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RECONSTITUTION OF MYCOPLASMA MEMBRANES

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Detergent-solubilized proteins and lipids of mycoplasma membranes reassemble spontaneously into membranous structures on the removal or dilution of the detergent in the presence of divalent cations. The cations seem to function by neutralizing the negatively charged groups on membrane lipids and proteins which interfere by electrostatic repulsion with membrane reassembly. Moreover, salt bridges formed by the divalent cation between acidic groups on membrane proteins and lipids seem to play an important role in the reconstituted membrane stability. Electron transport activity, as measured by the transport of electrons from NADH to oxygen, has been demonstrated in reconstituted Acholeplasma laidlawii membranes. However, restoration of active transport of sugars or ions has not been achieved so far. The conditions for obtaining properly sealed vesicles, which are obligatory for demonstrating transport activity, are still rather poorly defined. The reassembled membranous structures cannot be distinguished from the native membranes in chemical composition, density, and thin sections. However, probe techniques, x-ray diffraction, and freeze-fracturing electron microscopy indicate that the proteins are organized differently in the reassembled membranes, though the lipid bilayer is restored. The results obtained so far leave little hope for successfully reconstituting the molecular organization of membranes as complex as those of mycoplasmas by a single-step reassembly of detergent-solubilized membrane components. The prospects appear brighter with membranes having only a few protein species, such as the outer membrane of gram-negative bacteria. In spite of the failure to reconstitute fully active mycoplasma membranes, the reassembly procedure was found valuable in studying the interactions of detergent-solubilized membrane proteins with lipids, the effects of a hydrophobic environment on hydrophilic enzymes, and the production of "hybrid" membranes having selected membrane components.

INTRODUCTION

Membrane reconstitution offers a most promising approach for elucidating the molecular organization of the protein and lipid in biomembranes, the nature of the building blocks participating in membrane assembly, and the type of bonds holding them

together. Reconstitution studies should, ideally, include the disaggregation of the membrane into its building blocks, the biochemical and biophysical characterization of the solubilization products, and their reassembly to a membrane identical to the original as regards structure and function (1). If biomembranes actually consisted of homogeneous lipoprotein subunits, according to the theories prevailing in the middle 1960's (2), then such a task should not be too difficult. All that would have to be done would be to disaggregate the membrane into its component subunits, e.g., by detergent action, which would then reassemble spontaneously reforming membranous structures following the removal of the dissociative agent, since the structure-determining information was supposed to be innate in the subunits themselves (2). The first reconstitution experiments on mycoplasma membranes (3) appeared to support the subunit hypothesis, showing a single symmetrical schlieren peak of about 3S in the solution of Acholeplasma laidlawii membranes in sodium dodecyl sulfate (SDS). A similar peak was shown with a variety of biomembranes solubilized by the same detergent, lending support to the idea prevailing, at that time, that biomembranes are built of lipoprotein subunits of about the same size (2, 4). Further support came from the finding that, following the removal of the detergent, the solubilized membrane material from A. laidlawii and other membranes reaggregated into membranous structures resembling the native membranes in thin sections and in chemical composition (3, 5). Soon after, however, doubts about the validity of the subunit interpretation were raised by Engelman et al. (6), who showed that membrane protein could be separated from membrane lipid by the prolonged centrifugation of the solubilized membrane material on sucrose gradients. Further studies (7–9) showed conclusively that membrane material in SDS consists not of lipoprotein subunits, but rather of protein-SDS complexes and lipid-SDS micelles separable by various techniques.

Paralleling the dwindling of support for the subunit model of membrane structure over the past few years have been the difficulties in the single-step reconstruction of biomembranes from their detergent-solubilized components. The chances for the correct self-assembly of all the numerous detergent-treated membrane proteins in one step under any given set of conditions appear to be very slight in light of the present knowledge of membrane organization. Nevertheless, a survey of the extensive literature on the reconstitution of detergent-solubilized mycoplasma membranes raises many points of interest, in particular concerning the interactions of detergent-treated membrane proteins with membrane lipids.

