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SELECTIVE REAGGREGATION OF SOLUBILIZED MYCOPLASMA-MEMBRANE PROTEINS AND THE KINETICS OF MEMBRANE REFORMATION

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SUMMARY

1. Proteins and lipids of mycoplasma membranes solubilized in sodium dodecyl sulfate reaggregated to membraneous structures when the detergent was diluted by dialysis against a Mg^{2+}-containing buffer. The Mg^{2+} concentration determined the degree of reaggregation, the lipid-to-protein ratio in the reaggregate and, as shown by electrophoretic and enzymic analyses, the species of membrane proteins aggregated.

2. The reassembly of the solubilized membrane components to membraneous structures proceeded rapidly. After 40 min of dialysis against 20 mM Mg^{2+}, the reaggregate already contained membranes of a similar triple-layered structure and thickness as the original ones, but with a smaller number of protein species and a higher lipid-to-protein ratio.

3. On density-gradient analysis reaggregates of *Mycoplasma laidlawii* membranes were found to be heterogeneous, while at 5 mM Mg^{2+} only a "light" lipid-rich band ($d = 1.140$) was obtained. At higher Mg^{2+} concentrations this was accompanied by one or two heavier bands. The "light" band was transformed into a heavier one when the Mg^{2+} concentration was increased.

4. Our data suggest that the reaggregated membranes are assembled by a multistep process and not by the single-step assembly of lipoprotein subunits.

5. The application of the selective aggregation of solubilized membrane proteins to the fractionation and characterization of membrane enzymes and antigens is suggested and discussed.

INTRODUCTION

The structural organization of the protein and lipid in biological membranes is still a matter of controversy. Its investigation has been hampered by the lack of suitable means for the controlled solubilization of the membrane which is essential in order to study the macromolecular associations of the proteins and lipids it contains. Though detergents have been widely used for this purpose, the attempted characterization of the products was frequently misleading. Thus, it was shown that sodium

dodecyl sulfate does not, as was originally thought, disaggregate mycoplasma membranes to lipoprotein subunits[1] as the lipid was found to be separable from the protein[2,3]. Still, the separate protein and lipid components were able to reaggregate spontaneously to form the typical triple-layered membranes when the detergent was removed by dialysis against a Mg^{2+}-containing buffer[1,3,4]. The spontaneous reassociation of solubilized membrane protein and lipid appears to be a general property of biological membranes[5–9] and its investigation might disclose some of the mechanisms of the assembly of proteins and lipids into membraneous structures. The information obtained from *in vitro* studies may help in the understanding of membrane assembly *in vivo*. The present study is primarily concerned with the kinetics of the reaggregation process and the selective incorporation of structural and catalytic membrane proteins into membrane reaggregates. Extensive use was made of the finding that the concentration of Mg^{2+} in the dialysis buffer determines both the degree of reaggregation and the ratio of lipid to protein in the reaggregates[3,8] for regulating the reaggregation process.

MATERIALS AND METHODS

Organisms and growth conditions

Mycoplasma laidlawii (oral strain), *Mycoplasma gallisepticum* (strain A5969) and *Mycoplasma mycoides* var. *mycoides* (strain PG-46) were grown statically in a modified Edward medium[10]. The organisms were harvested after 18–20 h of incubation at 37° and washed twice in the cold with 0.25 M NaCl. Since oleic acid has been shown to be incorporated into the phospholipids and glycolipids which constitute over 90 % of the total membrane lipids of *M. laidlawii*[11] and the bulk of the membrane lipids of the other two mycoplasmas[12,13], 1 μC of [1-^{14}C]oleic acid (The Radiochemical Centre, Amersham, England) was added to each liter of the growth medium to label membrane lipids. The proportion of lipid in the membrane fractions could be conveniently measured by radioactivity determinations[2,3].

Isolation of cell membranes

Cell membranes were isolated from the cytoplasmic constituents by osmotic lysis of the organisms[3]. For lysis of the more osmotically resistant *M. gallisepticum*, the cells were loaded with glycerol prior to the osmotic shock. The membranes were washed 5–10 times as described before[3] and were resuspended in β-buffer (0.15 M NaCl, 0.05 M Tris, 0.01 M 2-mercaptoethanol, in deionized water adjusted to pH 7.4 with HCl; ref. 1), diluted 1:20 in deionized water (referred to below as "dilute β-buffer") and kept at −20° until used.

Membrane solubilization

Sodium dodecyl sulfate was added to the membrane suspension (containing 4 mg membrane protein per ml) to a final concentration of 0.01 M. After 15 min of incubation at 37°, the clear solution obtained was centrifuged at 35000 × *g* for 30 min to remove nonsoluble material, the amount of which was usually negligible.

Reaggregation of solubilized membrane components

The solubilized membrane material was dialyzed against 1000 vol. of cold dilute

β-buffer containing various concentrations of Mg^{2+}. Dialysis was carried out at 4° without stirring for various periods of time up to 3 days. The reaggregated material was collected by centrifuging the contents of the dialysis bags at 35000 × *g* for 1 h in the cold[1]. Both the sedimented reaggregate and the supernatant fluid were assayed for proteins and radioactivity. The ratio of lipid to protein in the fractions analyzed was expressed as counts/min per mg protein.

Isopycnic density-gradient analysis

Samples (0.15 ml, containing 0.5 mg protein) of membranes or membrane reaggregates were layered over 4.8 ml of linear sucrose gradients (351 to 512 g sucrose per l). The gradients were centrifuged at 49000 rev./min in the SW 50 rotor of a Spinco model L-2 ultracentrifuge for 2 h at 4°. The density of the bands was estimated according to their location in the gradient. Protein and radioactivity in the bands were determined after the material had been collected by puncturing the bottom of the centrifuge tubes. For the separation of the larger quantities of reaggregated membrane material required for electrophoretic and electron microscopic analyses, samples (1–2 ml, containing up to 20 mg protein) were layered over 50 ml of linear sucrose gradients. The gradients were centrifuged at 25000 rev./min in the SW 25.2 rotor of the Spinco ultracentrifuge for 4.5 h at 4° and the material separated in bands was collected.

