M_0 (Fig. 1). Amino acid analysis shows that the protein and amino sugar content of the three preparations is generally similar (Table I), and the observation of yellow color in all preparations indicates that the carotenoid portion of the membrane appears in M_r and M_r'. Electron mircoscope views of the three materials (Figs. 2 and 4 and previous papers[1,2]) show each to be composed of membranous material showing regions of three-layered "unit membrane" structure,

although M_r' is not in such large aggregates as M_o and M_r. Furthermore, M_o and M_r respond similarly to solubilization by sodium dodecyl sulfate as judged from sucrose gradient profiles (Fig. 3). The structural and compositional observations combine to indicate few differences between the M_o, M_r and M_r', with the strong reservation that no functional attributes have been examined. The point to be made is that separated membrane components can interact spontaneously to form membrane-like material in the absence of pre-existing membrane. Although the use of detergent removal to observe the interaction of separated lipid and protein clearly creates a situation different from membrane synthesis *in vivo*, it is possible that the interaction of individual lipid and protein molecules to form membrane in the intact cell might be dictated by the intrinsic properties of the molecules themselves. The existence of a small lipoprotein aggregate which is soluble in water and which can be assembled into a membranous structure opens the possibility that sites of synthesis of membrane components could be physically removed from points of membrane assembly. The possibility that the small lipoprotein aggregates observed constitute subunits of the membrane cannot be evaluated in the absence of evidence that their structural integrity is maintained upon assembly into membrane-like structures.

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It has come to our attention that the presence of glucosamine in hydrolysates of whole cells of *M. laidlawii* B has been reported previously[13]. Slightly more than 1 % of the dry weight of the organisms was found to be glucosamine, and no galactosamine or mannosamine were observed to be present. It is pointed out by the author that the hexosamine content of the organisms may vary in different growth media, which may explain the absence of galactosamine since the medium used was rather different from ours.

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