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ELECTRON MICROSCOPY OF SOLUBILIZED *ACHOLEPLASMA LAIDLAWII* MEMBRANE PROTEINS REAGGREGATED WITH *MYCOPLASMA PNEUMONIAE* GLYCOLIPIDS

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SUMMARY

1. The purified glycolipid haptens of *Mycoplasma pneumoniae* were reaggregated with *Acholeplasma laidlawii* membrane proteins. The process consisted of the solubilization of lipid-depleted *A. laidlawii* membranes and *M. pneumoniae* glycolipids in 20 mM sodium dodecyl sulfate, and dialysis of the solution separately or in mixtures against 20 mM Mg^{2+}.

2. The reaggregated material collected by centrifugation of the dialyzed solution of lipid-depleted *A. laidlawii* membrane proteins consisted of amorphous clumps, while the reaggreated *M. pneumoniae* glycolipids consisted of "myelin-like" globules and sheets composed of lamellae with a mean center-to-center distance of 37 Å. The reaggregated material of a mixture of lipid-depleted *A. laidlawii* membrane proteins and *M. pneumoniae* glycolipids contained, in addition to the amorphous clumps representing reaggregated proteins and the "myelin-like" structures representing reaggregated glycolipids, also long membranous sheets having a triple-layered structure with a mean center-to-center distance of the dense lines of 54 Å. The appearance and dimensions are closely similar to those of the original or reagregated *A. laidlawii* membranes.

3. It is suggested that these membrane-like structures are formed by the association of *A. laidlawii* membrane protein and *M. pneumoniae* glycolipids, and that these "hybrid" structures are responsible for the increased antigenicity of the reaggregated glycolipids.

INTRODUCTION

Sodium dodecyl sulfate has been shown to solubilize mycoplasma membranes to separate protein-detergent and lipid-detergent micelles[1,2]. In spite of the complete separation of the protein from the lipid by sodium dodecyl sulfate, these components could reaggregate spontaneously to form typical triple-layered membranes on removal of the detergent by dialysis against a Mg^{2+}-containing buffer[2-4]. Further

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studies have shown that solubilized membrane components from one mycoplasma reaggregated with those of another mycoplasma to form a hybrid aggregate which possessed antigens of both organisms[5].

This finding has recently been utilized to bind the glycolipid haptens of *M. pneumoniae* to the membrane proteins of *A. laidlawii*[6]. The reaggregated material elicited the production in rabbits of high titer of antibodies which inhibited the metabolism of *M. pneumoniae*, fixed complement with *M. pneumoniae* glycolipids or whole cells, precipitated *M. pneumoniae* glycolipids and agglutinated *M. pneumoniae* cells[6,7]. The efficient production of antibodies to the glycolipid haptens suggested that they were intimately bound to the membrane proteins of *A. laidlawii* during reaggregation[6]. Electron microscopy of the reaggregated membrane components seemed therefore necessary for the better understanding of the interactions which take place between the membrane proteins and lipids during the reaggregation process.

MATERIALS AND METHODS

Preparation of reaggregated membrane material

Purification of the serologically active *M. pneumoniae* glycolipids and the preparation of lipid-depleted *A. laidlawii* membranes by extraction with aqueous acetone have been described in detail elsewhere[6,8,9]. The lipid-depleted membrane suspension (10 mg protein per ml) was treated with 0.02 M sodium dodecyl sulfate for 15 min at 37°. The resulting clear solution was centrifuged at 36000 × *g* for 2 h to remove any non-solubilized membrane fragments. The amount of sedimentable material was usually negligible. The supernatant fluid was separated and used in the reaggregation experiments. The purified *M. pneumoniae* glycolipids (10 mg) were dissolved in 0.5 ml of chloroform–methanol (2:1, by vol.). 2 ml of deionized water and 0.5 ml of 0.1 M sodium dodecyl sulfate were added to the lipid solution. The resulting emulsion was heated under a stream of N_2 until all the chloroform evaporated and the emulsion cleared. The solutions of membrane proteins and glycolipids were dialyzed separately or in combination (1 mg glycolipids per 2 mg of protein) for 4 days in the cold without stirring against 3 l of dilute β-buffer[3] containing 20 mM Mg^{2+}. As a membrane reaggregation control, protein and lipid, both from *A. laidlawii*, were treated, combined, and dialysed as above to re-form membrane-like structures as previously described[4]. The contents of the dialysis bags were centrifuged at 36000 × *g* for 90 min and the pellets were processed for electron microscopy.