Polyacrylamide-gel electrophoresis of membrane proteins

Freeze-dried membranes or membrane reaggregates were solubilized in a phenol–acetic acid–water mixture (2:1:0.5, w/v/v) by adding 1 ml of the mixture per 10 mg protein in the sample. The electrophoretic run was carried out for 3 h in polyacrylamide gels containing 7.5 % acrylamide, 35 % acetic acid and 5 M urea, prepared as previously described[14]. A drop of a 1 % Rhodamine 6G solution in the phenol–acetic acid–water mixture was added as a marker dye to indicate the migrating front. The gels were stained with 1 % Amido Black 10B and densitometer tracings of the stained gels were made in a Gilford spectrophotometer equipped with a Model 2410 Scanner.

Assay procedures

Protein was determined by the Folin–phenol method of LOWRY *et al.*[15] using crystalline bovine plasma albumin as standard. Membrane lipids were extracted with chloroform–methanol (2:1, v/v) according to FOLCH *et al.*[16]. Nucleic acids and total carbohydrate were estimated as described by RAZIN AND BOSCHWITZ[8]. Radioactivity was determined in a Packard Tri-Carb scintillation spectrometer using the scintillation mixture described previously[11]. NADH oxidase (EC 1.6.99.3) activity was measured spectrophotometrically by determining the decrease in absorbance at 340 mμ on addition of NADH to the reaction mixture[17]. Data are expressed as the decrease in absorbance at 340 mμ per min per mg of protein. Ribonuclease and deoxyribonuclease activities were estimated as described by RAZIN *et al.*[18] by determining the release of uranyl acetate-soluble material absorbing at 260 mμ from reaction mixtures containing highly polymerized yeast ribonucleic acid or highly polymerized sperm deoxyribonucleic acid, both from Calbiochem. Data are expressed as increase in absorbance at 260 mμ per h per mg of protein.

Electron microscopy

The pellets of sedimented membranes and membrane reaggregates were initially fixed in 4 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 4 h at 0°, then washed in the phosphate buffer and post-fixed in 2 % OsO_4 in the same buffer for 8 h. The material was embedded in Epon according to LUFT[19] and sectioned with a Porter–Blum Servall MT-2 microtome. The sections were mounted on uncoated 400-mesh grids, stained with uranyl acetate and lead citrate[20] and examined in a Philips EM 300 electron microscope operated at 60 kV with a condenser aperture of 200 μ and an objective aperture of 30 μ. Electron magnifications were between 45000 × and 180000 ×. For the estimation of membrane thickness, negatives were enlarged in print to 500000 × on transparent film and densitometer tracings across the triple-layered membranes were made with the Spinco Analytrol Type RB Densitometer using a vertical slit of 0.6 mm long and 0.2 mm wide. The distance between the two density peaks of the membrane on the densitometer tracing was measured (see Fig. 12) and expressed in Ångström units with standard deviation.

RESULTS

Composition of reaggregates formed at various Mg^{2+} concentrations

The finding that the Mg^{2+} concentration of the dialysis buffer profoundly affects the lipid-to-protein ratio in reaggregates of solubilized *M. laidlawii*, *M. gallisepticum*[3] and the *Streptobacillus moniliformis* L-phase membranes[8] has been extended to *M. mycoides* var. *mycoides*. In composition the membrane of this mycoplasma was similar to that of the other mycoplasmas, particularly of *M. mycoides* var. *capri*[21]. It consisted (% dry weight) of: 51.2 % protein, 39.3 % lipid, 5.6 % RNA, 0.8 % DNA and 3.2 % carbohydrate. Fig. 1 shows the dependency of the reaggregation of solubilized *M. mycoides* var. *mycoides* membranes on the Mg^{2+} concentration. The concen-

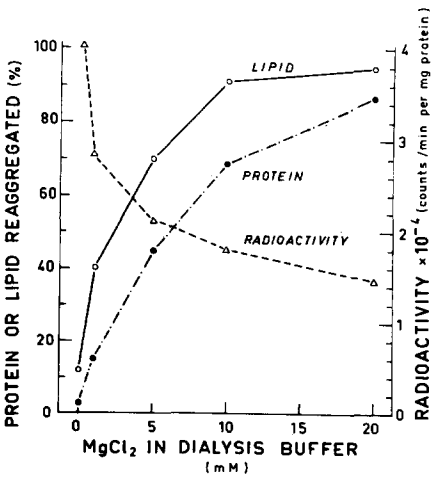


Fig. 1. Effect of Mg^{2+} on the reaggregation of protein and lipid of solubilized *M. mycoides* var. *mycoides* membranes. The membranes were solubilized in 0.01 M sodium dodecyl sulfate and dialysed against dilute β-buffer containing various Mg^{2+} concentrations for 3 days at 4°. ●–·–●, percentage of membrane protein reaggregated; ○—○, percentage of membrane lipid reaggregated; △---△, ratio of labeled lipid (expressed as radioactivity) to protein in reaggregates.

TABLE I

EFFECT OF THE Mg^{2+} CONCENTRATION AND DETERGENT REMOVAL ON REAGGREGATION OF SOLUBILIZED *M. laidlawii* MEMBRANES

The membranes containing labeled lipid were solubilized in 0.01 M sodium dodecyl sulfate and reaggregated by dialysis against dilute buffer containing various Mg^{2+} concentrations for 3 days at 4°. Part of the solubilized membrane material was dialyzed before the reaggregation experiment against dilute buffer without Mg^{2+} for 36 h at 4° to remove the detergent[26].

Mg^{2+} concn. in dialysis buffer (mM)	*Reaggregation without prior removal of sodium dodecyl sulfate*			*Reaggregation after removal of sodium dodecyl sulfate*		
	Protein reaggregated (%)	*Lipid reaggregated (%)*	*Lipid/protein (counts/min per mg protein)*	*Protein reaggregated (%)*	*Lipid reaggregated (%)*	*Lipid/protein (counts/min per mg protein)*
5	13	34	114 300	6	21	121 000
10	38	70	87 000	23	41	83 000
20	83	90	45 000	87	98	46 000

tration of the cation in the dialysis buffer determined both the degree of reaggregation of membrane material and its composition. Reaggregates obtained at a low Mg^{2+} concentration contained a higher percentage of lipid, as expressed in higher radioactivity values.

