

# Electron Microscopic Observations of Reconstituted Proteoliposomes with the Purified Major Intrinsic Membrane Protein of Eye Lens Fibers

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**Abstract.** The purified major intrinsic protein of the lens fiber plasma membrane (MP26) reconstituted into liposomes favored membrane-to-membrane close contacts as visualized by freeze fracture and immunoelectron microscopy.

Reconstituted apposed unilamellar vesicles formed pentalamellar profiles, and multilamellar liposomes showed regions of stacked bilayers.

Immunogold labeling, using antibody directed against MP26, demonstrated that this polypeptide is present in

regions of membrane-to-membrane close interaction.

Fracture faces displayed both randomly distributed clusters of 8-nm polygonal intramembrane particles and membrane domains where a bidimensional lattice of repeating subunits was present.

The structural pleomorphism which characterized the MP26-reconstituted proteoliposomes seems quite comparable to that visualized in natural fiber plasma membrane domains.

**A** KEY step of the terminal differentiation of the eye lens epithelium into fiber is the biosynthesis of a novel protein of 26 kD (MP26) (3). While the epithelial cell elongates and loses most of the intracellular organelles, with a large increase of the surface of the plasma membrane surrounding each individual fiber, a process of junctional assembly takes place (2).

The intrinsic membranous nature of MP26 is consistent with the results of experiments on translation *in vitro* of messenger RNA encoding MP26. This polypeptide is cotranslationally inserted into microsomal membranes (1, 28) and into isolated lens fiber plasma membranes (8). The most convincing line of evidence that MP26 is a potential candidate for being the constituent of the communicating pathway between fibers is the amino acid analysis (7) and the sequence of MP26 based on cDNA cloning (13). These interesting data suggest that this polypeptide with a prevailing degree of hydrophobicity commonly found in transmembrane proteins, comprises among other helical domains an amphiphilic helix that according to Revel et al. (32) may easily accommodate either in tetramers or hexamers to form a pore-like structure.

MP26 undergoes posttranslational modifications in the lens fibers. The phosphorylation of this polypeptide mediated by a cyclic AMP protein kinase has been recently shown (16, 20, 23). On the other hand, MP26 proteolytic degradation by endogenous proteases generates a product of 22 kD (MP22) which accumulates in the lenticular nucleus during aging (7, 8).

A number of experiments have demonstrated that MP26 is the major protein component of the lens fiber plasma membrane (3, 31), but conflicting results have been ob-

tained, by immunocytochemistry, from different laboratories concerning the accumulation of this protein in the lens fiber junctional domains (3, 4, 17, 33, 37; however, see 18, 27). In particular, it has not been clear whether MP26 alone or other polypeptides form the lens permeable pathway (18). This problem is even more complicated because different subclasses of the pentalamellar structure, characteristic of closely apposed bilayers, have been identified according to their thickness, by thin section electron microscopy of cortical and nuclear lens fibers (41). Freeze-fracture studies of lens fiber membranes have shown that the intramembrane particles (IMPs)<sup>1</sup> occur either in nongeometrically packed assemblies or in an orthogonal lattice. The orthogonal pattern is more evident in the plasma membranes of the lenticular nucleus (3, 8). However, it is not easily apparent whether these two different types of IMP arrangements are formed by MP26 and/or its degradation product MP22 in a different lipid environment (cf. 8).

A number of studies have already shown that MP26 solubilized and reconstituted into liposomes is a channel-forming protein (12, 26), and that MP26 channels (in one single vesicle), are regulated by voltage and by calmodulin-dependent Ca<sup>++</sup> concentration (11, 40).

The gap junction is a stable contact between two adjoining membranes; a continuous aqueous pathway is formed from two axially aligned and oppositely facing protein oligomers which must be linked one to another in the middle of the extracellular gap. In this view, it remains to be established

1. *Abbreviations used in this paper:* Bic, bicarbonate buffer; IMP, intramembrane particles.

whether MP26 by itself is capable of forming membrane-to-membrane close contacts and organized domains in the plane of the bilayer. In this study, we have addressed this question by reconstituting solubilized MP26 into artificial lipid bilayers. The resulting proteoliposomes were frequently joined by extensive bilayer contacts at which MP26 was located. These contacts shared ultrastructural features with lens fiber junctional domains in situ.

## Materials and Methods

### Preparation of Membranes

Fresh cow lenses were decapsulated and the cortical region was isolated and homogenized in bicarbonate buffer (Bic) (1 mM NaHCO<sub>3</sub>, pH 7.4). After three washes by centrifugation in the same buffer, at 8,000 g for 10 min at 4°C (50 g of wet weight cortex in 1 liter of buffer for each run) the pellets (Bic-plasma membranes) were resuspended in 100 ml of 6 M urea, stirred for 30 min at 4°C, diluted with 1 vol of distilled water, and centrifuged at 27,000 g for 10 min. Resuspension and centrifugation were repeated two times and the final urea-insoluble material was treated with 50 ml of 100 mM NaOH (pH 11) for 10 min on ice, diluted with 1 vol of distilled water, and centrifuged at 27,000 g for 20 min. The final membrane pellet (U-Na membrane) was thoroughly washed with distilled water by repeated centrifugations at the same speed. The yield obtained was 30 mg protein/50 g wet cortex. Samples of U-Na membranes were treated with V8 *Staphylococcus aureus* protease, 25 mg membrane protein/mg protease, in 0.5% ammonium bicarbonate at pH 8, during 2 h (cf. 7).

### Solubilization and Reconstitution Experiments

30 mg of the U-Na membranes were solubilized in 12 ml of 10% non-ionic detergent *n*-octyl  $\beta$ -D-glucopyranoside (octyl glucoside) (cf. 14) in Tris-HCl buffer (50 mM Tris, 100 mM NaCl, 5 mM EDTA, pH 7.3) overnight at 4°C. This solution, diluted with 1 vol of the same buffer, was centrifuged for 1 h at 127,000 g. The supernatant, containing 70–80% of the protein, with a phospholipid/protein ratio of 29:1 (mol/mol), was used for reconstitution.

One aliquot, containing 2.6 mg protein, was mixed with a solution of azolectin and cholesterol (6 mg of each) already solubilized with 2 ml of the same Tris buffer containing 5% octyl glucoside. The molar ratio phospholipid/protein was then 110:1.

Dialysis of this clear solution against 7×1 liter of Tris buffer (20 mM Tris, 100 mM NaCl, 5 mM EDTA, pH 7.3) for 24 h at 4°C, yielded a whitish suspension.

### Characterization of MP26 Antiserum

The antiserum to MP26 was raised in rabbits injected with MP26 purified by chloroform-methanol extraction of U-Na membranes. 1 vol of membranes at 30 mg/ml was extracted with 20 vol of chloroform-methanol mixture (1:1) for 30 min at 4°C. After centrifugation (10 min at 27,000 g) the supernatant, enriched in MP26, was stored at –20°C.

1 vol of chloroform-methanol solution (1 mg of protein) was emulsified in an equal vol of complete Freund's adjuvant for the initial injection of the animal. 1 mo after, the same amount, in incomplete adjuvant, was given as a boost. After 6 mo of resting, a new booster injection was given, and the antiserum was collected 1 mo after.

The appropriate concentration of the antiserum was determined after ELISA test using as antigen the U-Na membranes and the chloroform-methanol extract.