Electron microscopy

Pelleted preparations were fixed in osmium and treated subsequently by the methods of KELLENBERGER *et al.*[10], except that embedment from propylene oxide dehydration was made in Epon 812 (ref. 11). Silver to gold sections were cut with a diamond knife on an LKB Ultratome I, and stained with lead citrate[12]. Examination and photography utilized an Hitachi electron microscope, Model HU-11C, operating at 75 kV and using condenser and objective apertures of 500 and 50 μm, respectively. Photographs were made on 3¼ × 4 inch Kodak Electron Image Plates.

Measurements

A standard grid (54800 lines/inch) and appropriate sections of membranes or lipid aggregates, were photographed at a plate magnification of × 23000. Selected

areas of the negatives were then printed at the same final magnification (× 150000) on Kodak Electron Microscope Film No. 4489 (Thick Estar Base: 2 inch × 10 inch strips). The selected areas were oriented so that the long axis of membranes or lipid aggregates was at right angles to the long axis of the film strip. Development was for 2 min in Dektol (Kodak D-72), diluted 1:2; and fixation for 3–6 min in Kodak Rapid Fixer.

These film strips exactly fit the carrier, for 2 inch × 10 inch acetate strips, of the Photovolt Densicord Electrophoresis Densitometer, Model 542A, by which densitometric tracings were made. After calibration by use of the control grid photo, peak-to-peak distances on tracings of the membrane or lipid electromicrographs were measured as indicating center-to-center distances of the densely staining lines in the sections. At least ten measurements, for each sample were made directly on the tracing by use of a Bausch and Lomb measuring magnifier with a scale marked in 0.1 mm gradations. Although no precise statistical validity is claimed, measurements were consistent within a narrow range of variation for either membrane or glycolipid, and agreed closely with results obtained with difficulty by direct measurements on electron micrograph negatives or prints. It should be emphasized that the measurements represent center-to-center distances, not total width of triple-layered structures.

RESULTS

The clear solution of *A. laidlawii* membrane proteins in 0.02 M sodium dodecyl sulfate became very turbid during dialysis against 20 mM Mg^{2+}. The reaggregated material collected by centrifugation at the end of the dialysis period produced a white opaque sediment. Thin sections (Fig. 1) showed it to consist of clumps of amor-

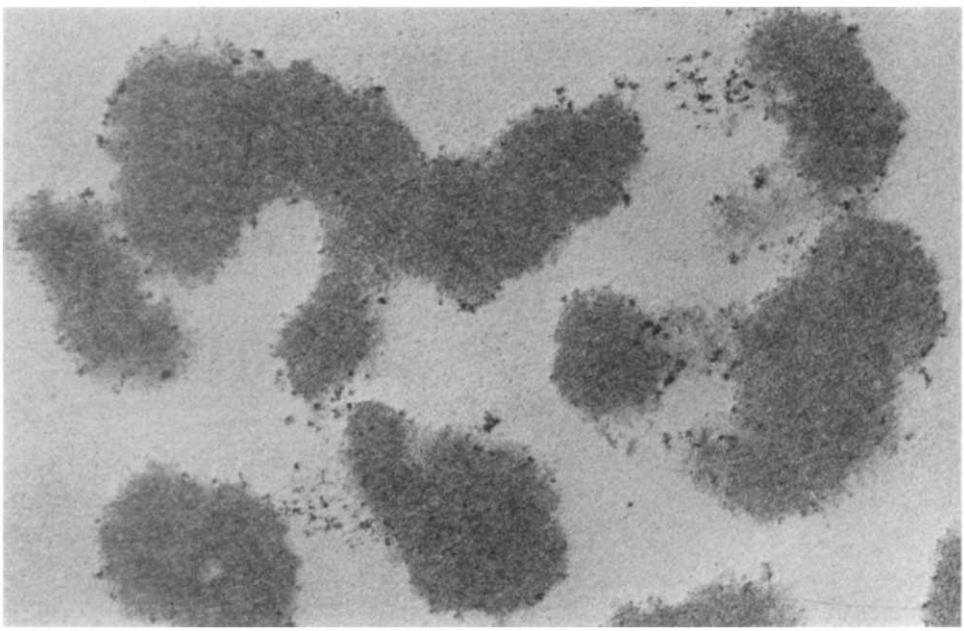

Fig. 1. Reaggregated membrane proteins of *A. laidlawii*. × 80500.