The effect of Mg^{2+} on the degree of reaggregation of solubilized *M. laidlawii* membranes and on the lipid-to-protein ratio in the reaggregates is shown in Table I. The variations in the composition of the reaggregates caused by changing the Mg^{2+} concentration were not affected by the prior removal of the sodium dodecyl sulfate. The effect of Mg^{2+} on the reaggregation of solubilized *M. gallisepticum* was the same as on the other membranes: at 5 mM Mg^{2+} only 49 % of the membrane proteins were incorporated into the reaggregate, while at 20 mM Mg^{2+} over 89 % of the proteins were aggregated (see also Table II in ref. 3). The amount of solubilized membrane protein incorporated into the reaggregates can thus be regulated by changing the Mg^{2+} concentration in the dialysis buffer used for reaggregation. To ascertain whether the Mg^{2+} concentration also determines the species of the proteins incorporated into the reaggregates, polyacrylamide-gel electrophoresis was carried out on reaggregates obtained at various Mg^{2+} concentrations (Fig. 2). The number of protein species incorporated into the reaggregates increased with the Mg^{2+} concentration, and predictably the reaggregates obtained at a high Mg^{2+} concentration (20 mM), which contained about 90 % of the original membrane protein, exhibited almost all the protein bands characterizing the electrophoretic profile of the original membranes. Since the reaggregates produced at low Mg^{2+} concentrations contained only a few of the protein species of the original membranes, the bands of these proteins predominated in the electrophoretic profiles (*e.g.* the bands marked by arrows in Figs. 2B and 2C) as they constituted a much higher proportion of the total protein than in the original membranes. The heavy protein band which could be seen at the origin of all the gels was shown to represent all the protein species which had entered the gel. This was demonstrated by eluting unstained protein at the gel origin with phenol–acetic acid–water and reapplying the extracted protein to a different gel. The resulting electrophoretic profile was identical with that of the original sample (see also refs. 22 and 23).

Density-gradient analysis of the reaggregates formed at various Mg^{2+} concentrations (Fig. 3) indicated that their density depended on the Mg^{2+} concentration in the dialysis buffer, increasing with the Mg^{2+} concentration. At 20 mM Mg^{2+} the density of the reaggregates was close to that of the original membranes. However, the reaggregates of *M. laidlawii* formed at 10 and 20 mM Mg^{2+} contained in addition to the major band a minor band resembling the "light" band ($d = 1.140$) characterizing the reaggregate formed at 5 mM Mg^{2+}. Table II shows that the ratio of lipid-to-protein was higher in the low density bands. Electrophoretic analysis of the proteins in the bands of the reaggregates formed at various Mg^{2+} concentrations (Fig. 4) showed that the highest density band ($d = 1.168$), which was closest to the density of the original membranes, was richer in protein species than the "light" lipid-rich band ($d = 1.140$).

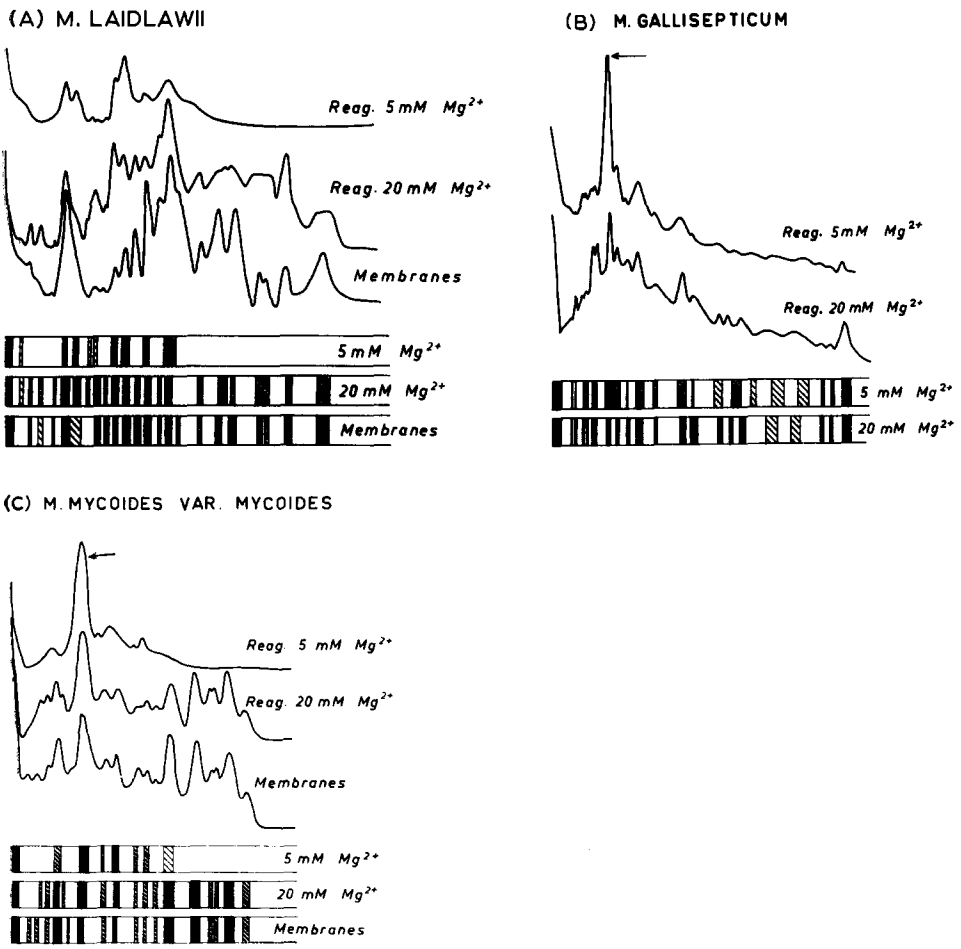


Fig. 2. Densitometer tracings and schematic representation of the electrophoretic patterns of the proteins of membranes and reaggregates obtained at different Mg^{2+} concentrations. The reaggregates were obtained by dialysis of solubilized membranes against dilute β-buffer containing various Mg^{2+} concentrations (indicated in figures) for 3 days at 4°. The arrows point to the protein bands which became predominant in reaggregates obtained at low Mg^{2+}. The top of the polyacrylamide gels is on the left.

