Compaction and Particle Segregation in Myelin Membrane Arrays

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ABSTRACT Compacted membrane arrays are formed in the nerve myelin sheath by lowering the water activity (through evaporation or immersion in hypertonic solutions of nonelectrolytes or monovalent salts) or by binding specific cations (Ca^{++}, La^{+++}, and tetracaine at concentrations above 5–10 mM). X-ray diffraction observations on intact, hydrated nerves treated to induce compaction provide a control to assess the significance of structural changes seen by freeze-fracture electron microscopy. Compaction inevitably leads to lateral segregation of particles away from the closely packed membrane arrays into contiguous normal, or slightly expanded, period arrays. In the particle-enriched layers, the E fracture face is more particle-dense than the P face, whereas no particles are found on either face in the compacted layers. Morphologically, compaction induced by the all-or-nothing, relatively irreversible action of specific cations cannot be distinguished from compaction to the same extent induced by the graded, reversible effects of nonelectrolytes. Compaction by sodium chloride resembles that by specific-cation binding in that the repeat period is independent of reagent concentration; but, like dehydration by nonelectrolytes, the extent of compaction is reversibly related to reagent concentration. Sodium chloride-compacted myelin can be distinguished morphologically by a lack of the elongated border particles at the boundary between smooth and particle-enriched membrane observed for other compacting treatments. Fracture faces in compacted arrays are not always smooth, but the unusual appearances can be duplicated in purified myelin lipid multilayers subjected to similar treatments, which indicates that the particle-free membrane fracture faces are uninterrupted lipid hydrocarbon layers. Correlation of x-ray diffraction and electron microscopy observations provides a direct basis for identifying the intramembrane particles with transmembrane protein. The transmembrane protein appears to play a significant role in maintaining the normal membrane separation; swelling of the particle-enriched arrays in myelin compacted by tetracaine at low ionic strength provides information about the charge distribution on the transmembrane protein. Swelling of the compacted arrays following irreversible particle segregation shows that the interaction properties of the particle-free membranes are similar to those of pure lipid multilayers. Compaction and the consequent particle segregation in myelin results from conditions stabilizing close apposition of the lipid bilayers. Particle segregation in areas of close contact between other cell membranes may also be driven by interbilayer attractive forces.

The crystal-like array of membranes in the nerve myelin sheath can be compacted or swollen by controlled changes in the environment. Compaction occurs when the water activity is lowered (7, 8, 10, 14, 16, 32) or when the concentration of specific cations is raised above a threshold level (23, 38); swelling results when the ionic strength is reduced (11, 31, 37).

The normal repeat period measured by x-ray diffraction for the membrane pair in intact mammalian peripheral nerves is

~180 Å (2, 8). At low ionic strength, the repeat period increases, reaching 250–300 Å in water (11, 37). In myelin compacted by dehydration, repeat periods in the range 118–130 Å have been measured (7, 10, 14, 16, 32); the smaller values correspond to lower water activity. The 126-Å repeat period of calcium- or tetracaine-compacted myelin is independent of the reagent concentration above a critical threshold level (23).

When myelin is compacted by any treatment, the diffraction patterns usually show reflections from at least one other array having a normal or slightly expanded period (7, 10, 14, 16, 23). Electron micrographs of sectioned tissue show continuity between normal and compacted period domains (8, 10, 14). Some morphological features seen in thin sections may, however, be misleading because conventional fixation and embedding procedures for microscopy introduce substantial shrinkage, disorder, and distortion of contrast (10, 19, 24).

Replicas of fresh-frozen, untreated myelin show that intramembrane particles are randomly dispersed on the bilayer fracture faces (20, 28). After standard preparative procedures for freeze-fracture (glutaraldehyde fixation and/or glycerol impregnation), the particles form a network surrounding particle-free membrane zones (28). We have previously observed that membrane compaction induced by dimethylsulfoxide (DMSO), glycerol, or calcium leads to the lateral displacement of particles from closely packed arrays into particle-enriched domains having a near-to-normal repeat period (1, 20, 23).