### Electrophoresis and Immunoblotting

The SDS-PAGE was carried out in 12.5% polyacrylamide slab gels according to Laemmli (19). Aliquots of the different samples were solubilized in sample buffer (2% SDS, 5% 2- $\beta$  mercaptoethanol) and heated at 37°C for several minutes. After electrophoresis, the gels were stained with Coomassie Blue and/or counterstained with silver to reveal the presence of minor bands.

Immunoblotting analyses, using the anti-MP26 antiserum were performed, on Bic-plasma membranes and on reconstituted proteoliposomes.

After SDS-PAGE, the polypeptides were transferred to nitrocellulose paper by Western blotting (10). Nonfat dry milk solids (5% wt/vol) and gelatin (0.2% wt/vol) PBS were used to saturate the paper and also for antibody dilution and washing steps. Incubation with MP26 antisera diluted 1,000-fold was followed by incubation with goat anti-rabbit IgG conjugated to peroxidase (Nordic Laboratories, Tilburg, The Netherlands). The complex antibody-peroxidase was visualized with 4-chloro-1-naphthol 0.5 mg/ml in the presence of 0.1% of 30% hydrogen peroxide.

### Analytical Ultracentrifugation

Sedimentation velocity of octyl glucoside-soluble fraction was performed in an analytical ultracentrifuge (model E; Beckman Instruments, Inc., Palo Alto, CA) with photoelectric scanner at 280 nm. 1 vol of the octyl glucoside-solubilized fraction was diluted with 1 vol of Tris buffer before running. The final solution contained 0.5 mg/ml of protein and 2.5% octyl glucoside. Running was performed stabilizing rotor velocity at different speeds, from 8,000–44,000 rpm.  $S_{20,w}$  was calculated from data obtained during 1 h of centrifugation at 44,000 rpm as described by Møller et al. (25). By this technique the cell is completely filled with the sample, and the presence of monomeric/oligomeric detergent protein complexes is observed by the gradual disappearance of protein from the upper part of the sample in the cell.

### Electron Microscopy

The reconstituted material, obtained after dialysis, was rapidly frozen using either a propane jet or Freon 22. The frozen samples were transferred to the precooled stage of a Balzers BA 301 (Balzers, Liechtenstein) freeze-fracture device. They were fractured at –150°C in a vacuum of  $\sim 10^{-7}$  Torr, immediately replicated with Pt and C at angles of 45 and 90°, respectively. Experiments done using reconstituted material which had been fixed (1% glutaraldehyde in dialysis buffer) and impregnated with 30% glycerol gave similar results. Replicas were also made of U-Na membranes and of U-Na membranes treated by V8 *Staphylococcus aureus* protease.

Octyl glucoside-soluble fraction was also studied by freeze fracture according to Le Maire et al. (22). Control liposomes made of pure lipids (azolectin and cholesterol 1:1) were also studied by freeze fracture. For conventional thin sectioning, the reconstituted material was spread on glass slides, previously coated with polylysine and fixed in 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), for 30 min. The samples were then post-fixed in 1% osmium tetroxide in the same buffer for 45 min, dehydrated, and embedded in Epon/Araldite mixture.

Gold immunolabeling was done after a mild fixation (0.25% glutaraldehyde in PBS) of the reconstituted material spread on glass slides, followed by incubation in a quenching solution (50 mM, NH<sub>4</sub>Cl in PBS) for 10 min and incubation in the blocking buffer (0.2% gelatin in PBS) for 30 min. The samples were then incubated with the anti-MP26, diluted 100-fold, followed by repeated washing with gelatin-PBS, and incubation with goat-antirabbit IgG conjugated with 5-nm gold particles (Janssen Pharmaceutica, Beerse, Belgium). After immunolabeling, the material was processed for thin sections as described before.

Freeze-fracture replicas and thin sections were examined in a Philips EM 400 operating at 80 kV.

The computation of particle density and size and the estimation of the relative proportion and sizes of the different classes of reconstituted lipid vesicles and multilamellar liposomes, were performed on micrographs taken of standard magnification (20,000 or 40,000) with a coordinate analyzer and computer (Tektronix, Inc., Beaverton, OR). Computations were made on a great number of micrographs taken on at least four different replicas of each five different reconstitution experiments.

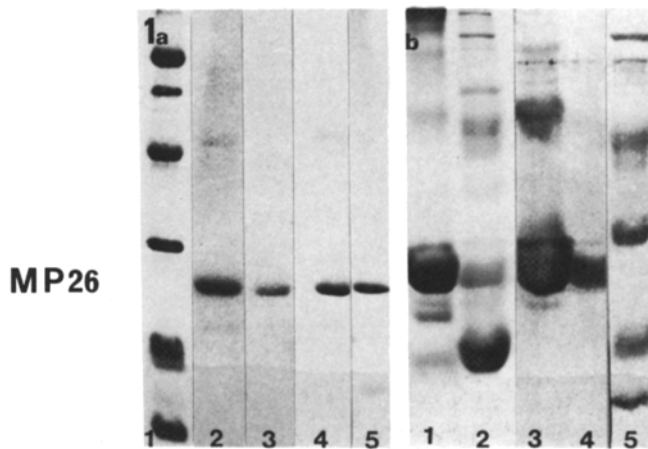
All the chemicals used for this work were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

## Results

### SDS-PAGE Experiments and Immunoblotting

In Fig. 1 *a*, we show the results obtained by SDS-PAGE on isolated plasma membrane fractions, the reconstituted proteoliposomes, the octyl glucoside-insoluble material, and the purified MP26.

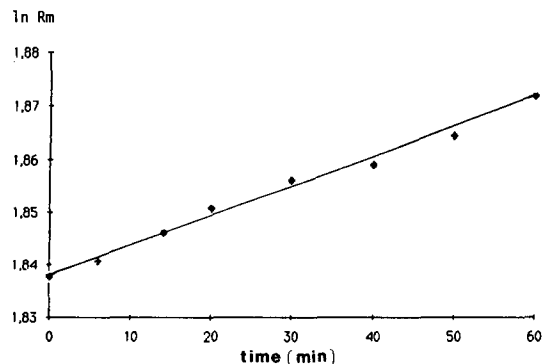
The electrophoretic pattern of U-Na membranes (Fig. 1 *a*,



**Figure 1.** SDS-PAGE protein profiles of different samples and immunoblotting of reconstituted proteoliposomes and Bic-plasma membranes probed with the antibody directed against MP26. (a) (Lane 1) Markers—phosphorylase B: 92.5 kD; BSA: 66.2 kD; ovalbumin: 45kD; carbonic anhydrase: 31 kD; soybean trypsin inhibitor: 21.5 kD; and lysozyme: 14.4 kD (BioRad Laboratories, Richmond, CA); (lane 2) U-Na membranes; (lane 3) MP26 purified by chloroform-methanol extraction; (lane 4) protein profile of the reconstituted proteoliposomes; (lane 5) octyl glucoside-insoluble pellet; in all samples the protein concentration was 50  $\mu\text{g}/\text{well}$  (b) (Lane 1) Protein profile of the reconstituted proteoliposomes (250  $\mu\text{g}$  protein/well); (lane 2) protein profile of the Bic-plasma membranes (250  $\mu\text{g}/\text{well}$ ); (lane 3) immunoblotting of the same material represented in lane 1 probed with the antibody directed against MP26; (lane 4) immunoblotting of the same material represented in lane 2 probed with the antibody directed against MP26; (lane 5) markers.

lane 2), of the reconstituted proteoliposomes (Fig. 1 a, lane 4), and of the octyl glucoside-insoluble material (Fig. 1 a, lane 5), showed that the major protein constituent of these preparations is the MP26 (cf. also Fig. 1 a, lane 3 which corresponds to the chloroform-methanol-purified MP26). Furthermore, U-Na membranes and the reconstituted proteoliposomes showed the presence of a weak band migrating in the region of 22 kD.