RECONSTITUTION CONDITIONS

Obviously, the solubilizing agent must be removed for the solubilized membrane components to reassociate. The complete removal of detergents, which is usually extremely difficult, is, however, unnecessary. Reconstitution may be induced merely by reducing the detergent concentration below a certain value by simple dilution, by Sephadex filtration, or by dialysis, providing that divalent cations are also supplied. Dialysis against a buffer containing a divalent cation can play the dual role of diluting the detergent and of slowly adding the required cation (1, 3).

The need for a divalent cation, such as Mg^{2+}, in the reconstitution of mycoplasma membranes solubilized by SDS as noted in the early stage of reconstitution studies (3) has

not only been confirmed since then (8, 10–14), but has also been extended to a variety of other biomembranes (1, 15, 16).

Neutralizing the negative charges of the SDS molecules bound to the membrane material is unlikely to be the primary function of the divalent cations, since it was found that Mg^{2+} was still needed for reconstitution when SDS was removed by Sephadex G-25 (8) from the solubilized membrane material. Moreover, Mg^{2+} was also required for reconstituting membrane material solubilized by nonionic detergents (8, 17). It appears that the divalent cations facilitate membrane reconstitution by neutralizing those negatively charged groups on membrane lipids and proteins which might interfere with membrane reassembly due to their electrostatic repulsion. At neutrality acidic phospholipids will bear a net negative charge as will the mycoplasma membrane proteins. Lowering the pH restricts the dissociation of the acidic groups and the quantity of Mg^{2+} needed for reassembly is much smaller (8).

High Na^{+} concentrations were found to be a substitute for Mg^{2+} requirement in reconstitution (8). It seems clear that in this case too, the cation acts by neutralizing repulsive negative charges on proteins and lipids. Moreover, the increased ionic strength of the reaction mixture due to the addition of Na^{+} would also be expected to enhance hydrophobic bonding between proteins and lipids (18). However, the bonds holding protein and lipid in membranes reconstituted in the presence of Na^{+} can be broken by reactivating the repulsive charges by washing the cation off with deionized water (8). This is not so in the case of membranes reformed in the presence of Mg^{2+}, so that salt bridges formed by the divalent cation seem to play an important role in the stability of the reconstituted membranes.

How would Mg^{2+} removal affect the reconstituted membranes? Dialysis against EDTA was found to remove more than 95% of the Mg^{2+} bound to reconstituted A. laidlawii membranes, yet these membranes did not disintegrate (19). It seems that the small amounts of Mg^{2+} not available for EDTA chelation may, in fact, suffice to maintain membrane integrity. However, more drastic EDTA treatment according to the procedure of Marchesi et al. (20) resulted in the solubilization of some 60% of the reconstituted membrane proteins as against only 8 to 14% of the proteins of native membranes treated in the same way (19, 21). It thus appears that the Mg^{2+} concentration required for the initiation of the reconstitution process is much higher than that needed to maintain the membranous structures formed, since the primary role of the cation is to facilitate hydrophobic bond formation by keeping the electrostatic repulsion between the interacting components to a minimum. Once these bonds are formed, much less Mg^{2+} is needed (see also Ref. 15).

The concentration of Mg^{2+} in the dialysis buffer has a marked effect on the lipid-to-protein ratio of the reconstituted membranes (8, 10, 13). The solubilized membrane lipids tend to reaggregate at lower Mg^{2+} concentrations than the solubilized proteins, producing "lipid-rich" membranes, lighter than the native membranes.

RESTORATION OF FUNCTION IN RECONSTITUTED MEMBRANES

Restoration of the native membrane functions, particularly those associated with multienzyme complexes, may serve as an excellent indicator for the successful recon-

stitution of the original molecular organization. The electron-transport chain located in microbial cell membranes is a convenient candidate for such studies. The solubilization of Bacillus stearothermophilus membranes with low SDS concentrations interrupted electron flow because there was fragmentation of the membrane into particles containing different sections of the electron transport chain. Dialysis of the solubilized membrane material against Mg^{2+} resulted in the "proper" reassembly of the fragments into membranes exhibiting the activity of a complete electron-transport chain (15).

