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CHARACTERIZATION OF THE MYCOPLASMA MEMBRANE PROTEINS

I. REAGGREGATION OF SOLUBILIZED MEMBRANE PROTEINS OF *ACHOLEPLASMA LAIDLAWII*

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SUMMARY

Reaggregation of solubilized membranes as a means for the fractionation of membrane proteins was tested with *Acholeplasma laidlawii* membranes. The dialysis of membranes solubilized by 10 mM sodium sulfate against 20 mM Mg^{2+} for 1 h, or against 5 mM Mg^{2+} for 72 h, yielded reaggregated membranes exhibiting a different protein profile in polyacrylamide gels from that of the native membranes. The reaggregated membranes obtained under similar conditions from membranes solubilized by 20 mM sodium dodecyl sulfate did not significantly differ from the native membranes in protein composition. The membrane material solubilized by 20 mM sodium dodecyl sulfate met several criteria for complete membrane solubilization as distinct from the material solubilized by 10 mM sodium dodecyl sulfate, which contained minute membrane fragments sedimentable at 100000 $\times$ *g* for 1 h with a higher content of certain membrane proteins. It was stipulated that the incorporation of these fragments into the reaggregated membranes was responsible for the difference in protein composition between them and the native membranes. No significant differences in lipid types could be detected between membranes reaggregated under different dialysis conditions. It is concluded that the incorporation of the various membrane proteins and lipids into the reaggregated membranes is not selective. The ultrastructure of the reaggregated membranes is discussed in light of these and other findings and the hypothesis is put forward that the reaggregated membranes essentially consist of a bimolecular leaflet of lipid covered with protein on both sides.

INTRODUCTION

The reconstitution of membrane-like structures by the reaggregation of biological membrane components solubilized by detergents has attracted much attention[1] primarily because its investigation was expected to shed light on the mode of assembly of protein and lipid in the biological membrane. Recent studies carried out on *Acholeplasma laidlawii* membranes solubilized by sodium dodecyl sulfate showed differences in the protein composition of the reaggregated membranes formed by dialysis of the

solubilized material against different Mg^{2+} concentrations[2]. This was taken to indicate that the incorporation of the membrane proteins into the reaggregated material was selective, a property which could be applied to the fractionation of several membrane enzymes[2] and antigens[3]. The reaggregation process was consequently reexamined with a view to its furnishing a method for the fractionation of the mycoplasma membrane proteins.

It soon became evident that the selectivity of the incorporation of the solubilized membrane proteins into the reaggregated material could be properly tested only when the solubilized membrane material contained no membrane fragments. In early studies on the solubilization and reaggregation of mycoplasma membranes a concentration of 20 mM (about 0.58 %) was used[4–6]. The solubilized membrane material was centrifuged at 37000 × *g* for 30 min to remove nonsoluble material, the amount of which was usually negligible[5]. Later, the concentration of sodium dodecyl sulfate used for membrane solubilization was reduced to 10 mM[2,7] to minimize the denaturing effects of the detergent. However, the centrifugal force employed to remove nonsoluble material was still kept at 37000 × *g*, never exceeding 48000 × *g* for 30 min[8]. According to the findings of BONT *et al.*[9] this centrifugal force could not be expected to remove all nonsoluble membrane fragments. The following operational criteria for complete membrane solubilization were accordingly set up: (1) no sediment should be formed on centrifugation of the solubilized membrane material at 100000 × *g* for 1 h; (2) the solubilized membrane material should not be excluded in the void volume of a Sepharose 4B column, equilibrated with the detergent at the concentration used for membrane solubilization (exclusion limit of Sepharose 4B approx. $3 \cdot 10^6$ mol.wt.); (3) the solubilized membrane material should not contain any membranous structures detectable by electron microscopy.

MATERIALS AND METHODS

Organisms and growth conditions

A. laidlawii (oral strain, formerly *Mycoplasma laidlawii*) was grown statically in a modified Edward medium[10]. To label the membrane lipids 25 to 50 μC of [9,10-$^3H_2$]oleic acid (The Radiochemical Centre, Amersham, England) were added to each liter of the growth medium. The organisms were harvested after 18–20 h of incubation at 37° and were washed twice in the cold with 0.25 M NaCl. Cell membranes were isolated by osmotic lysis of the organisms and washed 10 times as described by ROTTEM *et al.*[6].