The protein composition of the "light" band formed at 5 mM Mg^{2+} very much resembled that of the corresponding band isolated from the reaggregate formed at 20 mM Mg^{2+}. The dependence of the reaggregate density on the Mg^{2+} concentration was also shown with *M. mycoides* var. *mycoides* membranes. Unlike the *M. laidlawii* reaggregates, those of *M. mycoides* var. *mycoides* were homogeneous and sedimented as a single band having a density ranging from 1.123 at 5 mM Mg^{2+} to 1.154 at 20 mM Mg^{2+}.

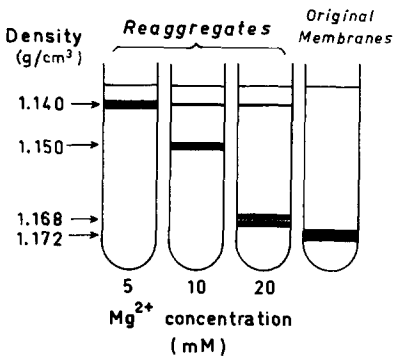


Fig. 3. Density-gradient analysis of *M. laidlawii* membranes and reaggregates. The reaggregates were obtained by dialysis of the solubilized membranes against dilute β-buffer containing different concentrations of Mg^{2+} for 3 days at 4°. Linear sucrose gradients (35–51 % sucrose) were used.

TABLE II

THE DENSITY AND COMPOSITION OF REAGGREGATE BANDS SEPARATED ON SUCROSE-DENSITY GRADIENTS

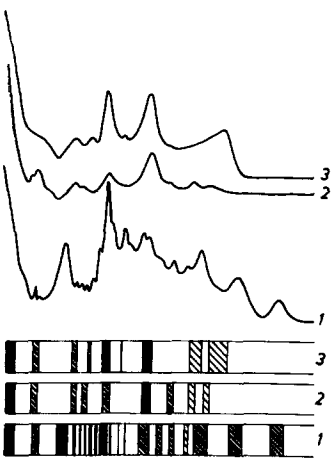
Analysis was carried out on material from bands obtained as shown in Fig. 3.

Mg^{2+} concn. in dialysis buffer (mM)	*Density of reaggregate bands (g/cm³)*	*Lipid/protein (counts/min per mg protein)*
5	1.140	109 000
10	1.140	120 100
	1.150	47 400
20	1.140	99 700
	1.168	32 800

Reaggregation kinetics and membrane formation

Since the "light" band (d = 1.140) appeared in all the reaggregates of *M. laidlawii* formed at different Mg^{2+} concentrations, it seemed possible that this band might have been formed in the dialysis bag at the beginning of the reaggregation process, when the concentration of Mg^{2+} in the bag was still low. To test this point and to investigate the kinetics of the reassembly of membrane protein and lipid to membraneous structures, the reaggregation process was checked at various dialysis periods against a constant Mg^{2+} concentration (20 mM). Fig. 5 shows that after 1 h of dialysis about 45 % of the lipid and 30 % of the protein had aggregated and the aggregation process nearly reached its completion after 6 h. At each of the time intervals tested, a higher

percentage of lipid than of protein was found to be aggregated. Electrophoretic analysis of the reaggregates formed after various dialysis periods (Fig. 6) showed the gradual and selective incorporation of the different membrane proteins into the re-aggregates. Thus, the reaggregate formed after 1 h of dialysis contained only a few protein species, while the reaggregate formed after 20 h of dialysis contained almost all the protein species of the original membrane. The heterogeneity of the reaggregates obtained at various times of dialysis was demonstrated by density-gradient analysis (Fig. 7). Several bands appeared among which the "light" band ($d = 1.140$) was most pronounced at the early stage of reaggregation. Table III shows that the amount of membrane protein incorporated into the heavier bands increased with the dialysis time, but that already after 1 h of dialysis a band having a density of 1.167 could be noted.



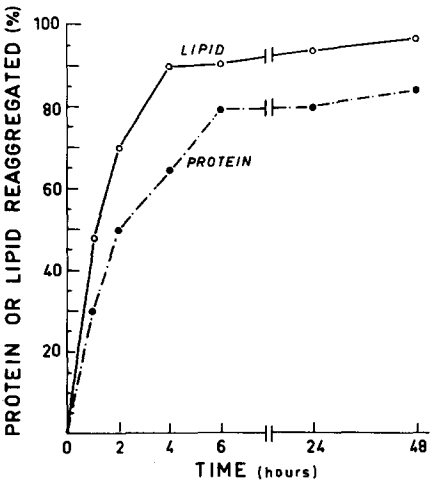


Fig. 4. Densitometer tracings and schematic representation of the electrophoretic patterns of proteins of reaggregate bands separated on sucrose-density gradients. *M. laidlawii* membranes solubilized in 0.01 M sodium dodecyl sulfate were dialyzed for 3 days at 4° against dilute β-buffer containing either 20 or 5 mM Mg^{2+}. The resulting reaggregates were centrifuged on linear sucrose-density gradients. The reaggregate obtained at 20 mM Mg^{2+} separated into two bands. A major band having a density of 1.168 and a minor band having a density of 1.140. The reaggregate obtained at 5 mM Mg^{2+} sedimented as a single band having a density of 1.140. 1, the pattern of the 1.168-density band; 2, the pattern of the 1.140-density band obtained at 20 mM Mg^{2+}; 3, the pattern of the 1.140-density band obtained at 5 mM Mg^{2+}.

Fig. 5. Effect of dialysis time on the reaggregation of protein and lipid of solubilized *M. laidlawii* membranes. Dialysis was carried out without stirring, at 4° against dilute β-buffer containing 20 mM Mg^{2+}.

Electron microscopical observation disclosed the presence of triple-layered membrane-like structures in sections of all the bands separated by density-gradient centrifugation of reaggregates formed after various dialysis periods. The membrane profiles appearing in reaggregates formed after short dialysis periods varied in length and had free ends (Fig. 8). After 20 min of dialysis, when only 3 % of the solubilized membrane protein had aggregated, the thin sections showed some short faint images of triple-layered membranes embedded in an amorphous matrix. After 40 min of dialysis, about 10 % of the protein had aggregated and presented the picture shown

in Fig. 8. The membranes showed a tendency to form side-by-side aggregates. Non-organized amorphous material could also be seen at these early phases of reaggregation (Fig. 9). Later on, at least some of the membranes closed up to form vesicles (Fig. 10). A highly magnified section through a reformed membrane (Fig. 11) displays the triple-layered structure, but the layers seem to be built of globules. The thickness of the reformed membranes from the various bands did not vary significantly (Table IV).