Comparison of x-ray diffraction patterns from nerves at room temperature and at liquid nitrogen temperature show that, with cryoprotection, it is possible to preserve, with only small changes, the myelin structure that exists at room temperature (20). Treatments that lower water activity protect the tissue from ice damage; thus, freeze-fracture can be used to study the effects of lowering the water activity in myelin without additional treatment to preserve the structure; and x-ray diffraction measurements on the intact treated nerves provide a control to assess the significance of structural changes seen in the replicas of freeze-fractured specimens.

We have used information from x-ray diffraction to design electron microscopy experiments to explore the following questions. Is membrane compaction always correlated with lateral segregation of particles? Can compacted myelin membrane arrays produced by different treatments be morphologically distinguished? How similar are the hydrocarbon layers of compacted myelin membranes and purified myelin lipid multilayers? Do the intramembrane particles represent a protein component that is excluded from the compacted arrays? Can compaction and particle segregation be manipulated to characterize distinctive properties of the membrane bilayer and intramembrane-proteins? Correlation of myelin compaction with pure lipid bilayer interactions provides information about interbilayer force that must act in all close membrane contacts.

MATERIALS AND METHODS

Sciatic nerves were dissected from ether-intoxicated adult albino mice. In some cases optic nerve or brain white matter was also taken.

Specimen Preparation

UNTREATED CONTROLS: Whole nerves were immersed in buffered Ringer's solution (145 mM NaCl, 6 mM KCl, 2 mM CaCl₂, and 2 mM MgCl₂ buffered to pH 7.4 with 1.5 mM NaHCO₃/NaH₂PO₄ buffer) for periods ranging from 2 to 24 h.

DEHYDRATION BY EVAPORATION: Pieces of nerve mounted on cardboard disks were placed in a desiccating jar and equilibrated for 24 h against the vapor of sucrose solutions having water activities of 0.98, 0.95, or 0.90. Some

samples which had been at 0.90 water activity for 24 h were equilibrated an additional 24 h at 0.99 water activity.

AIR-DRYING AND REHYDRATION: Nerves were air-dried at ambient relative humidity (~0.4–0.5 water activity) for 12 h and rehydrated in buffered Ringer's solution for 12 h. Samples were then immersed for 2 h in either 20% glycerol in Ringer's or 20% DMSO in Ringer's.

POLYETHYLENE GLYCOL: Nerves were immersed in 15% polyethylene glycol (PEG) 6000 in buffered Ringer's solution for 2 h.

DMSO: Nerves were immersed either in 8, 15, 20, or 45% DMSO in buffered Ringer's solution for 2 h or in 45% DMSO in Ringer's for 8 h. Some samples that had been in 45% DMSO for 2 h were transferred to 15% DMSO for a further 2 h.

DIMETHYL FORMAMIDE (DMF): Nerves were immersed in 20% DMF in buffered Ringer's solution for 2 h.

ACETONE: Nerves were immersed in 15, 50, or 75% acetone in buffered Ringer's solution at 0°C for 4 h or a 50% solution at room temperature for 4 h.

GLYCEROL: Nerves were immersed in 20% glycerol in buffered Ringer's solution for either 15 min or 2–3 h.

GLUCOSE: Nerves were immersed in 20% glucose in buffered Ringer's solution for 2 or 24 h.

SODIUM CHLORIDE: Nerves were immersed in 0.6, 1.7, or 5.0 M NaCl in buffered Ringer's solution for 2 h.

CALCIUM CHLORIDE: Nerves were immersed in 33 mM CaCl₂ plus 8% DMSO in Ringer's for 5 h, in 33 mM CaCl₂ in Ringer's at 37°C for 3.5 h, or in 33 mM CaCl₂ in Ringer's overnight. Samples from the last two treatments were immersed a further 2 h in 20% glycerol plus 33 mM CaCl₂ in Ringer's.

LANTHANUM NITRATE: Nerves were immersed in 10 mM La(NO₃)₃ in buffered Ringer's solution for 3.5 h followed by 2 h in the same solution plus 20% glycerol.

TETRACAINE: Nerves were immersed in 20 mM tetracaine in buffered Ringer's solution for 10 min or 3.5 h. Some samples were immersed an additional 2 h with 20% glycerol added to the solution. Other nerves were immersed in 20 mM tetracaine plus 20% glycerol in buffered Ringer's solution for 30 min or 3.5 h.