Membrane solubilization and gel filtration

Various concentrations of sodium dodecyl sulfate were added to the membrane suspension (3.6 mg membrane protein per ml) and incubated for 15 min or for 16 h at 37°. The clear solution obtained was centrifuged at 37000 × *g* for 30 min to remove the nonsoluble material. In some experiments higher centrifugal forces were employed. In other experiments the solubilized membrane material was filtered on Sepharose 4B (Pharmacia, Uppsala, Sweden) columns (30 cm × 1.5 cm) equilibrated with dilute NaCl–Tris–β-mercaptoethanol buffer (0.15 M NaCl, 0.05 M Tris, 0.01 M 2-mercaptoethanol, adjusted to pH 7.4 with HCl and diluted 1:20 in deionized water) containing 20 mM sodium dodecyl sulfate and 0.02 % NaN_3. The void volume of the columns

was determined with highly polymerized coliphage T_2 DNA, dissolved in the same elution buffer. The column flow rate was 30 ml/h and 4-ml fractions were collected. Protein and radioactivity in the fractions were determined as described under assay procedures.

Reaggregation of solubilized membrane components

The solubilized membrane material was dialyzed against 1000 vol. of cold dilute NaCl–Tris–β-mercaptoethanol buffer containing various concentrations of Mg^{2+} (ref. 6). The reaggregated material was collected by centrifuging the contents of the dialysis bags at 25000 × *g* for 1 h.

Assay procedures

Protein was determined by the Folin–phenol method of LOWRY *et al.*[11] or by absorbance at 280 nm. Electrophoretic analysis of the proteins was carried out by a modification of the Takayama technique[2]. The polyacrylamide gels were stained with 0.05 % of Coomassie Brilliant Blue R250 (ref. 12). Densitometer tracings of the stained gels were made in a Gilford spectrophotometer equipped with a model 2410 Scanner. Total membrane lipid was estimated by the colorimetric method of SAITO AND SATO[13] using the bichromate reagent and cholesterol as standard. Lipid analysis was carried out by thin-layer chromatography[14]. Radioactivity in membranes, reaggregates or in lipid fractions was determined in a Packard Tri-Carb liquid scintillation spectrometer model 3380 as described by KAHANE AND RAZIN[14]. Membrane density was determined by isopycnic sucrose density analysis[2].

Electron microscopy

Samples were fixed and sectioned as described by RAZIN *et al.*[2]. The sections were examined in a Philips EM300 electron microscope operated at 60 kV.

RESULTS

Membrane solubilization by sodium dodecyl sulfate

The criteria set up in the INTRODUCTION were applied to the *A. laidlawii* membranes solubilized by 10 mM sodium dodecyl sulfate. Centrifugation at 100000 × *g* for 1 h of the clear solution of membrane material in 10 mM sodium dodecyl sulfate yielded a yellowish translucent sediment containing about 10–20 % of the total membrane protein. As indicated by labeled lipid content, the sedimented material had a 2–3 times higher lipid to protein ratio than the native membrane. This was also borne out by its density being 1.13 g/cm^3 as against 1.17 g/cm^3 of the native membranes. The gradient of loosely-sedimented carotenoid-colored material found on top of the translucent sediment indicated that the centrifugal force employed was incapable of sedimenting all the nonsoluble material in the sample. In fact, centrifugation of the same solubilized membrane sample at 160000 × *g* for 7 h sedimented about 70 % of the membrane protein and about 85 % of the membrane lipid. Thin sections of the sediment formed by centrifugation of the solubilized membrane material at 100000 × *g* for 1 h were found to consist of amorphous material and short membrane fragments partly retaining their triple-layered structure (Fig. 1). On electrophoretic analysis of the same material it was shown to contain only some of the protein species of the native membranes (Fig. 3B).