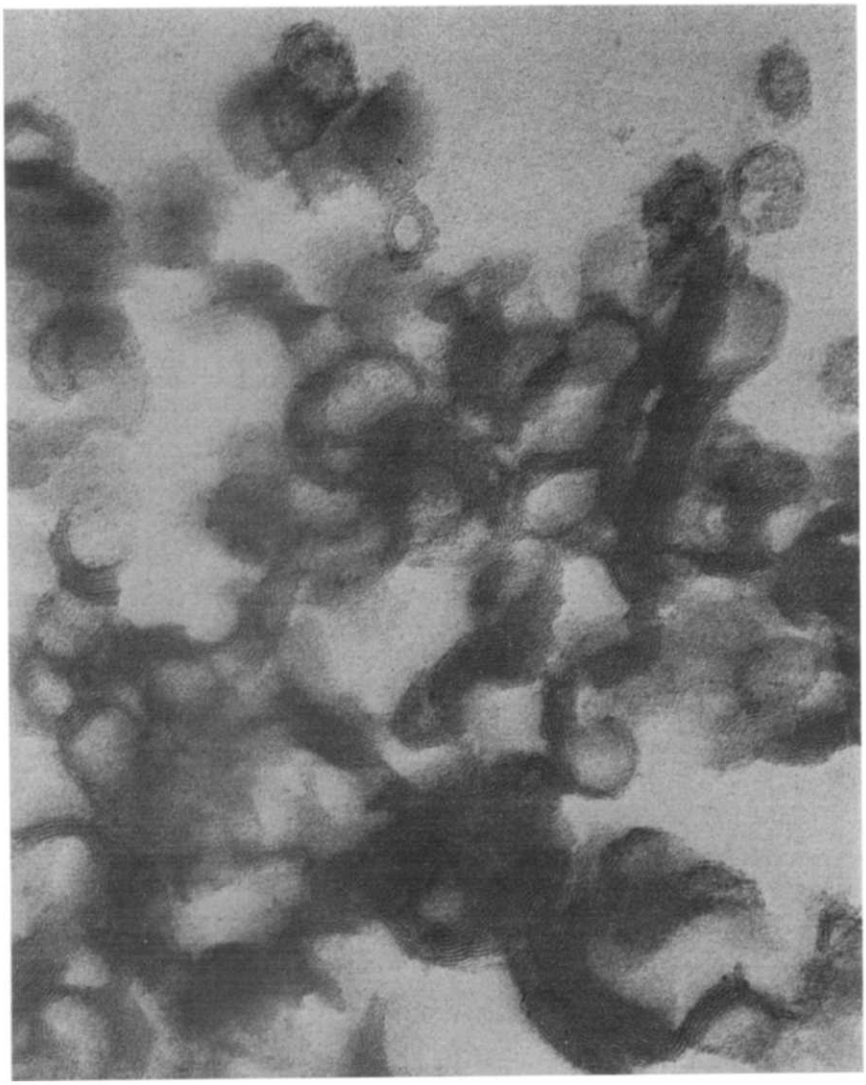

Fig. 2. Reaggregated glycolipids of *M. pneumoniae*. The concentric lamellar striations in the globules can be seen. × 135000.

phous material. The clear *M. pneumoniae* glycolipid solution in sodium dodecyl sulfate became opalescent during the dialysis period. Thin sections of the centrifuged material showed it to consist mostly of globules composed of several or more concentric lamellar striations with a center-to-center distance between dark layers of 34–42 Å ($m = 37$ Å) (Fig. 2). Large lamellar sheets resembling in ultrastructure the "myelin-like" globules could also be seen.