GLUTARALDEHYDE FIXATION: Nerves were fixed by immersion for 30 min in 2.5% glutaraldehyde buffered with cacodylate to pH 7.2, then rinsed in the buffer for 30 min. Some of this tissue was transferred to 20% glycerol in cacodylate buffer for 2 h. Some nerves were fixed by perfusion through the heart before further immersion in the fixative.

OSMIUM TETROXIDE FIXATION (OsO₄): Nerves were fixed by immersion in 1% OsO₄ in cacodylate buffer for 2 h, then rinsed in the buffer for 30 min and transferred to 20% glycerol in cacodylate buffer for 1 h.

SWELLING: Nerves were immersed in 20% glycerol in water for 24 h.

SWELLING WITH TETRACAINE: Nerves were immersed in 20 mM tetracaine in buffered Ringer's solution for 2 h followed by 2 h in 20 mM tetracaine plus 20% glycerol in water.

SWELLING AFTER COMPACTION AND FIXATION: Nerves were immersed in 0.6 M NaCl in Ringer's for 1 h. Glutaraldehyde was added to make up 3% of the solution and left for 30 min. The nerves were then transferred to 20% glycerol in water for 3 h.

Freeze-fracture Electron Microscopy

After the treatments, small pieces of nerve were mounted on cardboard disks, frozen at liquid nitrogen temperature in a slush of Freon 22, and stored in liquid nitrogen. Samples were fractured and immediately replicated with platinum and carbon at −115°C in a Balzers BAF301 (Balzers Corp., Nashua, N. H.). After overnight storage in methanol, the replicas were cleaned in Clorox and mounted on uncoated grids. All observations were made on a Philips 301 electron microscope. Freeze-fracture nomenclature is according to Branton et al. (3).

Thin-section Electron Microscopy

Sciatic nerves that were air-dried and rehydrated, or treated with PEG, NaCl, CaCl₂, acetone, glucose, or tetracaine, or immersed only in buffered Ringer's solution as a control were transferred for 1 h to a similar solution containing, in addition, 1% OsO₄. Several samples were fixed only with 2.5% glutaraldehyde or with 2.5% glutaraldehyde, followed by 1% OsO₄. After a brief rinse in the buffer, the samples were dehydrated through a graded series of alcohol to propylene oxide and embedded in Epon. Thin sections cut with a diamond knife were stained with uranyl acetate and lead citrate.

Freeze-fracture of Purified Myelin Lipid Multilayers

Myelin membranes were purified and the lipids extracted as previously described (23). Multilayers of myelin lipids from adult mouse brain were formed

on gold disks by a method developed by Franks (12). Some disks were frozen directly; others were immersed in 50% acetone in buffered Ringer's at 0°C for 4 h; a drop of 20% DMSO was placed on others which were allowed to sit in a closed container for 2 h before freezing. After fracturing, as described above, replicas were cleaned in chloroform before mounting on grids.

X-ray Diffraction

After the treatments listed in Table I, nerves were mounted in quartz capillaries. Diffraction patterns were obtained at room temperature using a single focusing mirror camera with the line source of a Philips fine-focus x-ray tube and recorded on Ilford Industrial-G x-ray film. Diffraction patterns were recorded in 15–30 min, and successive short exposures were used to establish the kinetics of compaction.

RESULTS

Compaction and Particle Segregation

Cross-fractured replicas or thin sections of myelin compacted by different treatments show that compacted membrane domains are always continuous with normal or expanded period domains (Fig. 1). It is clear in oblique fractures that membrane compaction is always coupled with particle segregation (Fig. 2). The membrane faces in compacted domains have no detectable intramembrane particles, whereas those in the normal or slightly expanded domains are enriched with particles. Seen in cross fracture, the particle-rich membranes in the arrays with a near-to-normal period of ~180 Å have a rough appearance. The particle-free membranes that are closely packed with a period of ~120–135 Å appear very smooth. Compacted do-