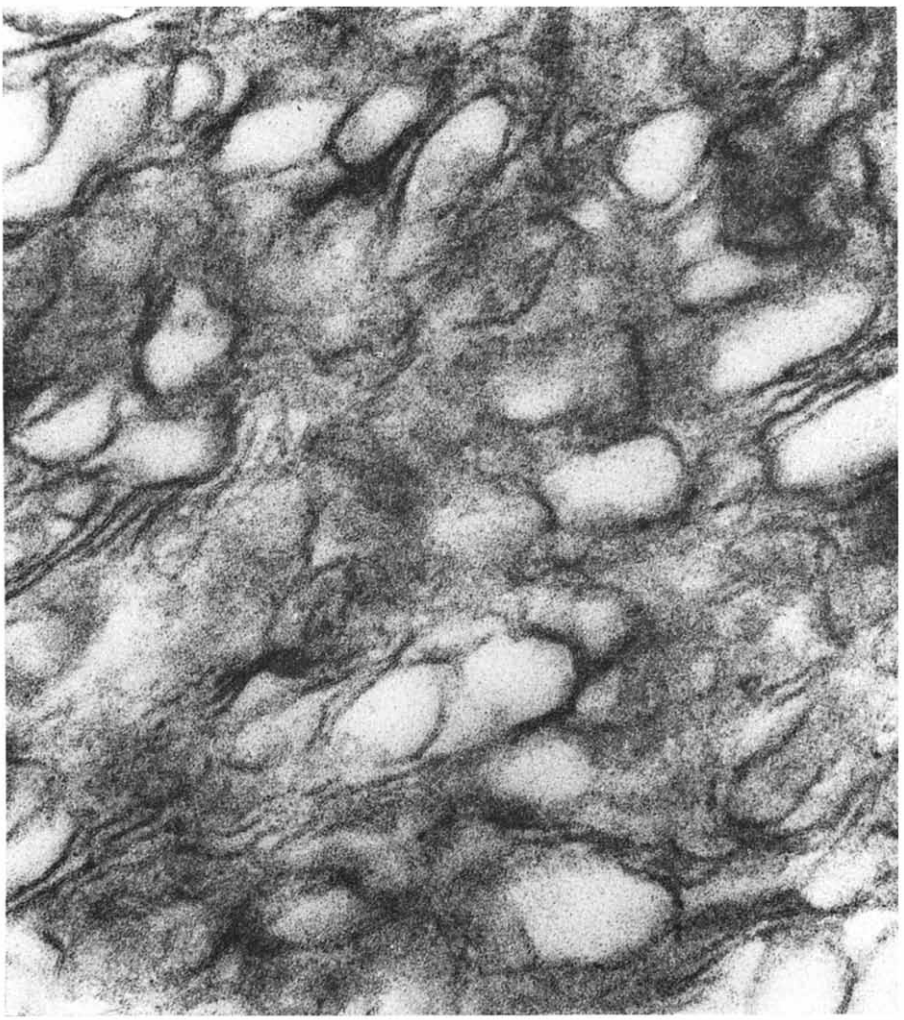

Fig. 1. Thin section of sediment formed by centrifugation at 100 000 × *g* for 1 h of *A. laidlawii* membranes solubilized by 10 mM sodium dodecyl sulfate. Small membrane fragments retaining the triple-layer structure are visible. × 97 500.

Hence the membranes solubilized by 10 mM sodium dodecyl sulfate did not meet the criteria for complete solubilization. The concentration of the detergent was, therefore, increased to 20 mM. Here centrifugation at 100000 × *g* for 1 h failed to produce any sediment, but filtration through a Sepharose 4B column equilibrated with 20 mM sodium dodecyl sulfate revealed a small portion of membrane protein and lipid excluded in the void volume of the column, pointing to its possible particulate nature (Fig. 2). The length of the solubilization period was, therefore, increased from 15 min to 16 h at 37°. This time, none of the solubilized material sedimented even when centrifuged at 160000 × *g* for 7 h, or was excluded in the void volume of the Sepharose 4B column.