The fragmentation of the electron-transport chain by detergents and the complete restoration of the chain by the dialysis of the solubilized products against Mg^{2+} have also been reported for membranes of Bacillus megaterium (22) and Micrococcus lysodeikticus (23). The picture is different with A. laidlawii membranes. Here, the electron flow to oxygen is much more resistant to detergent action, and NADH oxidase activity survives the complete solubilization of the membranes in fairly high bile salt concentrations, or even in SDS (21). It is not surprising, therefore, that reconstituted A. laidlawii membranes are capable of transporting electrons from NADH to oxygen (3, 13). The absence of cytochromes, together with the resistance of NADH oxidase activity to most electron-transport inhibitors and its nondependence on membrane lipids may indicate that the electron-transport chain of A. laidlawii is extremely simple, possibly consisting of one or two enzymes only (24, 25). Hence, the demonstration of electron-transport activity in reconstituted A. laidlawii membranes may be of little significance with regard to the successful restoration of the original membrane organization.

The restoration of active transport systems may also serve as a good index of the successful reconstitution of molecular organization, but these have not been demonstrated so far in reconstituted mycoplasma membranes. The phosphoenolpyruvate-dependent phosphotransferase system responsible for sugar transport in Mycoplasma mycoides var. capri was inactivated when the membranes were solubilized by a variety of detergents and was not reactivated by the reaggregation of the solubilized membranes (V. P. Cirillo, unpublished data). Likewise, the property of active K^+ uptake in intact A. laidlawii cells was not recovered in their reconstituted membranes (E. Giberman and S. Razin, unpublished data).

It is evident that the presence of vesicles is a prerequisite for demonstrating any transport activity in reconstituted membranes. Yet, the conditions and factors affecting vesicularization in reconstituted material are poorly defined. Time appears to be a major factor in vesicle formation. With SDS-solubilized mycoplasma membrane material, the reconstituted membrane profiles produced after short dialysis periods appeared to vary in length and had free ends. The membranous sheets folded into vesicles only after several hours' dialysis, with almost quantitative vesicularization after 72 hours (13). Divalent cations may possibly affect vesicularization since the Mg^{2+} content in the dialysis bag increases with decreasing detergent concentration. While there is no direct evidence that divalent cations play a direct role in vesicularization, dialysis of SDS-solubilized mycoplasma membranes against a buffer containing a low Mg^{2+} concentration (5 mM) usually resulted in the formation of membranous sheets (13, 26). However, removing the SDS before adding Mg^{2+} resulted in the formation of short membranous sheets (11). Thus, for vesicle formation, the removal of the detergent and the addition of Mg^{2+} should be simultaneous. Mg^{2+} and Ca^{2+} have been shown to facilitate the resealing of erythrocyte ghosts (27, 28).

The similarity between the resealing process and vesicularization has recently led to

awareness of another possible influence on vesicularization. The resealing of erythrocyte ghosts is temperature-dependent, being very rapid at 37°C and extremely slow at 0°C (27, 28). This temperature effect suggests that the resealing process involves some rearrangement of the lipid structure of the membrane which takes place only when this structure is in a fluid state. Accordingly, sealed vesicles should only form when the lipids of reconstituted membranes are in the liquid-crystalline phase, i.e., above their phase transition temperature. This conclusion is supported by the findings of Kagawa (29) that successful reconstitution of the coupling device of the inner mitochondrial membrane, which depends on vesicle formation, can be achieved only with unsaturated phospholipids, saturated phospholipids being inactive. At the reconstitution temperature (between 0°C and 4°C), the unsaturated phospholipids would still be in the fluid phase. The reconstitution of mycoplasma membranes is usually carried out between 0°C and 4°C to minimize enzyme denaturation by the detergent. It seems probable that the extent of vesicle formation would decrease most markedly when the membrane polar lipids are of the saturated type. Such a possibility ought to be tested experimentally.