In Fig. 1 b, the overloaded profile of the reconstituted proteoliposomes (lane 1) showed a predominant band corresponding to MP26 and a minor constituent of  $\sim 22$  kD. High molecular mass components are also present. The electrophoretic profile of Bic-plasma membranes (Fig. 1 b, lane 2) is characterized by various protein components including MP26 and  $\alpha$ -crystallin. If we compare the electrophoretic profile of this latter membrane fraction (Fig. 1 b, lane 2) with that of reconstituted proteoliposomes (Fig. 1 b, lane 1), it is apparent that the high molecular mass components present in these two fractions are migrating at different regions of the gels. MP26, MP22, and the high molecular mass constituents of the reconstituted proteoliposomes, which have been transferred to nitrocellulose paper and probed with anti-MP26, strongly react with this monospecific antibody (Fig. 1 b, lane 3). Fig. 1 b, lane 4, which corresponds to the immunoblotting of Bic-plasma membrane constituents clearly showed that the same antibody reacts exclusively with MP26.



**Figure 2.** Analytical ultracentrifugation of the octyl glucoside-soluble fraction. Axis  $\ln R_m$  was calculated from graphs of  $A_{280}$  vs. position obtained between 0 and 1 h of centrifugation. A value of Sapp. 4.42S was obtained from the slope of  $\ln R_m$  vs. time.

### Analytical Ultracentrifugation and Electron Microscopy of the Octyl Glucoside-soluble Fraction

Sedimentation velocity by analytical ultracentrifugation allowed us to demonstrate that no large aggregates were present in the octyl glucoside-soluble fraction. We obtained this result by showing that during the run-up of the centrifuge to reach the highest speed, the total amount of soluble protein did not change, as followed by optical density measurements done before, during, and after the run-up. Furthermore, we calculated an  $\sim S_{20,w}$  of 4.6 s after a sedimentation velocity analysis of 1 h at 44,000 rpm.

By measurements performed from 0 to 1 h of run, we could establish a plot of  $\ln R_m$  vs.  $t$ , presented in Fig. 2.

An Sapp. of  $4.42 \times 10^{-13}$ S was calculated from this data.  $S_{20,w}$  was then calculated by the equation:

$$S_{20,w} = \text{Sapp.} \times \frac{\eta_{20,\text{Tris-NaCl}}}{\eta_{20,w}} \times \frac{1 - \bar{V}^* \rho_{20,w}}{1 - \bar{V}^* \rho_{20,\text{Tris-NaCl}}}$$

where  $\eta_{20,\text{Tris-NaCl}}$  and  $\rho_{20,\text{Tris-NaCl}}$  were calculated from tables (6) and  $\bar{V}^*$  was

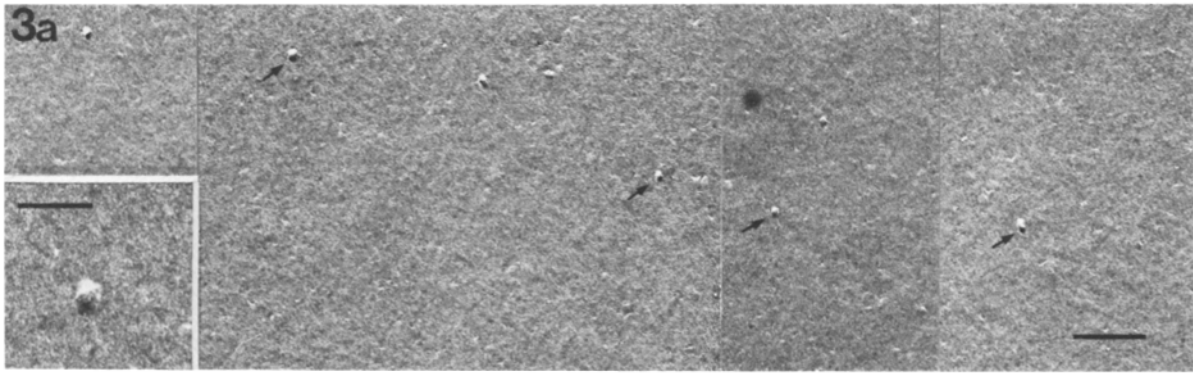
$$\frac{\bar{V} \text{ MP26} + \delta \bar{V} \text{ octyl glucoside}}{1 + \delta}$$

$\delta$  was estimated to 0.5 g/g, by comparison with other non-ionic detergents (21).  $\bar{V} \text{ MP26} = 0.735$ , was calculated from partial volumes of the amino acids composition and the sequence of the protein  $\bar{V}$  octyl glucoside is of 0.859.

It should be noted that the amount of bound detergent cannot be easily measured because of the high micellar critical concentration of the octyl glucoside, which implies a high detergent concentration in the experiments. However, detergent binding has little effect on the conversion of Sapp. to  $S_{20,w}$ . Indeed if we considered an octyl glucoside binding of 1 g/g of protein, the  $S_{20,w}$  value would change from 4.6 to 4.61S.

Finally, the profile of optical density as a function of position during sedimentation was that expected for a solution containing a single main component and a small amount of more rapidly sedimenting species.

Freeze-fracture study of the octyl glucoside-soluble fraction, which contains 70–80% of the initial U-Na membrane protein, indicates the absence of any organized structure resembling either membranes or intact junctions. The fracture



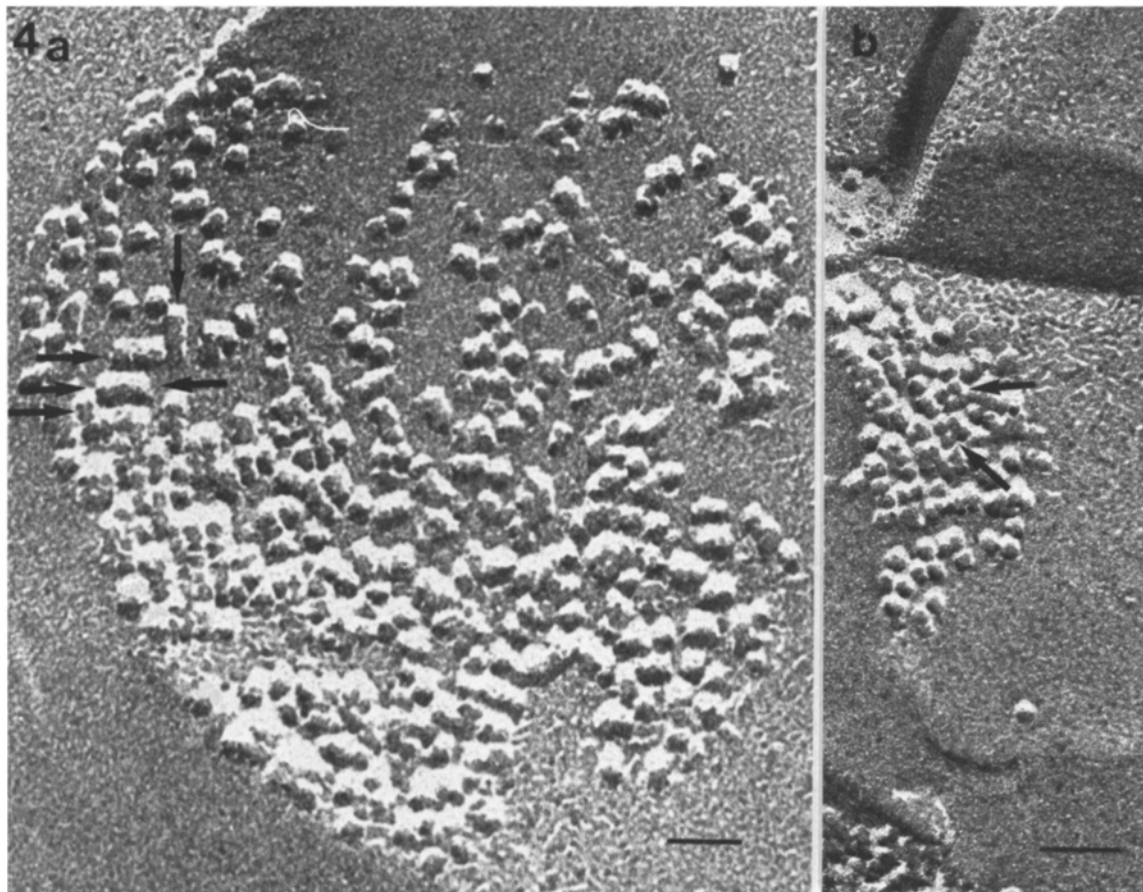
**Figure 3.** Freeze-fracture aspect of the octyl glucoside-soluble material. (a) The fracture surface is characterized by randomly dispersed particulate entities, ranging in diameter between 6 and 8 nm, and by pits. The protein concentration of the sample was 0.5 mg/ml. (Inset) High magnification of one individual 8 nm particle. Bars: (a) 100 nm; (inset) 20 nm.