Reaggregation of solubilized membrane material

Since the membrane material solubilized by incubation with 20 mM sodium dodecyl sulfate at 37° for 16 h met the criteria for complete solubilization, it could be used to answer a major question in membrane reaggregation — whether the completely solubilized membrane material can reaggregate to membrane-like structures in the absence of any membrane primer. As the completely solubilized membrane material was reaggregated by dialysis for 72 h against 20 mM Mg^{2+} to typical triple-layered membranous structures, the answer was in the affirmative. Our previous findings[2] had shown that reaggregated material formed under different dialysis conditions from *A. laidlawii* membranes solubilized by 10 mM sodium dodecyl sulfate varied in protein composition. Selective incorporation of solubilized proteins was

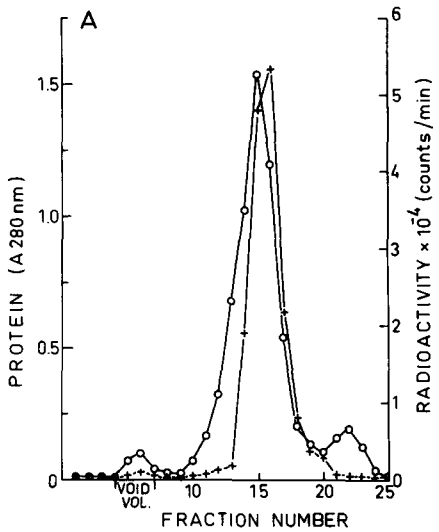

Fig. 2. Filtration of *A. laidlawii* membranes solubilized by 20 mM sodium dodecyl sulfate through a Sepharose 4B column equilibrated with the same concentration of detergent. O—O, membrane protein; +—+, labeled membrane lipid. Solubilization of membranes was carried out at 37° for 15 min.

TABLE I

COMPOSITION OF REAGGREGATED MEMBRANES FORMED AT DIFFERENT Mg^{2+} CONCENTRATIONS FROM *A. laidlawii* MEMBRANES SOLUBILIZED BY DIFFERENT DETERGENT CONCENTRATIONS

The membranes containing labeled lipid (132000 counts/min per mg protein) were solubilized by 10 mM sodium dodecyl sulfate (SDS) or by 20 mM SDS at 37° for 15 min. Dialysis was carried out for 72 h in the cold.

SDS conc. used for solubilization (mM)	*Mg^{2+} conc. in the dialysis buffer (mM)*	*Protein reaggregated (% of total)*	*Lipid/protein (counts/min per mg protein)*
10	5	13	183 000
10	20	90	137 000
20	5	6	158 000
20	20	66	147 000