Even when vesicles do form and can be seen by electron microscopy (Fig. 1), their permeability properties may still differ from those of intact cells. One usually finds that such vesicles leak. The reason for this may quite simply be the presence of minute holes in the reconstituted membranes detectable only by high power electron microscopy (Fig. 2), or it may be due to more subtle differences in membrane organization. Resealed erythrocyte ghosts, for example, never exhibit complete restoration of the diffusion barrier and their permeability to nonelectrolytes is 10- to 50-fold higher than that of intact erythrocytes (28). If reconstituted membrane vesicles are not sealed, then the proteins on their inner membrane surface may be accessible to exogenous macromolecules such as lactoperoxidase, which is not capable of penetrating intact cells. Recent experiments in our laboratory (Z. Ne'eman and S. Razin, unpublished data) suggest that lactoperoxidase has access to proteins on both sides of reconstituted A. laidlawii membrane vesicles, since the lactoperoxidase-mediated iodination values of the reconstituted membranes obtained by the method of Phillips and Morrison (30) were four to five times greater than those of membranes of intact cells. Nevertheless, the high iodination values of reconstituted membranes may also be due to factors other than imperfect vesicularization, such as the unfolding and different organization of the polypeptide chains in the reconstituted membranes, as will be discussed in the last section.

RECONSTITUTED "HYBRID" MEMBRANES

Although significant variations in the lipid-to-protein ratio and in lipid composition may be observed in many biomembranes, the composition and disposition of membrane proteins appear to be fairly stable characteristics for each membrane type (31, 32). Membrane assembly in vivo must, therefore, be a highly specific and controlled process, particularly with respect to the type of protein molecules incorporated into the membrane. Is such specificity retained during the reconstitution of membranes from detergent-solubilized membrane components? The answer appears to be negative, at least with regard to SDS-solubilized membrane components. Dialysis of a mixture of SDS-solubilized membranes from A. laidlawii and Mycoplasma gallisepticum against Mg^{2+} produced an

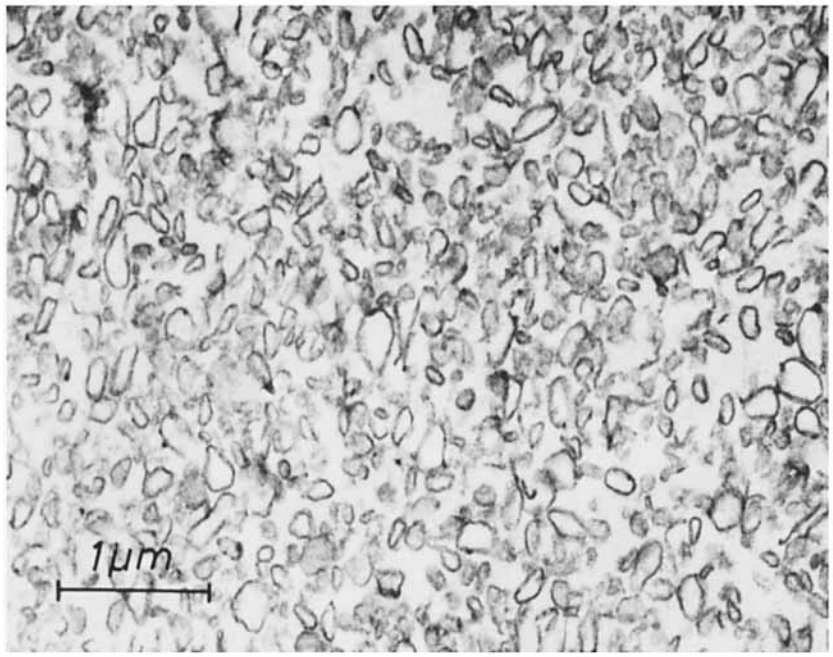

Fig. 1. Thin section of reaggregated material from Acholeplasma laidlawii membranes solubilized by taurocholate. The vesicular nature of the reconstituted membranes is evident. From Z. Ne'eman and S. Razin, unpublished observations.