face of this fraction is characterized by the presence of a few randomly dispersed particulate entities ranging in diameter from 6 to 8 nm (Fig. 3). Small pits are also visible.

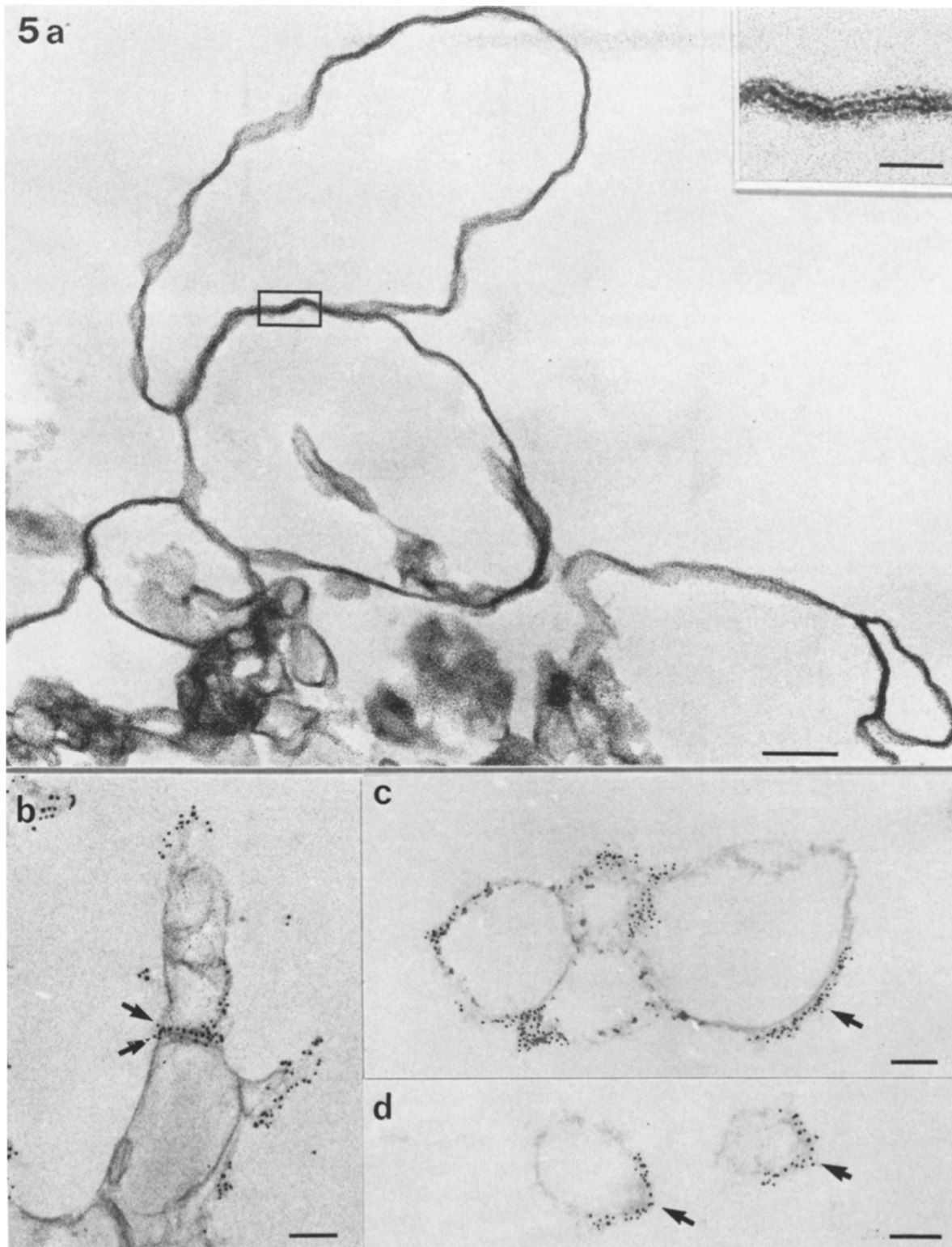
#### **Electron Microscopy of U-Na Membranes and Reconstituted Proteoliposomes**

**U-Na Membranes.** This membrane fraction is characterized on thin sections by pentalaminar profiles and single mem-

branes (not shown). Freeze fracture of this preparation shows that geometrically packed arrays of 8 nm polygonal IMPs are the predominant type of particle organization (Fig. 4 b). If U-Na membranes are further treated by V8 *Staphylococcus aureus* protease, the fracture faces are also characterized by the presence of the polygonal IMPs packed in orthorhombic array (Fig. 4 a). It is noteworthy that the SDS-PAGE of this protease-treated membrane fraction is characterized



**Figure 4.** Fracture aspect of U-Na membranes, treated with V8 *Staphylococcus aureus* protease. Note the polygonal shape of IMPs which in some regions are packed in orthogonal lattice (arrows). (b) Fracture aspect of U-Na membranes. The IMPs form geometrical packed arrays (arrows). Bars: (a) 22 nm; (b) 32 nm.