mains sometimes extend across the entire width of the myelin sheath, but more commonly are a half to two-thirds of the sheath thickness. Seen in cross fracture and in thin sections, the junction between compacted and native period domains is staggered in a stepwise fashion and frequently forms an arrowhead shape (Fig. 1). The smooth regions in the fracture faces are seen to be finite patches (which often have an oval shape) bounded by a network of rough-surfaced membrane (Fig. 3). In three dimensions, the compacted domains most often have a lenticular shape. With all of the treatments that lower water activity, the extensive compaction leads to puckering of the myelin sheath; the continuity of the spirally wrapped membrane pair is not, in general, disrupted. Control nerves bathed in buffered Ringer's solution for 2–24 h show no compaction by x-ray diffraction and no particle segregation by freeze-fracture. Nerves treated to induce compaction that are fixed only in glutaraldehyde and then processed for thin sectioning show large empty holes where compacted arrays would have been expected, but the particle-containing arrays are preserved. When osmium tetroxide is used as a fixative (alone, or following glutaraldehyde), both the compacted and uncompacted membranes survive processing for thin sectioning (Fig. 1 b).

Cryoprotection

The membrane fracture faces of fresh-frozen myelin are unevenly distorted as a result of ice damage (20, 28). If the nerves are fixed with glutaraldehyde before freezing, preser-
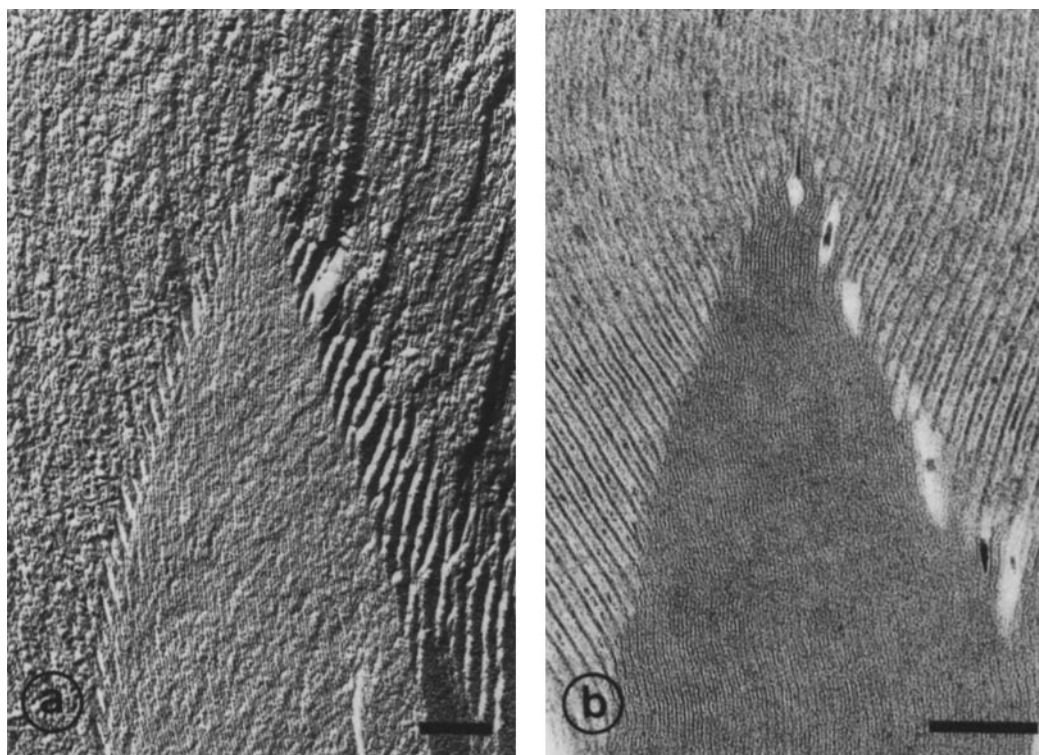