aggregate with a density intermediate between those of reconstituted A. laidlawii and M. gallisepticum membranes. It consisted of trilaminar membranes accompanied by amorphous material, and contained the antigens of both membrane species (33). In a similar way membranes were assembled from SDS-solubilized A. laidlawii membrane proteins and Mycoplasma pneumoniae glycolipids (34) or even with cytolipin H from bovine spleen (35).

The finding that reconstituted mycoplasma membranes retain their immunogenicity (36) has led to the use of hybrid membranes for the preparation of highly specific and potent antisera to serologically active membrane lipids (34, 35, 37). By themselves the lipids are unable to elicit an antibody response; hybrid membranes prepared from purified glycolipids of M. pneumoniae or cytolipin H from bovine spleen and membrane proteins of A. laidlawii were most effective in eliciting the production, in rabbits, of antibodies to the lipid component. The high immunogenicity of the lipid hapten when bound to membrane proteins by reconstitution is apparently due to intimate binding, probably by the same kind of bonds as are responsible for the association of proteins and lipids in native membranes. The membranous nature of a considerable part of the hybrid material supports this assumption (38). The main advantage of using reconstituted hybrid material for the production of antibodies to membrane lipids is that the lipid and protein components can be selected so that the type of antibodies produced is controllable.

It should be borne in mind that these hybrid membranes form from proteins which

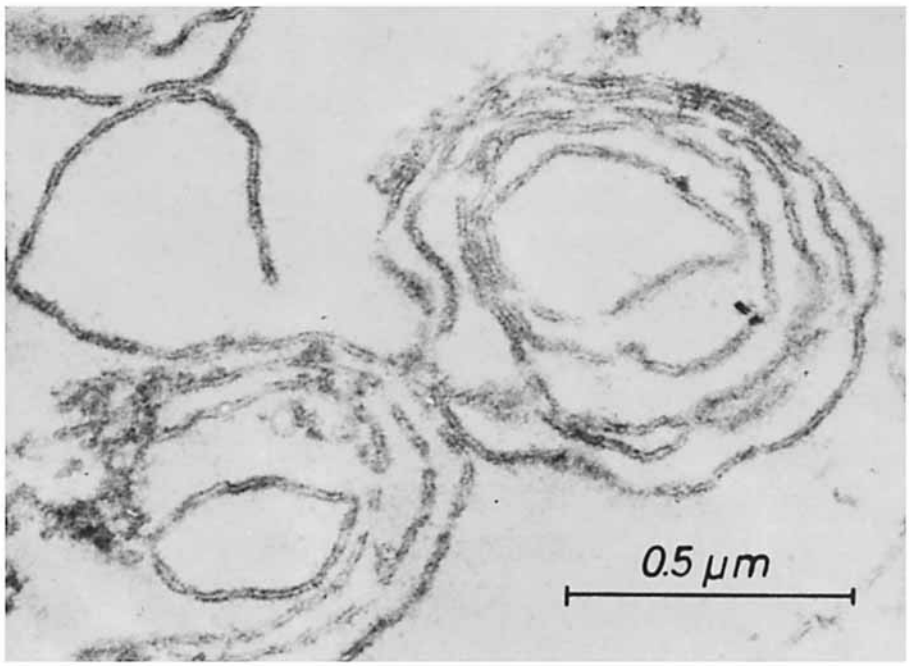

Fig. 2. Thin section of reaggregated material from Acholeplasma laidlawii membranes solubilized by cholate. The discontinuities in the reconstituted membranes indicate that the "onion-like" vesicles are not sealed. From. Z. Ne'eman and S. Razin, unpublished observations.