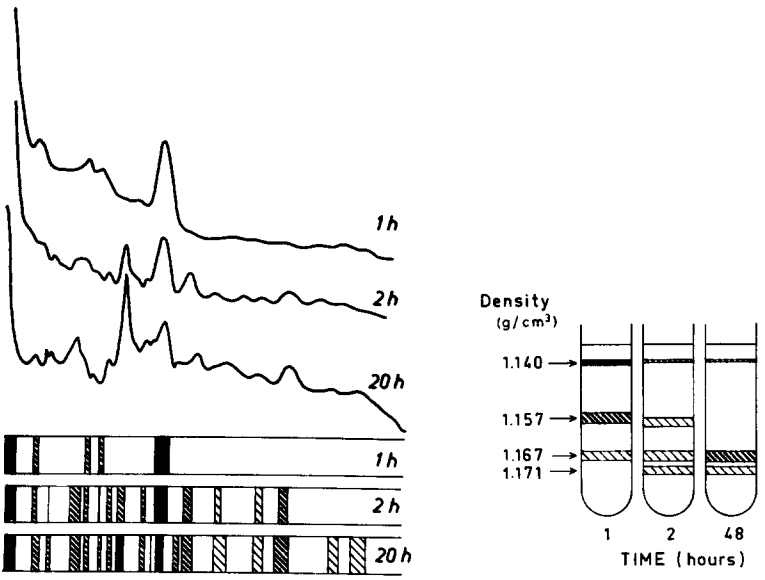


Fig. 6. Densitometer tracings and schematic representations of electrophoretic patterns of reaggregates obtained after various periods of dialysis (in hours) of solubilized *M. laidlawii* membranes against dilute β-buffer containing 20 mM Mg^{2+}.

Fig. 7. Density-gradient analysis of reaggregates of solubilized *M. laidlawii* membranes obtained after various periods of dialysis against dilute β-buffer containing 20 mM Mg^{2+}.

TABLE III

THE DENSITY AND COMPOSITION OF MEMBRANE REAGGREGATES FORMED AFTER VARIOUS DIALYSIS PERIODS

Analysis was carried out on material from bands obtained as shown in Fig. 7.

Band density (g/cm^3)	*Dialysis time*					
	1 h		*2 h*		*48 h*	
	Protein (% of total protein) reaggregated	*Lipid/protein (counts/min per mg protein)*	*Protein (% of total protein) reaggregated*	*Lipid/protein (counts/min per mg protein)*	*Protein (% of total protein) reaggregated*	*Lipid/protein (counts/min per mg protein)*
1.140	53	21 800	19	22 000	14	21 100
1.157	25	12 400	16	12 600	No band	No band
1.167	22	8 600	40	9 100	41	9 400
1.171	No band	No band	25	8 450	45	7 360

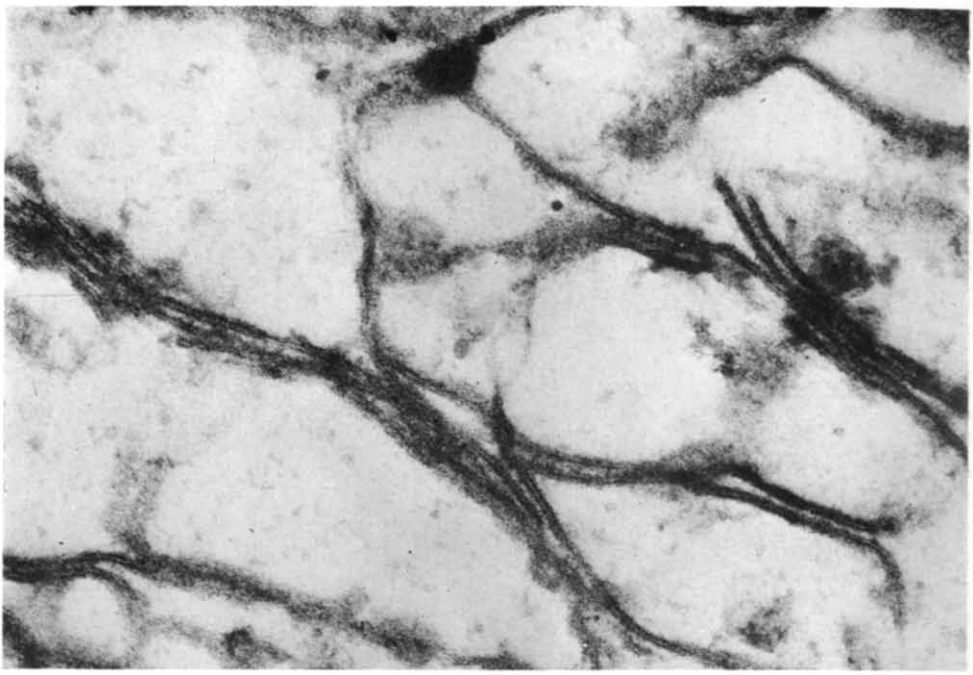


Fig. 8. Thin section of the light band ($d = 1.140$) obtained by dialysis of solubilized *M. laidlawii* membranes against dilute β-buffer containing 20 mM Mg^{2+} for 1 h at 4°. Triple-layered membrane profiles with free ends are the predominant feature. × 57000.

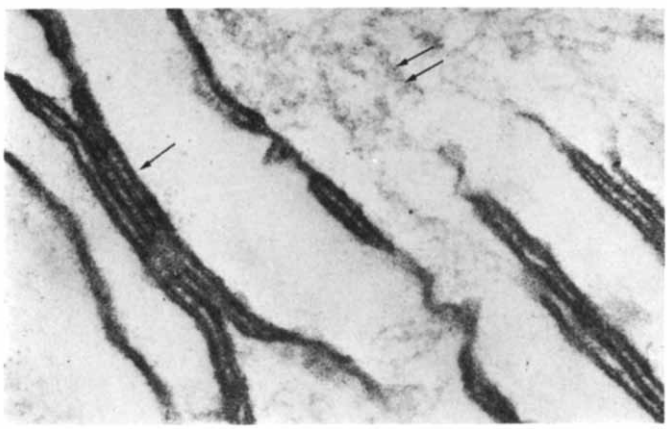


Fig. 9. Thin section of the light band ($d = 1.140$) obtained by dialysis of solubilized *M. laidlawii* membranes against dilute β-buffer containing 20 mM Mg^{2+} for 2 h at 4°. Side-by-side arrangement of membranes (single arrow) and amorphous material (double arrow) can be seen. × 74000.