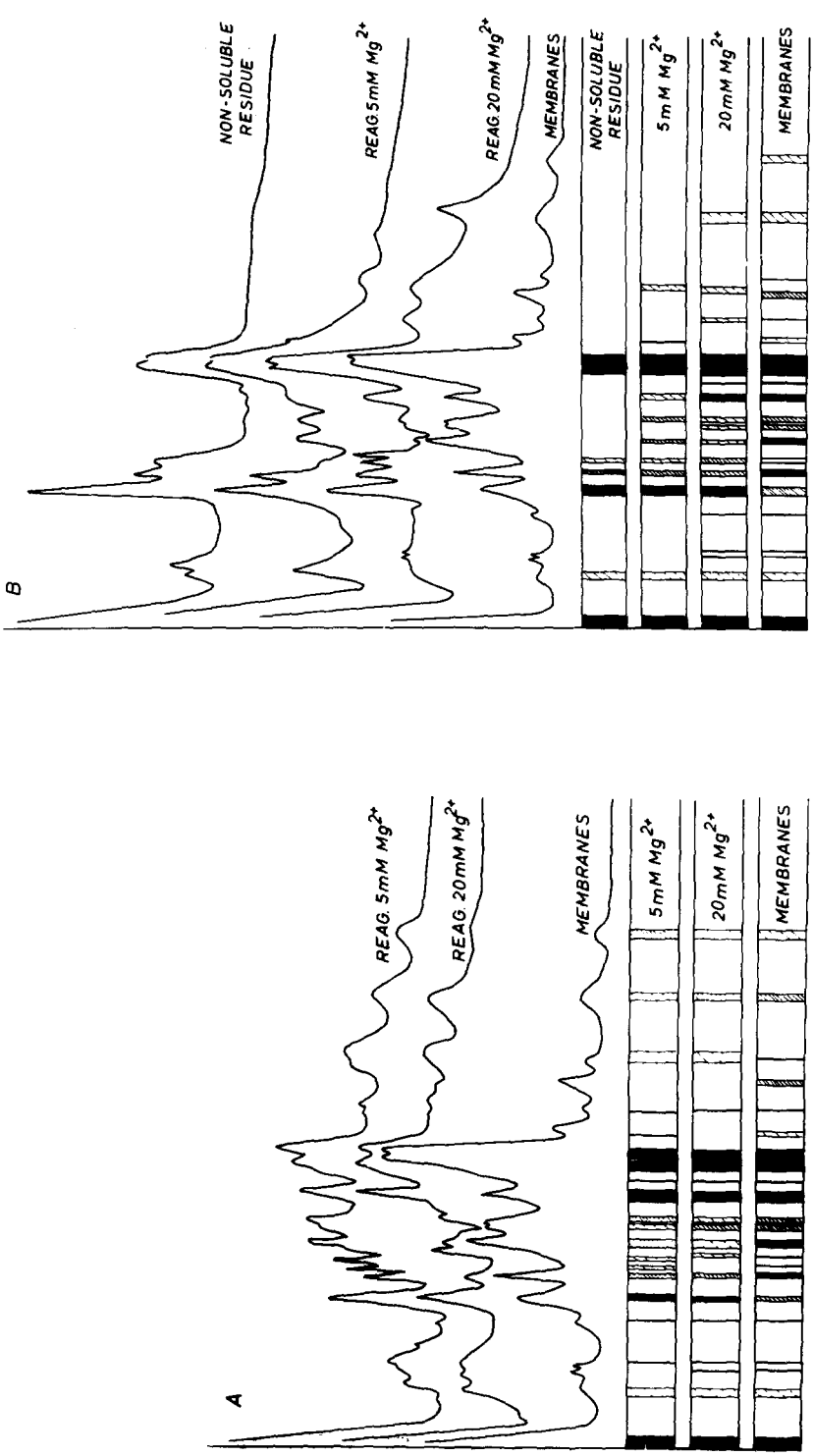

Fig. 3. Densitometer tracings and schematic representation of the electrophoretic patterns of native and reaggregated membranes formed at different Mg^{2+} concentration. The reaggregated membranes were obtained by dialysis of solubilized membranes against dilute NaCl–Tris–β-mercaptoethanol buffer containing various Mg^{2+} concentrations (indicated in figures) for 72 h at 4°. A. Membranes solubilized by 20 mM sodium dodecyl sulfate. B. Membranes solubilized by 10 mM sodium dodecyl sulfate. The electrophoretic pattern of the nonsoluble residue obtained by centrifugation of the solubilized membrane material for 1 h at 100000 × *g* is included for comparison.

demonstrated both with varying Mg^{2+} concentrations the time of dialysis being constant and with varying dialysis times the Mg^{2+} concentrations being constant. In view of the present findings that 10 mM sodium dodecyl sulfate was not sufficient for the complete solubilization of *A. laidlawii* membranes it seemed worthwhile to repeat the selective incorporation experiments using membrane material solubilized by 20 mM sodium dodecyl sulfate. A solubilization period of 15 min at 37° was found adequate for these experiments; lengthening the solubilization period to 16 h did not change the results. Table I summarizes the results of the reaggregation of membranes solubilized by 10 mM and 20 mM sodium dodecyl sulfate. More protein reaggregated from the material solubilized at the lower detergent concentration. The lipid-to-protein ratio was higher in the reaggregated material formed at the lower Mg^{2+} concentration. Electrophoretic analysis of the proteins showed that the reaggregated material formed at the different Mg^{2+} concentrations from membranes solubilized by 20 mM sodium dodecyl sulfate possessed an electrophoretic pattern very similar to that of the native membranes (Fig. 3A). On the other hand, the reaggregated material formed at 5 mM Mg^{2+} from membranes solubilized by 10 mM sodium dodecyl sulfate exhibited a pattern containing only part of the protein species of the native membranes, resembling to some extent the electrophoretic pattern of the nonsoluble residue collected by centrifugation at 100000 × *g* for 1 h of membranes solubilized by 10 mM sodium dodecyl sulfate (Fig. 3B).