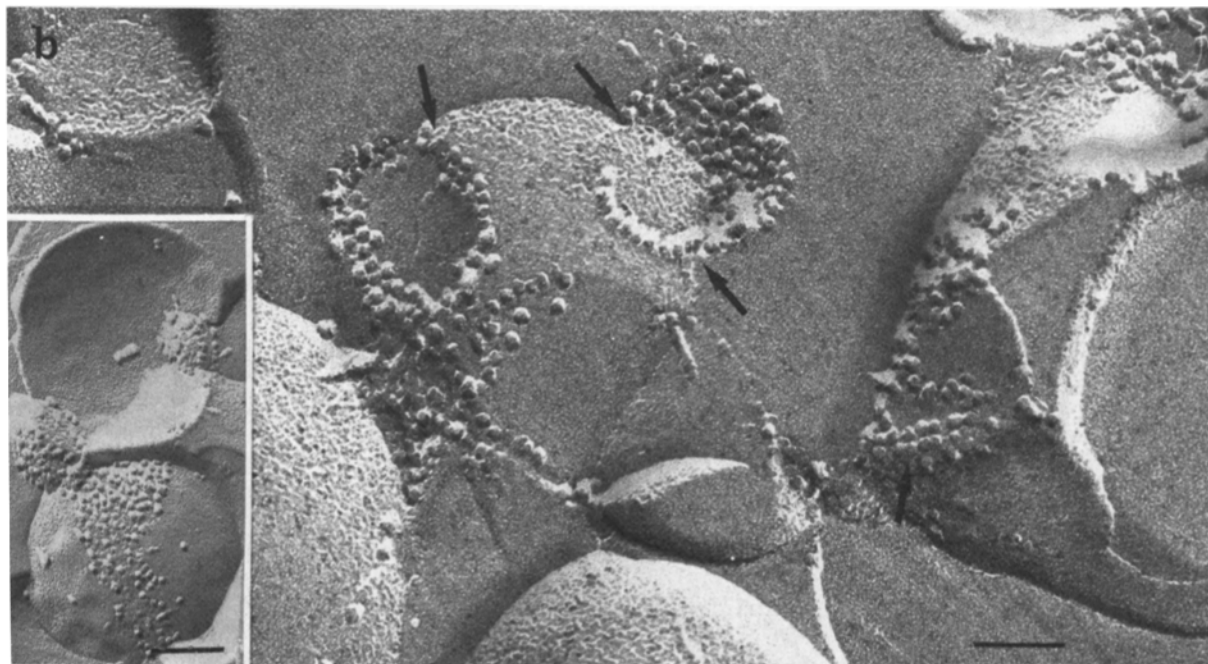
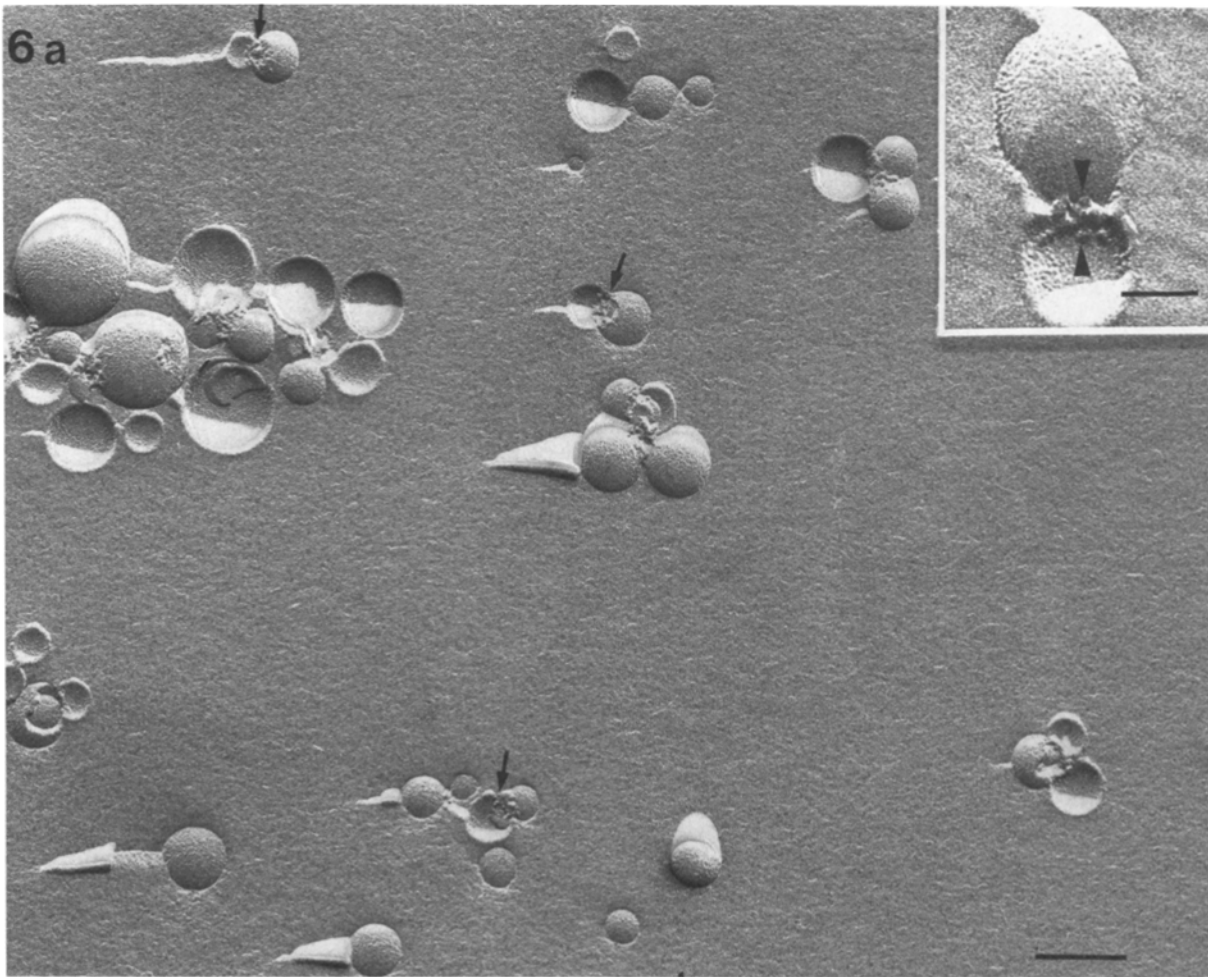


**Figure 5.** (a) Vesicular profiles of different sizes in thin sections (stained with Ur acetate and Pb citrate). Pentalaminar structures are visible in sites of vesicles in close contact. In the inset, the pentalaminar profile enclosed in the rectangle is shown at high magnification. (b–d) Immunogold labeling of the reconstituted proteoliposomes. The gold particles are either uniformly distributed at the vesicular surface or formed clusters of various dimensions (arrows in c and d). Note that MP26, labeled by the gold particles, was also accumulated in sites where two membranes were in close apposition (arrows in b). Bars: (a–d) 100 nm; (inset) 30 nm.