have suffered radical conformational changes as a result of their contact with SDS (39). The use of membrane material, solubilized by other means, which preserves the tertiary and quaternary structure of the proteins may yet reveal a stricter specificity in the incorporation of protein into the reconstituted membranes. Nevertheless, protein–lipid and lipid–lipid interactions, which play a major role in the reassembly of the solubilized membrane components, are known to be nonstoichiometric and to be less specific than protein–protein interactions (29). The formation of hybrid membranes from solubilized proteins and lipids originating in the membranes of different organisms is not, therefore, so surprising. The fusion of membranes of viable A. laidlawii cells with phosphatidylcholine (40) and the fusion of membranes from different cell types (41) demonstrate the low specificity and the lack of stoichiometry of the interaction of membrane proteins with lipids even more dramatically.

So far, our discussion has been limited to interaction between detergent-solubilized membrane proteins and lipids. What would happen if soluble proteins were added to the reconstitution mixture? Would they be incorporated into the reconstituted membranes? Would they interfere with the reaggregation of the solubilized membrane components? How would the detergent affect the binding of soluble proteins to the reaggregated membrane components? In recent studies in our laboratory (S. Rottem, M. Hasin, and S. Razin, unpublished data) such problems were investigated. Cytochrome c was bound to reconstituted A. laidlawii membranes in large quantities and its presence in the recon-

stitution mixture enhanced the reaggregation of the solubilized membrane proteins and lipids, acting probably in parallel with Mg^{2+}. Bovine serum albumin, on the other hand, was only bound in very small quantities and had little effect on the reaggregation of membrane components. The presence of SDS during the binding of the soluble proteins to the reaggregated membrane components affected both the quantity of the proteins bound as well as the nature of the bonds formed. The addition of cytochrome c, for example, to the reconstitution mixture after detergent removal reduced its tendency to be bound most markedly, the reverse being true for bovine albumin. Essentially all the cytochrome c bound in the absence of detergent was released by 1 M NaCl, as opposed to only 70% of that bound in the presence of detergent. This suggests that, after exposure to detergent, as least some of the cytochrome c molecules are bound to the reconstituted membranes by nonionic bonds. The much greater binding of cytochrome c in the presence of SDS may be explained in terms of higher numbers of residual SDS molecules, bound to reaggregated membrane components, providing additional binding sites for basic proteins, just as they do for Mg^{2+} (19). In the case of albumin, the reverse is true and the residual anionic detergent molecules will add to the repulsive effect of the acidic phospholipids and interfere with the binding of the protein which bears a net negative charge at neutrality. Hence it appears that soluble proteins which are negatively charged at neutrality will not be incorporated very well into reconstituted membranes.

Studies by Rottem et al. (42) provide an interesting application of the binding of a soluble protein into reconstituted membranes. They investigated the effects of environmental changes on the properties of the soluble penicillinase of Bacillus cereus by means of the reconstitution technique. When added to the reconstitution mixture of solubilized membranes from A. laidlawii, appreciable amounts of the enzyme were found to be bound to the reconstituted material. About two-thirds were obviously bound electrostatically since they were removed by 1 M NaCl washing. As the rest (8–10 μg penicillinase per mg membrane protein) could not be released in this manner, it must have been held by hydrophobic bonds, or by a combination of hydrophobic and electrostatic bonds. That tightly bound penicillinase was elutable with 1 M NaCl, after extraction of the lipid from the reconstituted membranes, shows that it had been embedded in a largely hydrophobic environment, a factor likely to modify its conformation. It actually turned out that the bound penicillinase was found to be much more resistant to iodination in the presence of methicillin and more sensitive to thermal inactivation than the soluble native enzyme (42).