Table II summarizes the results of an experiment in which reaggregation took place at various dialysis periods against 20 mM Mg^{2+}. As distinct from membrane material solubilized by 10 mM sodium dodecyl sulfate the membrane material solubilized by 20 mM sodium dodecyl sulfate did not reaggregate during the short dialysis periods. In accordance with the previous findings (Fig. 6 in ref. 2) electrophoretic analysis of the reaggregate formed after 1 h of dialysis of membrane material solubilized by 10 mM sodium dodecyl sulfate exhibited only a few protein bands, whereas the reaggregated material harvested after 2.5 h of dialysis showed a pattern which differed from that of the native membranes only by the absence of a few protein

TABLE II

COMPOSITION OF REAGGREGATED MEMBRANES FORMED AFTER VARIOUS DIALYSIS PERIODS

A. laidlawii membranes containing labeled lipid (24300 counts/min per mg protein) were solubilized by 10 mM sodium dodecyl sulfate (SDS) or by 20 mM SDS at 37° for 15 min. Dialysis was carried out for various periods of time against dilute NaCl–Tris–β-mercaptoethanol buffer containing 20 mM Mg^{2+}.

Dialysis time (h)	*Membranes solubilized by 10 mM SDS*		*Membranes solubilized by 20 mM SDS*	
	Protein reaggregated (% of total)	*Lipid/protein (counts/min per mg protein)*	*Protein reaggregated (% of total)*	*Lipid/protein (counts/min per mg protein)*
1.0	11	52 500	0	—
2.5	25	35 500	0	—
4.0	42	32 800	0	—
6.0	63	31 000	0	—
23.0	76	29 900	27	43 000

bands. Membrane material solubilized by 20 mM sodium dodecyl sulfate could be made to reaggregate after short dialysis periods by its dilution with buffer to bring the sodium dodecyl sulfate concentration down to 10 mM. The reaggregated membrane materials, including that obtained after 1 h of dialysis, showed in this case a protein pattern similar to that of the native membranes.

The reaggregated membranes obtained under different dialysis conditions differed markedly in their lipid-to-protein ratio as expressed in counts/min per mg protein (Tables I, II). This ratio was found to correspond nicely with the ratio obtained when total lipids were determined colorimetrically (Table III). Thin-layer chromatography showed, however, no significant difference in lipid types between the various reaggregates and native membranes.

TABLE III

LIPID-TO-PROTEIN RATIO IN NATIVE AND REAGGREGATED *A. laidlawii* MEMBRANES

Total membrane lipid was determined colorimetrically[13].

Preparation	*Mg^{2+} in dialysis buffer (mM)*	*Total lipid (% dry wt.)*	*Lipid/protein (counts/min per mg protein)*	*Ratio of lipid to protein compared to that of native membranes*	
				According to weight	*According to radioactivity*
Native membranes	–	32.0	25 400	1.00	1.00
Reaggregated membranes	5	49.0	51 800	2.03	2.04
Reaggregated membranes	20	33.2	26 800	1.06	1.03

DISCUSSION

The criteria set up by us for complete membrane solubilization by the detergent should be regarded as operational rather than absolute since we have no definitive proof that the solution of membrane material in sodium dodecyl sulfate, which fulfilled these criteria, really consisted of the basic units making up the membrane. Nevertheless, there is little doubt that prolonged solubilization of the membranes by 20 mM sodium dodecyl sulfate produced a solution devoid of membrane fragments, as evidenced by electron microscopy and by the retardation of this material in a Sepharose 4B column. That the solution reaggregated to membrane-like structures on dialysis against Mg^{2+} supports the previously made assumption[6] that no pre-existing membrane primer or seed is needed for the reassociation of the solubilized membrane protein and lipid to membraneous structures.