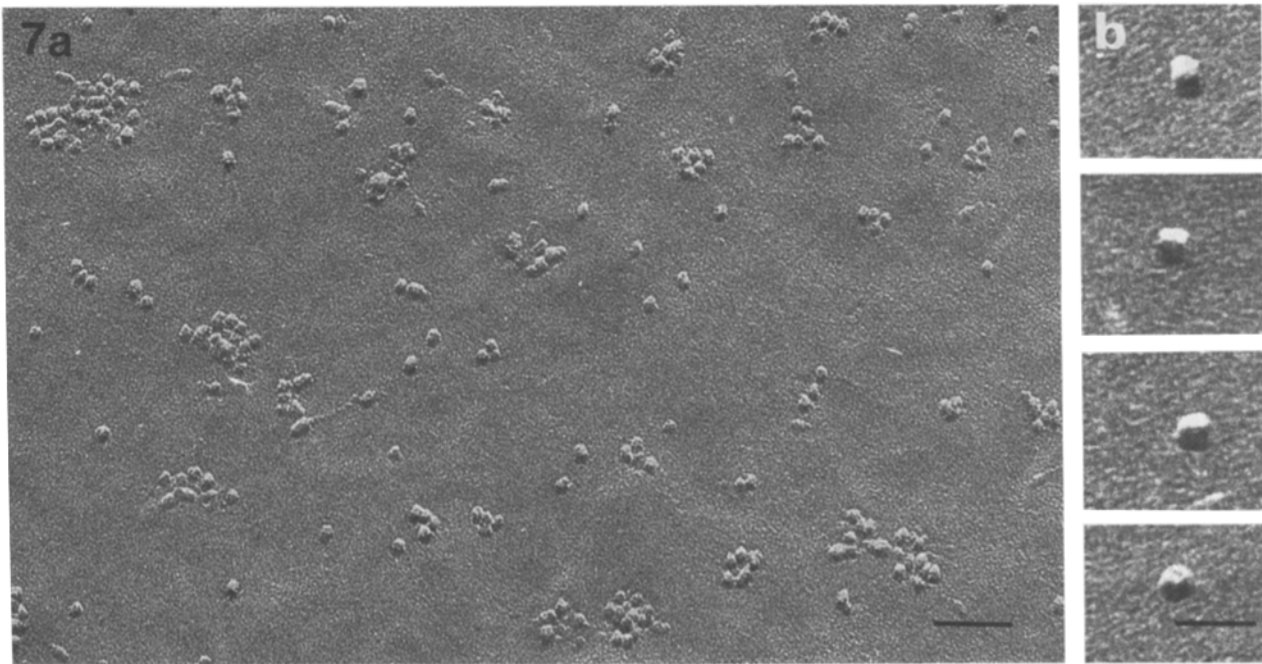
by the sole presence of MP22 (not shown, cf. 7 and Fig. 14 of 8).

**Reconstituted proteoliposomes.** (a) Thin sections of fixed

and embedded reconstituted proteoliposomes revealed the presence of unilamellar vesicular profiles of different sizes (Fig. 5) mixed together with multilamellar liposomes though



**Figure 6.** Freeze fracture of vesicular unilamellar reconstituted proteoliposomes. (a) The unilamellar vesicles aggregate into pairs and clusters (arrows). (Inset) The IMPs are localized in the site where two unilamellar vesicles are close (arrowheads). (b) Crescentlike array and clusters of IMPs are accumulated in a region of membrane-to-membrane contact (arrows). (Inset) Clusters of unilamellar vesicles displaying IMPs and pits on the fracture faces. Bars: (a) 140 nm; (inset) 50 nm; (b) 45 nm; (inset) 80 nm.



**Figure 7.** Freeze fracture of one large unilamellar proteoliposome. (a) The fracture surface is characterized by both randomly distributed isolated polygonal IMPs and particle clusters. (b) Gallery of isolated IMPs at high magnification. Note the tetragonal profile of the particles. Bars: (a) 50 nm; (b) 20 nm.

in a small proportion ( $\sim 20\%$ ). Both large and small vesicular elements revealed pentalaminar profiles in sites where membranes appeared close (Fig. 5 a, and *inset*). The immunogold labeling technique revealed that MP26 is accessible to the outer surface of the monolamellar liposomes (Fig. 5, b–d). The labeled MP26 was not evenly distributed but rather formed clusters of various extensions (Fig. 5 c, d). In particular, the exposed antigenic sites were also labeled in vesicular profiles where two membranes were in close apposition (*arrows* in Fig. 5 b).

(b) Freeze-fracture replicas of the reconstituted proteoliposomes also show the presence of a variety of vesicles having different sizes and multilamellar liposomes (Figs. 6–9).

The unilamellar vesicles, ranging in diameter between 50 and 1,000 nm, even in diluted preparations, appeared to aggregate into pairs or clusters (Fig. 6). Some were spherical; some others, especially the largest ones, were oval shaped. Both the convex- and concave-fracture faces of these unilamellar vesicles were characterized by the presence of particles which were either randomly dispersed or present as clusters without any apparent preferential partition coefficient between the two fracture faces. Complementary pits were also evident on both fracture faces (Fig. 6 b, *inset*). Clusters of particles were frequently localized in sites where two unilamellar vesicles were in close contact (Fig. 6). There the cleavage exposed convex- and concave-fracture faces of the two adjoining vesicles and the particulate entities were located where the intervesicular space seemed to be abruptly reduced (Fig. 6 a, *inset*). Fig. 7 a illustrates the presence of randomly dispersed particulate entities as well as clusters on a fracture face of a large reconstituted proteoliposome. Quantification of isolated and clustered particulate entities gave an approximate density of 350 particles per  $\mu\text{m}^2$ , where

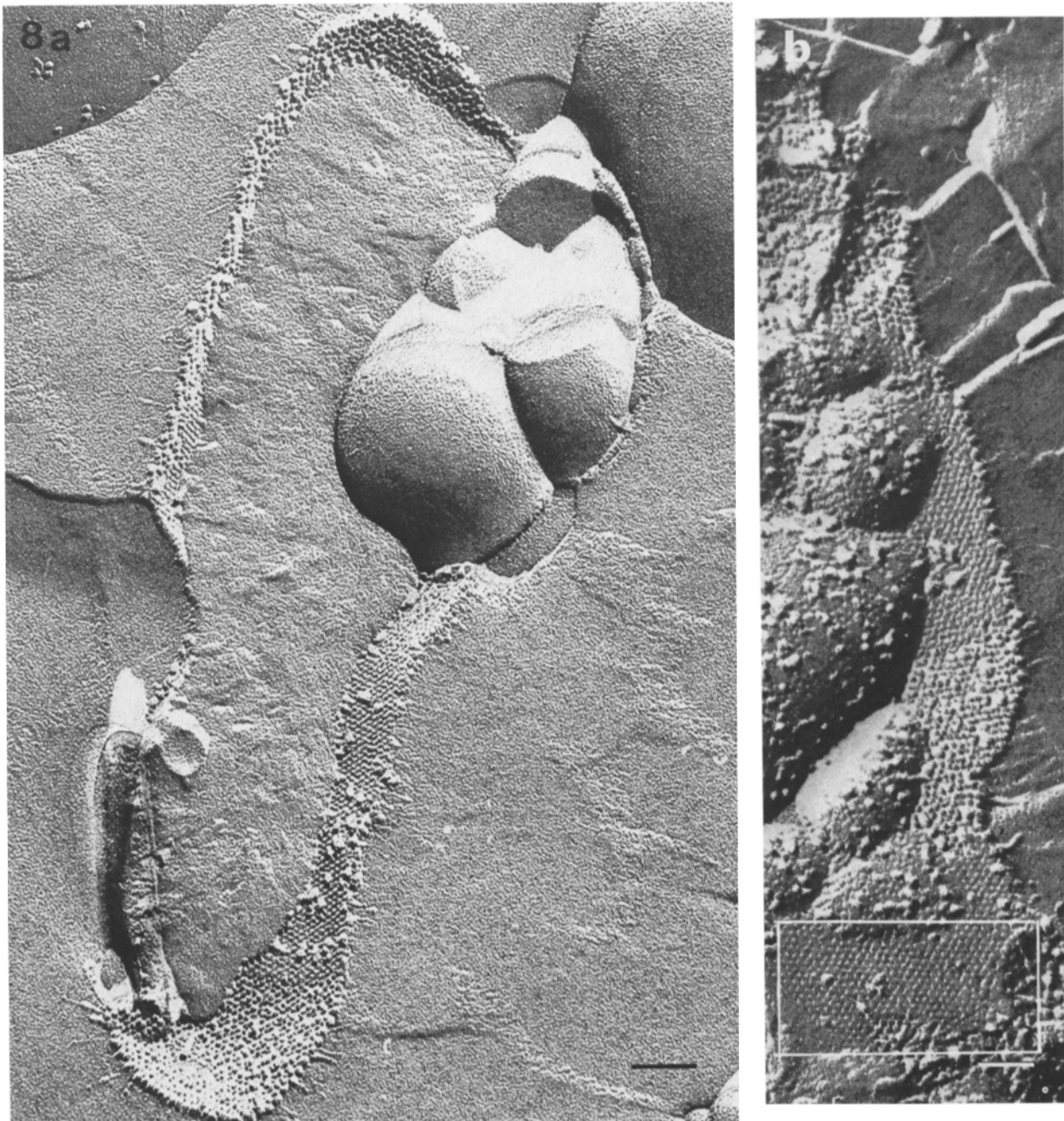
clusters (5–15 IMPs) and individual particulate entities were represented in the same proportion. The IMPs had a polygonal profile (Fig. 7 b, *inset*). Their average diameter is 8 nm.