THE MOLECULAR ORGANIZATION OF THE RECONSTITUTED MEMBRANES

Is the molecular organization of the reconstituted mycoplasma membrane identical to that of the native membrane? This is certainly the most relevant question to ask in reconstitution studies. The reconstituted mycoplasma membranes cannot be distinguished from native membranes in their chemical composition, density, and electron microscopical appearance when negative staining and thin sectioning are used (3, 8, 10, 13). However, the more recent application of probe techniques, x-ray diffraction, and freeze-fracturing electron microscopy clearly indicates that the reconstituted membranes differ in certain of their organizational details from those of native mycoplasma membranes, in particular

with respect to the organization of their proteins.

The results of the electron-spin resonance and x-ray diffraction studies suggest that the bilayer configuration of membrane lipids is restored in reconstituted membranes (43–45). This would be expected since, in aqueous solution, it is the most favorable configuration energetically. The question appears to be centered more on restoration of the original conformation and on the disposition of the membrane proteins. The solubilization of the proteins by detergents, in particular by SDS, may irreversibly alter their conformation and, consequently, their molecular properties and biological activity. The altered proteins may then be "incorrectly" assembled in the reconstituted membrane structure. That this is really what happens is indicated by data obtained by several independent methods. Both nuclear magnetic resonance measurements, with benzyl alcohol as the probe, and fluorescence measurements, with 1-anilinonaphthalene-8-sulfonate as the probe, showed many more binding sites for the probes on proteins in reconstituted membranes than were found in native membranes, indicating incorrect positioning of the proteins in the reconstituted structure (44, 45). Another indication of differences in protein organization between the reconstituted and native membranes comes from freeze-fracturing electron microscopy. Both the convex and concave fracture faces of reconstituted A. laidlawii membranes were fairly smooth, like those of myelin (46) or liposomes (47), lacking the characteristic particles seen in native membranes. The results were the same in the case of reconstituted membranes formed from SDS-solubilized material (26) and in the case of those from membrane material solubilized by bile salts (Fig. 3). There is strong evidence that the particles shown on the fracture faces of biomembranes consist of protein molecules or lipoprotein aggregates embedded within the hydrophobic region of the membrane (48). Hence, the lack of particles suggests the absence of proteins in the hydrophobic core of the reconstituted membranes.

All the evidence brought up so far, taken together with the higher susceptibility of the proteins of the reconstituted membranes to detachment by EDTA (19), supports our hypothesis (13, 49) that the reconstituted mycoplasma membranes form a bimolecular lipid leaflet coated on both sides with protein and with very little, if any, protein actually embedded within the lipid bilayer. According to this hypothesis, the reconstituted membranes build up in a multistep assembly process. First, a lipid-rich membrane is formed on which more protein is successively bound, depending on the Mg^{2+} concentration. The solubilized membrane proteins, lacking disulfide bonds (50), lose most of their tertiary and quaternary structure on solubilization by SDS (39), so that the polypeptide chains unfold. The open polypeptide chains are spread tangentially over the surfaces of the lipid bilayer, like the A_1 protein of myelin (51), and fit in well with the organization of proteins in the classical membrane model of Danielli and Davson as modified by Robertson (52).

One may conclude that the results obtained so far leave little hope for successfully reconstituting the molecular organization of membranes as complex as those of mycoplasmas by the spontaneous reassembly of detergent-solubilized membrane components. The prospects of success appear much brighter where the reconstitution procedure is applied to those membranes with only a few protein species, for example, the outer membrane of gram-negative bacteria. Bragg and Hou (53) claim to have demonstrated the successful restoration of the asymmetric arrangement of the proteins in the outer membrane of E. coli after subjecting it to SDS-solubilization and reconstitution procedures devised for mycoplasma membranes.