On the other hand, in reaggregation experiments carried out on the completely solubilized membrane material no selectivity in the incorporation of the various membrane proteins into the reaggregates formed at various Mg^{2+} concentrations or after different dialysis periods could be shown. The apparent selectivity in the reaggregation of the protein species previously demonstrated[2] should accordingly be

attributed to the use of a lower concentration of sodium dodecyl sulfate for membrane solubilization. Our present results show that the concentration of sodium dodecyl sulfate previously used (10 mM) does not solubilize the membranes completely, leaving minute membrane fragments, nonsedimentable at $35000 \times g$ for 30 min and enriched in certain membrane proteins, because the solubilization of the various membrane proteins by sodium dodecyl sulfate appears to be selective[15]. These fragments are apparently the first to aggregate at low Mg^{2+} concentrations or after short dialysis. In both instances the percentage of solubilized membrane protein reaggregated is low (less than 15 % of the total: Tables I, II). Hence the protein composition of the reaggregate is markedly influenced by the protein composition of the membrane fragments. The use of controlled reaggregation of solubilized membrane material as a technique for the fractionation of membrane proteins seems to be less promising.

No significant selectivity in the type of lipids incorporated into the reaggregated membranes could be demonstrated. Taken in conjunction with the absence of clear selectivity in the incorporation of membrane proteins, and the ability to produce "hybrid reaggregates" of protein and lipid from membranes of different organisms[16-19] this finding points to the conclusion that the reaggregation process has little specificity with regard to the membrane proteins and lipids incorporated. Recently, ROTTEM *et al.*[20] have shown that even a small percentage of soluble proteins, such as penicillinase from *Bacillus cereus* or bovine serum albumin could be incorporated into reaggregated *A. laidlawii* membrane material.

The low specificity of the reaggregation process leads us to consider the possible ultrastructure of the reaggregated membranes. The reformed membranes were recently shown to differ from native *A. laidlawii* membranes in that the particles which characterize the fracture faces of the native membranes are missing[21]. In this respect the reformed membranes resembled myelin membranes[22]. There is almost a general agreement that the ultrastructure of myelin conforms with the classical Danielli–Davson membrane model, in which the lipid forms a bimolecular leaflet covered on both sides with protein[23]. We would like to put forward the hypothesis that the reformed membranes are built on the same principle. The variations in the lipid-to-protein ratio in the reaggregated membrane material have led us to suggest[2] that the reformed membranes are built by a multi-step assembly process, with the initial formation of a lipid-rich primary membrane on which more protein is bound in several steps, depending on the Mg^{2+} concentration. According to our hypothesis the primary membrane is built of a bimolecular leaflet of lipid coated on both sides with protein. The membrane proteins lose most of their tertiary and quaternary structure during their solubilization in sodium dodecyl sulfate[24,25] though much of their secondary structure remains intact (S. ROTTEM AND L. HAYFLICK, unpublished results). The absence of S–S bonds in *A. laidlawii* membrane proteins[26] may enable the polypeptide chains to be unfolded by sodium dodecyl sulfate in the absence of a reducing agent such as 2-mercaptoethanol. The resulting polypeptide chains with their open conformation fit in well with the role conceived for proteins in the early Danielli–Davson model, spreading over the surfaces of the lipid bilayer. The unfolding of the polypeptide chains of the membrane proteins by the detergent may also be expected to expose more ionizable groups. Since membrane proteins are generally acidic rather than basic they are unable to attach themselves to the surfaces of the lipid bilayer, being repelled by the negatively-charged phosphate groups. Mg^{2+} or some other

divalent cation is therefore essential to neutralize these charge repulsions. Furthermore, Mg^{2+} may stabilize the structure by forming salt bridges between adjacent carboxyl and phosphate groups.

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