In few monolamellar large liposomes (representing  $\sim 10\%$ ), the particulate entities were packed in geometrical lattice with center-to-center distance of 6–7 nm. The bidimensional assembly of repeating subunits covered large areas of the fracture face (Fig. 8). It is noteworthy that in any given fracture face the lattice of particulate entities was contiguous with the area where the pits also formed geometrical packed arrays (Fig. 8).

As we examine micrographs of replicas of reconstituted large monolamellar liposomes, displaying geometrical arrays of repeating subunits, it is quite difficult in some areas to determine the type of symmetry which characterized the lattice, since different regions of the fracture surfaces are tilted in varying degrees with respect to electron beam. This fact is demonstrated in Fig. 8 a where the subunit pattern showed different types of packing continuous with each other. The change of the lattice arrangement from one type to the other seems, in some areas, to follow the tilt in the orientation of the fracture face. Hence, orthogonal and rhombic patterns are apparent. The center-to-center distance of the repeating subunit pattern, of  $\sim 7$  nm, apparently does not change in parallel with the variation of the type of geometrical lattice.

On the other hand (Fig. 8 b), the hexagonal pattern of repeating subunits and pits is also apparent in fracture faces nontilted in respect to the electron beam.

The fracture aspect of the multilamellar liposomes was characterized by the presence of IMPs displaying two different types of distribution (Fig. 9). One corresponded to few randomly dispersed particulate entities and complementary



**Figure 8.** (a and b) Freeze-fracture replicas of large unilamellar proteoliposomes. Notice that particles and pits are packed into orthogonal and rhombic arrays and that the two types of packing are continuous with each other. In some regions (*rectangle* in *b*) the pits are arranged into an hexagonal pattern. Bars: (a) 65 nm; (b) 50 nm.

pits; the other was represented by geometrical assemblies of repeating subunits which interconnect the bilayers in sites where the interlamellar space was reduced. These domains, which have common features with gap junctions, represented between 5 and 10% of the total area of the fracture face of a multilamellar liposome (Fig. 9).

Pure lipid liposomes showed fracture faces void of IMPs (not shown).

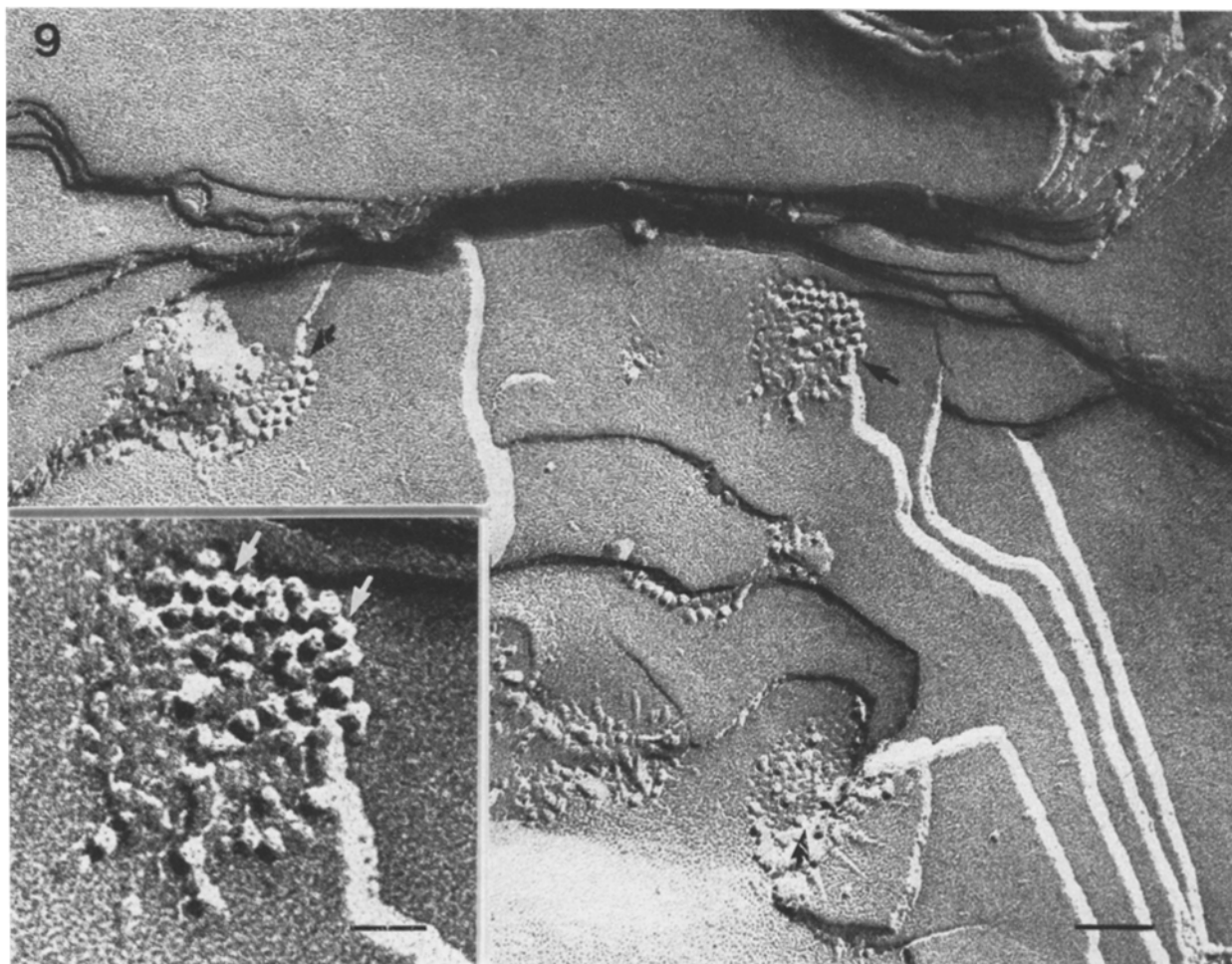
### Discussion

The data presented in this paper demonstrate that MP26,

similar to other channel-forming proteins (39), becomes incorporated into reconstituted lipid bilayers and that IMPs are found on the fracture faces of reconstituted liposomes only when MP26 becomes associated to the lipid bilayer.