The combined solution in sodium dodecyl sulfate of *A. laidlawii* membrane proteins and *M. pneumoniae* glycolipids became turbid upon dialysis, though to a lesser degree than that of the dialyzed solution of *A. laidlawii* membrane proteins alone. The sediment formed by centrifugation of the dialysis bag contents consisted

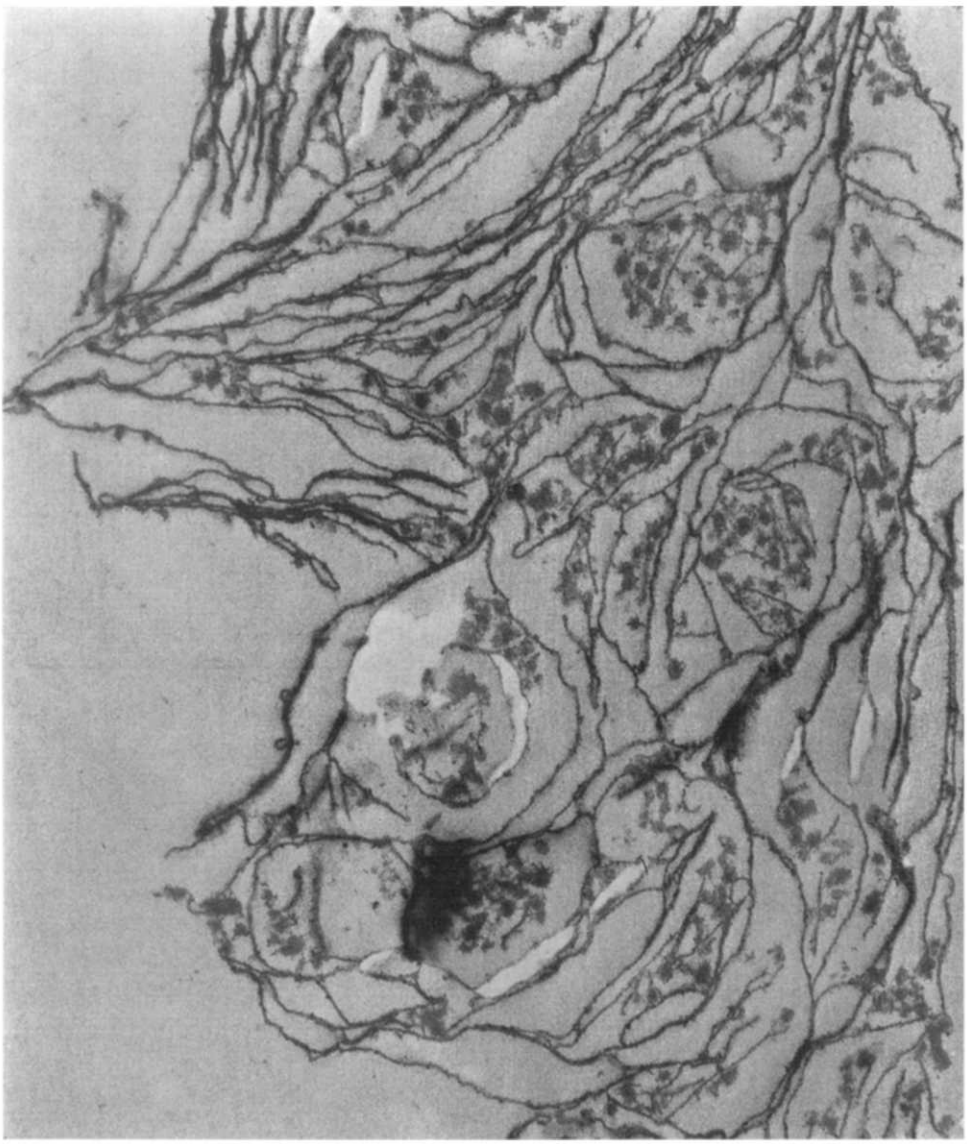

Fig. 3. *M. pneumoniae* glycolipids reaggregated with *A. laidlawii* membrane proteins. Very long triple-layered membranes entrapping protein clumps can be seen. × 31 500.

of an opaque white core overlaid with a large yellowish translucent pellet. This sections of the sediment confirmed its heterogeneity. Large areas of the sections contained amorphous clumps resembling those of the reaggregated membrane proteins. Other areas contained, in addition, the myelin-like structures characteristic of the reaggregated glycolipids. However, in certain areas of the sections (especially at the periphery), long membranous sheets could also be found (Fig. 3). These membranes exhibited the characteristic triple-layered structure (Figs. 4, 5) and resembled the