FIGURE 1 In transverse views of the myelin sheath treated to induce membrane compaction, the closely packed layers are continuous with layers arrayed with the normal or a slightly expanded period. The frozen, compacted arrays fracture smoothly, whereas the normal period arrays have a rough appearance and particles can be seen between lamellae. The boundary between domains is staggered in a stepwise fashion from layer to layer and frequently forms the arrowhead shape seen here. (a) Treated with 20% DMSO for 2 h before freezing. The period of the smooth compacted array is ~125 Å, whereas that of the rough particle-containing array is ~190 Å. The corresponding periods measured by x-ray diffraction on a similarly treated frozen nerve are 126 and 188 Å. × 96,000. (b) Treated with 1.7 M NaCl for 2 h before osmium tetroxide fixation and Epon embedding. The periods of the two domains are about 80 and 150 Å. The shrinkage is due to the fixation and embedding procedures, because the periods measured by x-ray diffraction on an intact nerve treated with the same hypertonic saline solution are 130 and 210 Å. Bar, 0.1 μm. × 145,000.
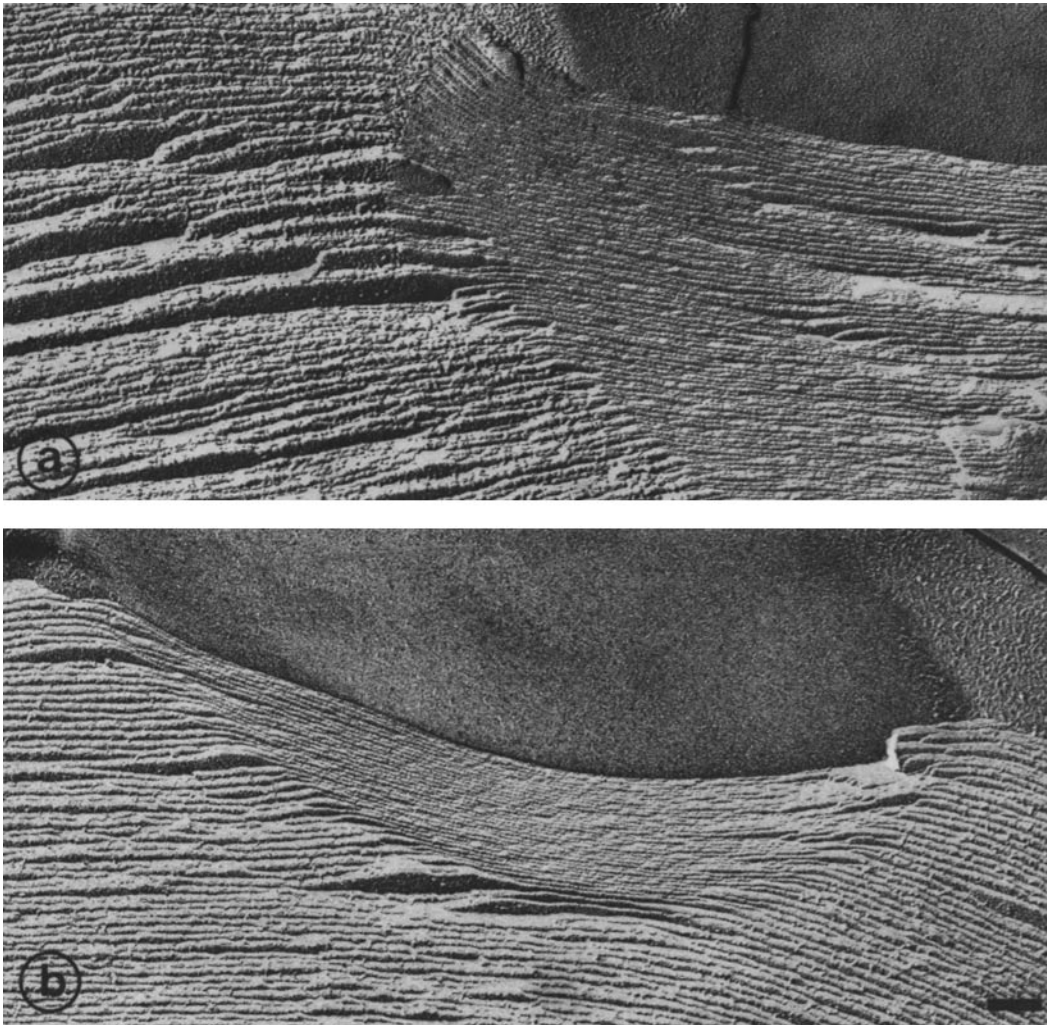
FIGURE 2   Oblique fractures of the myelin sheath clearly show that membrane compaction is coupled to the segregation of intramembrane particles out of the closely packed layers. (a) Treated with 20% DMSO for 2 h. (b) Treated with 0.6 M NaCl for 2 h. Bar, 0.1 μm. × 70,000.