Since the SDS-PAGE pattern of proteoliposomes shows that MP26 is the major protein component of the reconstituted material, we may assume that the particles found on the fracture faces are mainly composed of MP26. However, we cannot rule out that MP22, even though representing a minor constituent of the reconstituted material (10% max), may contribute to the formation of IMPs. A similar conclusion was reached by Zampighi et al. (40) in their elegant experi-





**Figure 9.** Freeze-fracture replica of multilamellar proteoliposomes. The IMPs and pits are assembled in geometrically packed arrays where the space between the bilayers is reduced (*arrows*). (*Inset*) Notice that the particulate entities have a tetragonal profile and show a central depression (*arrows*). Bars: (*a*) 50 nm; (*inset*) 20 nm.

ments showing that purified lens junctional protein (MP26 and its proteolytic derivatives) form channels in planar lipid bilayers.

The presence of higher molecular mass components than MP26 in our SDS-PAGE of proteoliposomes could account for the presence of other protein constituents. Immunoblot experiments indicated that these components are all recognized by the anti-MP26. Thus, they represent multimeric aggregates of MP26 rather than other constituents nondetectable in the starting material. The aggregation of MP26 and its degradation products probably reflects the instability of these "hydrophobic" polypeptides when extracted from their membrane environment (7, 15, 17).

The 70-kD polypeptide found by Kistler et al. (18) distributed along junctional domains in situ is probably extracted from the starting material, UNA-membranes, by the preparation procedure and appears to be absent from the electrophoretic profile of the reconstituted proteoliposomes. It has been shown that the 70-kD polypeptide represents a minor membrane constituent easily removed from the membrane by a mild detergent treatment, suggesting that this protein is more an extrinsic membrane polypeptide than a channel-forming protein (32).

In a variety of both synthetic and natural occurring lipids, particulate entities have been identified by freeze fracture (38) where nonbilayer configuration is likely to be present. These "lipidic" particles may also form geometrical assemblies, possibly in regions of membrane-to-membrane interactions. However, in our reconstitution experiments the protein nature of the IMPs either forming individual tetramers or lattice of repeating subunits, is supported by the fact that in the absence of MP26 the fracture faces of control reconstituted liposomes are void of particles.

It is likely that individual IMPs visualized on the fracture faces of reconstituted proteoliposomes contain more than one copy of MP26. The earlier observation of Segrest et al. (34) on reconstituted phospholipid bilayers with the membrane penetrating polypeptide segment of the human erythrocyte MN glycoprotein, have predicted that an 8-nm IMP would take up an area of 50 nm<sup>2</sup> and might accommodate ~25  $\alpha$ -helices. This evaluation is consistent with the more recent estimation that an 8-nm acetylcholine receptor oligomer, of five different subunits, may comprise at least 25  $\alpha$ -helices (5, 9, 30). From the analysis of MP26 sequence by hydrophobicity plots, six putative transmembrane  $\alpha$ -helices might be expected (13). A striking feature of this model is

an amphiphilic transmembrane helix which would allow MP26 copies to cluster and form a pore-like structure (32). If we assume that MP26 is the only protein component of the reconstituted liposomes, then each individual 8-nm IMP would contain no more than four MP26 copies (cf. also 32). One might expect that MP22 displaying the same transmembrane segments, and protected from further proteolysis, could, as well as MP26, make a transmembrane tetramer (see page 133 of reference 32).

It is not clearly understood whether the reconstitution of each individual MP26 oligomer relies upon the reassociation into the lipid bilayer of single copies of the polypeptide or to the existence of multimeric subunits in the octyl glucoside-soluble fraction before dialysis. The results of analytical ultracentrifugation of this soluble fraction and other experiments still in progress (Manenti, S., I. Dunia, M. Le Maire, and E. L. Benedetti, manuscript in preparation) indicate that a prevailing amount of the MP26 is in a monomeric form. However, a few multimeric subunits are revealed both by analytical ultracentrifugation and freeze-fracture experiments of the solubilized material.

The IMP distribution on either the convex- or the concave-fracture faces indicates that in the reconstituted proteoliposomes, there is little or no preferential orientation of the protein and that the insertion of MP26 and possibly MP22 occurs nonassisted by the morphopoietic factors which, during membrane assembly *in vivo*, govern the correct sidedness of the transmembrane polypeptide(s). In lens fiber plasma membranes MP26 is phosphorylated by a cyclic AMP dependent protein kinase (16, 20, 23). Likely this posttranslational event may represent an important factor for membrane domain formation favoring specific interactions between MP26 and extrinsic membrane polypeptides.

A striking observation that we describe is that an accumulation of IMPs is found in association with local membrane-to-membrane interaction between reconstituted lipid bilayers, both of unilamellar and multilamellar proteoliposomes, in sites where the space between bilayers is abruptly reduced. Furthermore, the immunogold labeling showing that MP26 is accumulated where reconstituted vesicles are in mutual contact, suggests that the inherent properties of this protein favor vertical interplay between MP26 oligomers associated with apposed reconstituted lipid bilayers. Likely this type of membrane-to-membrane contact could correspond to the pentalamellar structures visualized in thin sections of the reconstituted material.

Other interesting data concern the fracture aspect of unilamellar liposomes displaying large areas where repeating subunits are geometrically packed. These assemblies appeared in freeze fracture as contiguous lattices of particles and pits in the same fracture face. Since we were unable to find evidence for remnants of membranes and junctions in the octyl glucoside-soluble material before dialysis, we can assume that the bidimensional lattice of particulate entities reflects a process of self-assembly of MP26 subunits which occurs during the reconstitution of the proteoliposomes.

From the study of the freeze-fracture aspect of the particle lattice, various types of geometrical packing have been visualized. Likely single MP26 and MP22 oligomers have, as other channel-forming proteins, a cyclic symmetry (35, 36) and are likely formed by a ring of four subunits (cf. 29). These oligomers may accommodate in orthorhombic crystal-

lin arrangements reflecting the existence of preferential protein-to-protein interactions, when high local concentration of these polypeptides is reached. The variation of the microenvironment during the reconstitution of the proteoliposomes may also result in the formation of other types of the crystalline forms of the protein, for instance local transition from orthorhombic to hexagonal lattice. This transition may be of the type described by Michel et al. (24) during their elegant reconstitution experiment of purple membranes. The transition of one type of arrangement into the other (hexagonal to orthorhombic) does not necessarily imply a modification of the conformation of the individual protein molecules, but only a change of the bidimensional arrangement of the protein subunits in the plane of the reconstituted membranes. It is noteworthy that in the eye lens, the presence of orthorhombic lattice organization is found in parallel either with cortical plasma membrane extraction and/or proteolysis or with changes of the protein and lipid composition of plasma membranes, which are characteristic of the aging process in the lenticular nucleus (cf. 8).

Further experiments are needed to investigate the potential role of specific classes of lipids, of protease cleavage, of MP26, and of the interactions between MP26 and extrinsic lens fiber constituents, on the morphological and functional properties of the reconstituted proteoliposomes.

We are grateful to Professor H. Bloemendal, University of Nijmegen, for helpful discussion. We thank Dr. M. Le Maire, Centre de Génétique Moléculaire, Gif-sur-Yvette, for helping us with the analytical ultracentrifugation experiments and for valuable discussions. We thank Dr. R. Sealock, Department of Physiology, University of North Carolina, at Chapel Hill, for his valuable advice and suggestions during the preparation of the manuscript. The skilled technical assistance of Mr. M. Recouvreur and Mrs. M. A. Ludosky are gratefully acknowledged.

This work was supported by Institut National de la Santé et de la Recherche Médicale, France (INSERM) grant 509935 and Ministère de l'Industrie et de la Recherche grant 83V0639.

Received for publication 20 March 1987, and in revised form 27 May 1987.

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