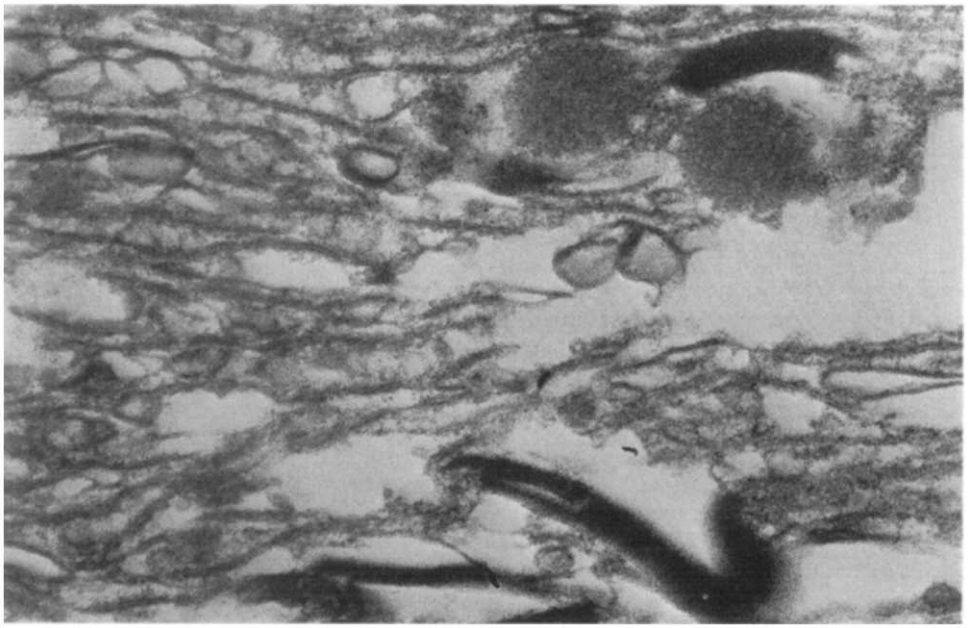

Fig. 4. *M. pneumoniae* glycolipids reaggregated with *A. laidlawii* membrane proteins. Long triple-layered membranes can be seen as well as protein clumps and glycolipid aggregates. × 80500.

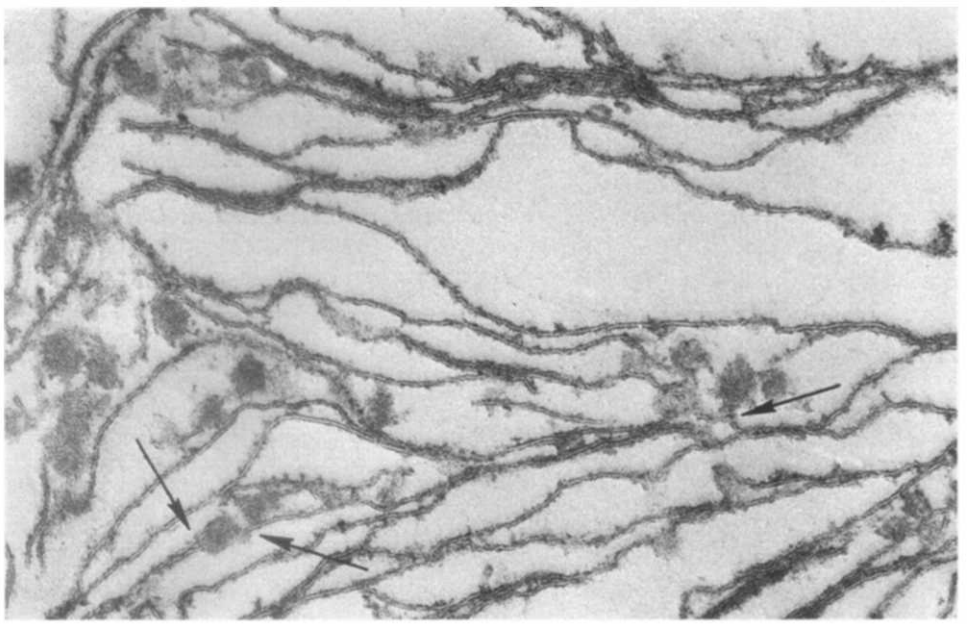

Fig. 5. A higher magnification of Fig. 3 showing clearly the triple-layered structure of the reaggregated membranes, the close association of the protein clumps with these membranes, and sites of association of membrane ends with protein clumps (arrows). × 80500.