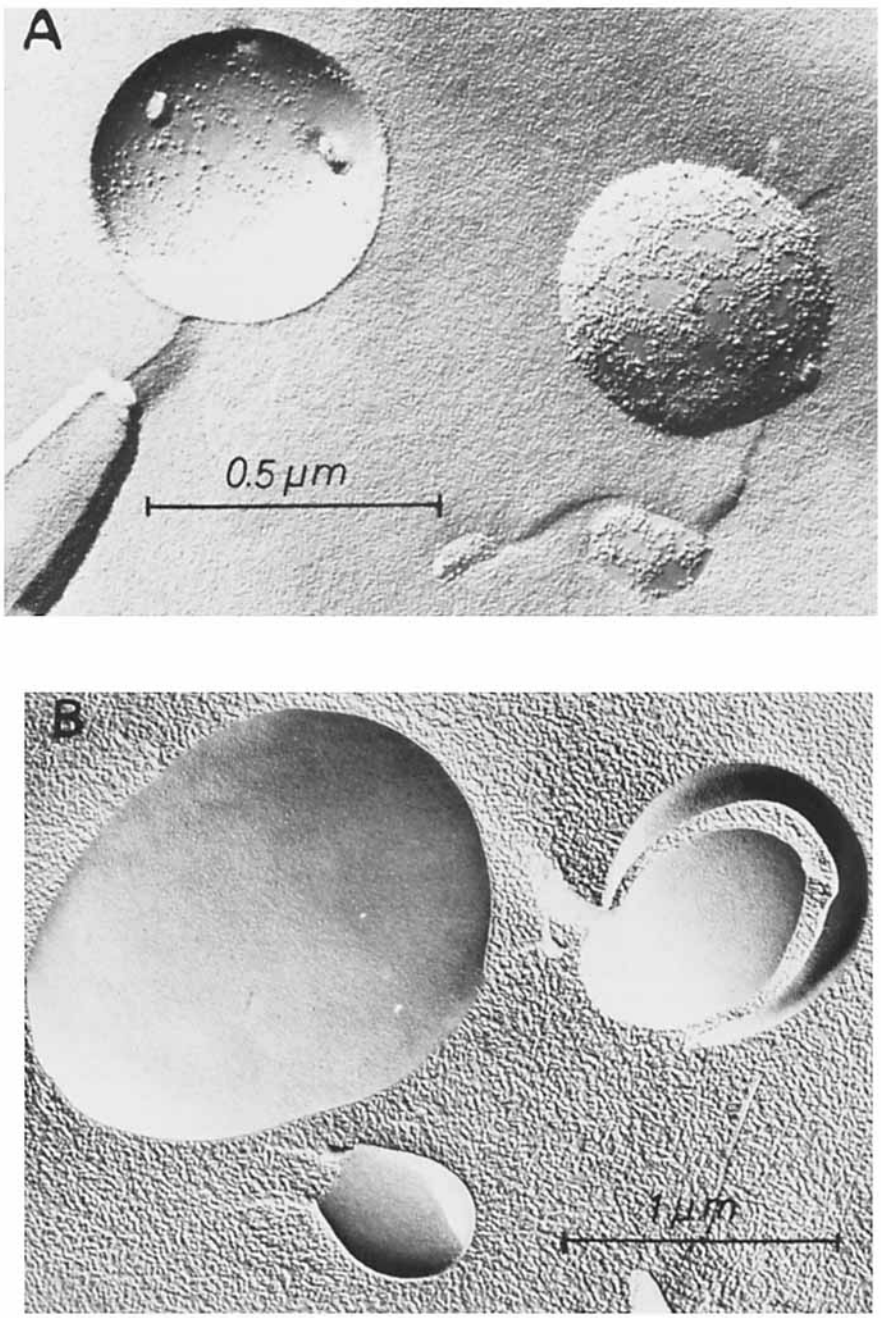

Fig. 3. Replicas of freeze-cleaved native (A) and reconstituted (B) Acholeplasma laidlawii membranes. Both the convex and concave fracture faces of the reconstituted membranes lack the particles seen on the fracture faces of the native membranes. The reconstituted membranes were formed from cholate-solubilized membrane material. The "onion-shaped" structures observable in thin sections (Fig. 2) are also evident here. From Z. Ne'eman and S. Razin, unpublished observations.

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