The distance between the two density peaks (Fig. 12) was about 80 Å in all the membranes examined, though the standard deviation of some of the measurements was quite considerable, probably due to the small number of membranes measured.

Changes in the composition of the reaggregates after their formation

The density-gradient analysis of reaggregates obtained at various dialysis

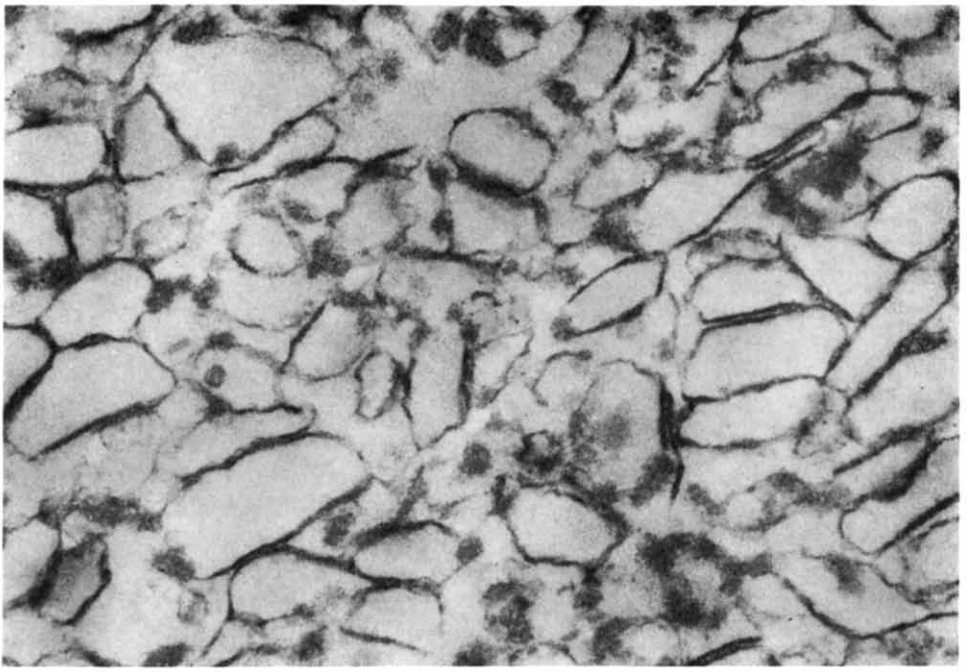


Fig. 10. Thin section of the light band ($d = 1.140$) obtained by dialysis of solubilized *M. laidlawii* membranes against dilute β-buffer containing 20 mM Mg^{2+} for 9 h at 4°. Many of the membranes closed up to form vesicles. × 35000.

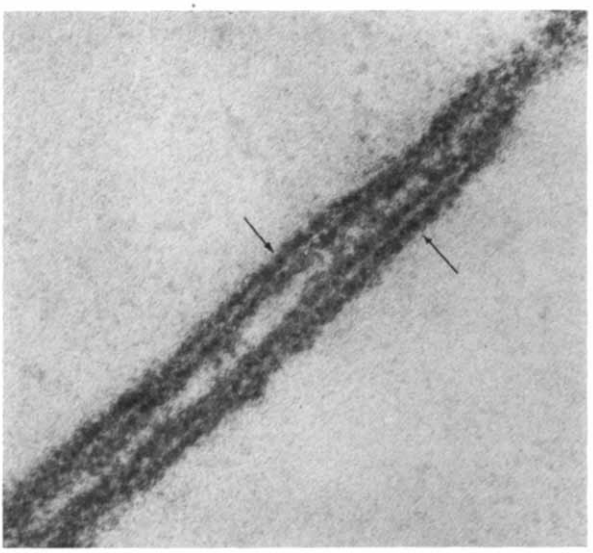


Fig. 11. High magnification of reformed membranes. The electron-dense bands present a globular structure. The diameter of the globules is about 60 Å (arrows). The sectioned material was the same as in Fig. 10. × 305000.

periods (Table III) indicated that the lipid-rich membranes of the "light" bands, appearing early in the reaggregation might later, with the rise of the Mg^{2+} concentration in the dialysis bag, be transformed into heavier membranes by incorporating additional protein. For a more direct investigation of this point, membranes of *M. laidlawii* solubilized in 0.01 M sodium dodecyl sulfate were reaggregated by dialysis against 5 mM Mg^{2+} for 3 days. The content of the dialysis bag was then divided into four equal parts. One part was centrifuged and the sediment was taken to represent

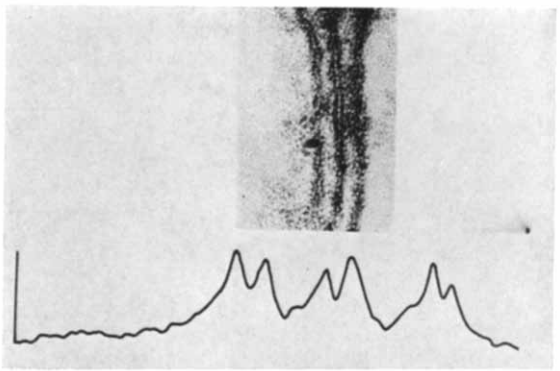


Fig. 12. An example of a densitometer tracing used for the measurement of center-to-center distance of the dense lines in sectioned reformed membranes.

TABLE IV

CENTER-TO-CENTER DISTANCE (Å) OF THE DENSE LINES IN SECTIONS OF REFORMED MEMBRANES IN REAGGREGATES OBTAINED AFTER VARIOUS DIALYSIS PERIODS

M. laidlawii membranes were solubilized in 0.01 M sodium dodecyl sulfate and dialyzed against 20 mM Mg^{2+} for various periods of time. The reaggregates were centrifuged on sucrose-density gradients and the various bands were separated and prepared for electron microscopy. For each band measurements were done on 10–20 profiles of membrane sections as described in MATERIALS AND METHODS.