original mycoplasma membrane in thickness. The center-to-center distance of the dense lines in sections of the reformed membranes, calculated from densitometer tracings, was 51–59 Å ($\bar{m}$ = 54 Å), resembling that of the native *A. laidlawii* membranes (44–74 Å; $\bar{m}$ = 54 Å). (Although not illustrated here, the membrane-like structures formed after dialysis of solubilized homologous *A. laidlawii* components were of similar appearance and dimensions: 42–66 Å, $\bar{m}$ = 52 Å). The electron micrographs (Figs. 3–5) also show that the amorphous clumps, representing apparently membrane proteins, are trapped within the large membranous sheets, and in some cases one gets the impression that membranes are originating from these amorphous clumps (Fig. 5, arrows). In some experiments (Fig. 4), but not in others (Figs. 3, 5), inclusion of uncoiled sheets of glycolipids was common.

DISCUSSION

The present study provides morphological evidence for the binding of *M. pneumoniae* glycolipids to *A. laidlawii* membrane proteins during the reaggregation process. Binding was indictated by the formation of long triple-layered membranous structures when the glycolipids were reaggregated in the presence of membrane proteins. Similar structures were not observed when the glycolipids or membrane proteins were reaggregated separately. Nevertheless, our studies show that under the conditions used for reaggregation a large portion of the solubilized membrane protein and glycolipids did not associate to form membrane-like structures. Hence, the reaggregated material is heterogenous, consisting of clumps of reaggregated protein, myelin-like arrangements (sometimes globular) of glycolipids, and membrane-like structures apparently produced by the association of the glycolipids with the proteins (Figs. 3–5).

Most of the membrane reaggregation studies have so far been carried out on *A. laidlawii* membranes. These studies[2–4,13] showed that it is possible to reaggregate almost all the solubilized *A. laidlawii* membrane material to membrane-like structures under strictly defined dialysis conditions and at certain concentrations of sodium dodecyl sulfate and Mg^{2+}. By changing the concentration of sodium dodecyl sulfate and Mg^{2+} the reaggregated material becomes heterogenous, as evidenced by electron microscopy and density gradient analysis[4,13]. Heterogeneity was expressed by the formation of several density bands and by the appearance of myelin-like globules and amorphous clumps in addition to the membrane-like structures, a picture resembling very much that obtained in the present study. Hence, our failure to bind all the *M. pneumoniae* glycolipids to *A. laidlawii* membrane protein may be due to inadequate dialysis conditions as well as to non-optimal sodium dodecyl sulfate and Mg^{2+} concentrations: these may also explain variations in the electron microscopic appearance (*cf.* Figs. 4 and 5). However, additional factors which could contribute to this failure should be considered. Thus, the acetone extraction of *A. laidlawii* membranes was done at room temperature, in order to free the membranes as completely as possible of *A. laidlawii* lipids. This extraction led to the denaturation of at least some of the membrane proteins as shown by inactivation of several enzymic activities associated with the membrane (ref. 14 and unpublished results of S. RAZIN AND *W. W.* GOTTFRIED). Denaturation of the proteins could affect their ability to reassociate with membrane lipids, but this aspect has not been investigated as yet.

The chemical composition of the lipids taking part in the reaggregation process should also be considered as another factor which may affect the reaggregation results. GRULA *et al.*[15] found that phospholipids from *Erwinia sp.* were incorporated into the membranous sheets formed by the reaggregated membrane proteins of *Micrococcus lysodeikticus*, indicating that the reaggregation process is not too specific with regards to the composition of the lipid component. Our results also indicate that lipids from one mycoplasma species can associate with membrane protein of another mycoplasma species and form membrane-like structures. This intimate binding is apparently responsible for the immunogenicity of *M. pneumoniae* glycolipids reaggregated with *A. laidlawii* membrane proteins. When the glycolipids were merely mixed with lipid-depleted *A. laidlawii* membranes, their immunogenic properties were much inferior to those of glycolipid reaggregated with protein[6]. Nevertheless, some restrictions imposed by the conformation of the membrane proteins used, or by other as yet undefined factors, may be expected to limit the type and amount of lipid bound to the protein by reaggregation. Thus preliminary experiments in our laboratory have failed so far to show the formation of characteristic triple-layered membranes by reaggregation of bovine cardiolipin or cerebrosides with *A. laidlawii* membrane proteins.

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