vation is better, but some ice damage can still be seen, particularly in the particle-free regions (Fig. 3 a). Addition of 8% DMSO is not sufficient to protect the nerve, although it is at the threshold level for detecting membrane compaction by x-ray diffraction (20). Specimens compacted by all other dehydrating treatments at the levels we have studied do not show damage from freezing. The inherent cryoprotection offered by these dehydrating conditions allows direct study of a particular treatment without the complications induced by adding glycerol or other cryoprotective agents. Compaction by calcium and tetracaine was examined with and without cryoprotection by glycerol (23). The size and shape of the compacted domains induced by divalent cations appear similar whether or not glycerol is added. The ice damage in divalent cation-compacted specimens without added glycerol occurs mainly in the particle-enriched domains (see Fig. 3 a in reference 23).

Repeat Periods and Extent of Compaction

Table I summarizes the repeat spacings for the myelin membrane pair found by x-ray diffraction and electron microscopy for nerves after corresponding treatments. The x-ray diffraction pattern is the summation from all the myelinated fibers in a whole nerve. For each treatment listed, the x-ray diffraction measurement of the compacted repeat period is reproducible to ~2 Å among different specimens, whereas the period of the particle-enriched phase may vary over a range of ~10 Å. The optical diffraction measurements are selective, each taken from a single membrane array. They are, on the whole, consistent with the x-ray diffraction measurements, as well as being self-consistent where more extensive measurements have been made.