Band density (g/cm^3)	*Dialysis time*				
	1 h	*2 h*	*3 h*	*9 h*	*24 h*
1.140	79 ± 9	Not done	79 ± 12	Not done	92 ± 20
1.150	No band	82 ± 12	Not done	70 ± 13	85 ± 12
1.167	No band	No band	82 ± 20	79 ± 11	82 ± 12

a normal reaggregate obtained at 5 mM Mg^{2+}. The second part was dialyzed for another 3 days against 5 mM Mg^{2+} and the third part was dialyzed for 3 days against 20 mM Mg^{2+}. The fourth part was centrifuged, and the supernatant fluid was dialyzed for 3 days against 20 mM Mg^{2+}. The results are summarized in Fig. 13 and Table V. It may be seen that the "light" band (d = 1.140) formed at 5 mM Mg^{2+} was replaced by a heavier band (d = 1.162) when the dialysis bag content was transferred from 5 to 20 mM Mg^{2+}. The transfer of the supernatant fluid separated from the reaggregate formed at 5 mM to 20 mM Mg^{2+} resulted in the formation of a single band having the density of 1.168, the same as of the heavy band of the reaggregate formed under

the usual dialysis conditions at 20 mM Mg^{2+}. On the other hand, when the dialysis period against 5 mM Mg^{2+} was prolonged from 3 to 6 days, the percentage of material reaggregated and the density of the reaggregate formed was not significantly altered.

Enzymic activities in membrane reaggregates

The selective incorporation of proteins into membrane reaggregates could also be demonstrated by the differential rates of incorporation of several enzymic activities associated with *M. laidlawii* membranes into the reaggregates. Only enzymic activities resisting inactivation by sodium dodecyl sulfate could be measured. The solubilization of *M. laidlawii* membranes by 0.01 M sodium dodecyl sulfate completely inactivated their adenosine triphosphatase and *p*-nitrophenyl phosphatase activities, but the solubilized membranes retained over 96% of their initial ribonuclease and deoxyribonuclease activities, and the NADH oxidase activity was about 140% higher than the initial activity as reported by POLLACK *et al.*[17]. Table VI shows that the nucleolytic activity of the solubilized membranes incorporated into the reaggregates formed at

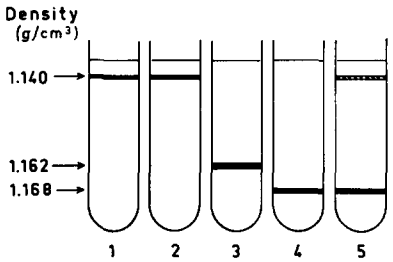


Fig. 13. Density-gradient analysis of reaggregated membrane material transferred from a low to a high Mg^{2+} concentration. For experimental details see text. 1 = reaggregate obtained after dialysis for 3 days against 5 mM Mg^{2+}; 2 = reaggregate obtained after dialysis for 6 days against 5 mM Mg^{2+}; 3 = reaggregate obtained after dialysis for 3 days against 5 mM Mg^{2+} and 3 additional days against 20 mM Mg^{2+}; 4 = reaggregate obtained by dialysis for 3 days against 5 mM Mg^{2+}, centrifugation and dialysis of the supernatant fluid for another 3 days against 20 mM Mg^{2+}; 5 = reaggregate obtained by dialysis for 3 days against 20 mM Mg^{2+}.

TABLE V

CHANGES IN THE REAGGREGATE COMPOSITION BY ITS TRANSFER FROM A LOW TO A HIGH Mg^{2+} CONCENTRATION

For details see text.

Dialysis conditions	*Protein reaggregated* (%)	*Lipid reaggregated* (%)	*Lipid/protein (counts/min per mg protein)*
3 days against 5 mM Mg^{2+}	15	28	66 300
6 days against 5 mM Mg^{2+}	16	31	70 500
3 days against 5 mM Mg^{2+} followed by 3 days against 20 mM Mg^{2+}	88	97	44 400
3 days against 5 mM Mg^{2+}, centrifugation and dialysis of the supernatant fluid against 20 mM Mg^{2+} for 3 days	86*	95*	39 100

* Percentage of total protein and lipid in the supernatant fluid after the sedimentation of the reaggregate formed at 5 mM Mg^{2+}.

different Mg^{2+} concentrations was lower than the percentage of the total membrane proteins aggregated, while the NADH oxidase activity incorporated was higher, similar to that of the lipid aggregated. Substantially the same results were obtained on examination of the incorporation of the three enzymic activities into reaggregates formed after various periods of dialysis against a constant Mg^{2+} concentration (Table VII).

DISCUSSION

The results of this study have indicated that the aggregation of solubilized membrane components is a much faster process than was previously assumed. Triple-layered membranes were the dominant feature in the reaggregate obtained after only 40 min of dialysis, and reaggregation was practically accomplished after 6 h when experiments using radioactive sodium dodecyl sulfate showed that most of the detergent was still in the dialysis bag[3]. Although no systematic electron microscopic study of the early phases of reaggregation has yet been made, our preliminary observations on the earliest reaggregate formed after 20 min of dialysis seem to indicate that the membranes are formed within a matrix of amorphous material, perhaps by its reorganization.

TABLE VI

ENZYMIC ACTIVITIES IN REAGGREGATES FORMED AT DIFFERENT Mg^{2+} CONCENTRATIONS

M. laidlawii membranes were solubilized in 0.01 M sodium dodecyl sulfate and dialyzed for 3 days at 4° against various Mg^{2+} concentrations. Protein, radioactive lipid and the enzymic activities were determined in the reaggregated material and in the supernatant fluids as described in MATERIALS AND METHODS.

Mg^{2+} concn. in dialysis buffer (mM)	*% Reaggregated*				
	Protein	*Lipid*	*NADH oxidase*	*Ribonuclease*	*Deoxy-ribonuclease*
5	9	24	21	13	9
10	36	52	53	23	26
20	80	93	92	29	32

The heterogeneous bands in the sucrose-density analyses of *M. laidlawii* reaggregates, recorded also by TERRY *et al.*[4], may provide the key for understanding the membrane reformation phenomenon. The varying ratio of lipid-to-protein in the bands and the fact that most of the membranes were not in the form of tightly closed vesicles leave little doubt that the difference in the buoyant density of the bands is the result of a difference in their matrix density rather than of the formation of vesicles impermeable to sucrose, for which the buoyant density need not necessarily be identical with matrix density[24,25]. The appearance of the "light" lipid-rich band, in addition to the heavier bands, in reaggregates of *M. laidlawii* membranes formed at 10 and 20 mM Mg^{2+} is of great theoretical interest. The reaggregation kinetics experiment, where the "light" band appeared at the early stage of reaggregation when the concentration of Mg^{2+} in the dialysis bag was apparently still low may provide

a simple explanation for its formation in reaggregates obtained at high Mg^{2+} concentrations. A fundamental problem is whether the lipid-rich membraneous material, constituting the "light" band, can be transformed into membranes of higher density when the concentration of Mg^{2+} in the dialysis bag increases. The transformation of the "light" band to a heavier one when the reaggregate was transferred from a low to a high Mg^{2+} concentration (Table V and Fig. 13) and the disappearance of the 1.157-density band when dialysis continued for 48 h (Fig. 7) tend to support the idea that the composition of the reassembled membranes may be changed after they have been formed. This change apparently involves the binding of more protein than lipid to the "light" membranes. The possibility that at the early stages of dialysis the reaggregate contains sodium dodecyl sulfate should also be considered. In this case changes in density might occur, at least in part, through the removal or displacement of the sodium dodecyl sulfate by protein. However, the pattern of density changes in reaggregates obtained after the removal of sodium dodecyl sulfate was similar to that obtained in the presence of sodium dodecyl sulfate (Table I). Additional data are required before drawing definite conclusions on this point.

TABLE VII

ENZYMIC ACTIVITIES IN REAGGREGATES FORMED AFTER VARIOUS DIALYSIS PERIODS

M. laidlawii membranes were solubilized in 0.01 M sodium dodecyl sulfate and dialyzed against 20 mM Mg^{2+} for different periods of time. Protein, radioactive lipid and the enzymic activities were determined in the reaggregated material and in the supernatant fluids as described in MATERIALS AND METHODS.

Dialysis time (*h*)	% *Reaggregated*				
	Protein	*Lipid*	*NADH oxidase*	*Ribonuclease*	*Deoxy-ribonuclease*
1	12	22	11	8	13
2	51	65	77	24	36
4	71	79	87	30	46

The appearance of bands of increasing density when the dialysis period is extended and the considerable variations in the lipid-to-protein ratio of the reformed membranes speak in favor of their being built by a multi-step assembly process. This process can perhaps best be conceived as consisting of the initial formation of a lipid-rich primary membrane (the "light" band) on which more protein is bound in quantities which may be dependent on the neutralization of repulsive negative charges on the primary membrane by the divalent cation. The presence of several discrete bands of different densities at any stage of the reaggregation process instead of one diffuse wide band speaks against the binding of the protein to the primary membrane being a continuous process. The binding of additional material to the lighter membranes may therefore conceivably be carried out in several steps, each of which requires a critical level of Mg^{2+}.

ENGELMAN AND MOROWITZ[26,27] claim that the removal of the sodium dodecyl sulfate from the solubilized membrane material in the absence of Mg^{2+} leads to the formation of lipoprotein subunits having a sedimentation coefficient of 4.8 S, which could aggregate to membraneous structures on dialysis against Mg^{2+}. This would

mean that the reformed membranes are built by a single-step assembly process and should have a constant lipid-to-protein ratio which is contradicted by our results (Table I). The reason for this discrepancy is not yet clear.

It is still largely unknown how the biomembranes are synthesized. The two possibilities of a single- or multi-step assembly process have been considered[28–30]. It is doubtful whether the conclusions drawn from the studies on membrane reformation *in vitro* may also apply to membrane assembly *in vivo*. One should always keep in mind that the system investigated by us is to a large extent artificial, as it contains a detergent and at least part of the membrane proteins are denatured. A recent finding[31] points to the nonspecificity of the reaggregation process, by showing the formation of a hybrid reaggregate of solubilized *M. laidlawii* and *M. gallisepticum* membranes, incorporating the proteins and lipids of both. Membrane assembly *in vivo* may be expected to be more specific, at least regarding the protein species incorporated into the different membrane types of the cell.

The electrophoretic and enzymic analyses clearly showed the selective incorporation of the various species of membrane proteins into the different reaggregates, as already recorded for the *Streptobacillus moniliformis* L-phase membrane[8]. Reaggregation of solubilized membrane material may thus be used for the isolation and characterization of different membrane proteins and serve as a new tool for the study of the enzymic and antigenic properties of biomembranes. Fractionation of mycoplasma membrane proteins by dialysis of solubilized membranes against various Mg^{2+} concentrations has already been successfully applied to their serological characterization (I. KAHANE AND S. RAZIN, in preparation). The differential incorporation of the ribonuclease and deoxyribonuclease activities into reaggregates formed after various dialysis periods (Table VII) indicates that different enzymes were responsible for the nucleolytic activity of *M. laidlawii* membranes. This was confirmed by further $(NH_4)_2SO_4$ fractionation of the solubilized membranes (S. RAZIN AND Z. NE'EMAN, unpublished data). The differential incorporation of enzymes may also be taken to indicate the affinity of the enzyme to membrane components. Thus the NADH oxidase activity of *M. laidlawii* membranes showed a much higher affinity to the basal lipid-rich membrane structure than the nucleases, having a higher rate of incorporation into the reaggregate than most of the other membrane proteins. Reaggregation under controlled conditions may also be applied for the enrichment of membrane enzymes in a certain fraction, an essential preliminary step in enzyme purification. Thus after reaggregation of solubilized *M. laidlawii* membranes at 20 mM Mg^{2+}, the non-reaggregated supernatant fluid contained about 70 % of the total ribonuclease activity and only 20 % of the total membrane protein. Furthermore, this ribonuclease-enriched fluid was essentially free of detergent so that the conventional protein fractionation procedures could be used for further purification.

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