With solutions of nonelectrolytes, such as DMSO, there is a progressive decrease in the repeat period of the compacted arrays from ~130 to 120 Å as the concentration of solute is increased from 10 to 50% (16). The proportion of membrane compacted increases as the water activity is decreased (or as the concentration of the solute lowering the water activity is increased). With 8% DMSO, very small patches of smooth, compacted membrane can be found. Larger smooth domains comprising about half of the total membrane surface area are observed after treatment with nonelectrolytes at concentrations of 15–20% and water vapor at a partial pressure of 0.95–0.98. At the extreme conditions of 45% DMSO, 0.90 water vapor activity, and 50% acetone, ≥80% of the fracture face is smooth and the particles are concentrated into narrow bands which
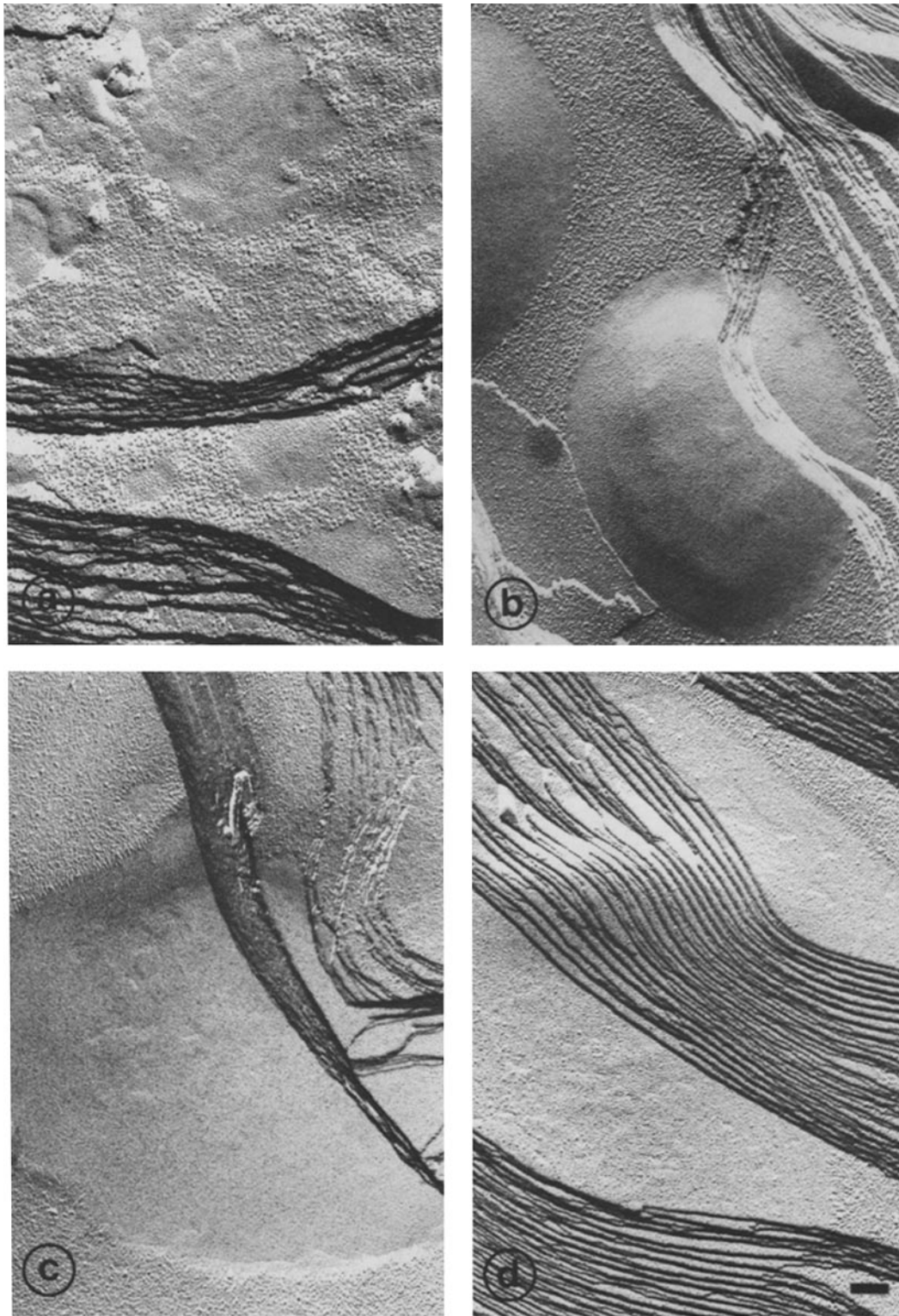
FIGURE 3 Particle segregation is induced by a variety of treatments. These oblique views show that the compacted domains of the myelin sheath consist of stacks of particle-free membrane patches. The compacted domains often have an oval outline. Damage from ice-crystal formation is present in the specimen fixed in glutaraldehyde (a), but the other compacting treatments (b, c, and d), which act by lowering the water activity, are cryoprotective. (a) Treated with 2.5% glutaraldehyde for 30 min. (b) Treated with 5.0 M NaCl for 3 h. (c) Treated with 15% PEG 6000 for 2 h. (d) Treated with 20% glucose for 2 h. Bar, 0.1 μm. × 61,000.

extend radially across the sheath (Fig. 4a, c, and d). The particles appear to have coalesced into rather large, irregular lumps. The 110-Å period array that appears after air-drying is probably a separated lipid phase rather than another form of compacted myelin.

The compacted phase obtained with >0.5 M NaCl solutions has a period of ~130 Å, independent of concentration. The repeat period of the noncompacted myelin, however, expands

TABLE I

Repeat Periods of the Membrane Pair Measured by X-ray Diffraction and Electron Microscopy

Treatment	Time	X-ray diffraction of intact nerve	Optical diffraction of cross-fractured sheath
		Å	Å
Untreated		177	--- ‡
20% DMSO/Ringer's	2 h	177, 121*	183, 120
20% DMF/Ringer's	40 min	199, 124*	---, 121
15% PEG/Ringer's	2 h	180*, 128	221, 127
20% glycerol/Ringer's	5–30 min	190*, 130	179, 125
20% glycerol/Ringer's	2–3 h	178	192 ‡
20% glucose/Ringer's	2 h	178*, 128	---, ---
20% glucose/Ringer's	24 h	178	185 ‡
0.6 M NaCl/Ringer's	2 h	188*, 129	190, 124
1.7 M NaCl/Ringer's	2 h	210, 131*	200, 118
5.0 M NaCl/Ringer's	2 h	219, 131*	221, 145
Dehydration at 0.95 water activity	24 h	224, 120*	201, 138
Air dried	12 h	152*, 129**, 110	---, ---
Air dried: rehydrated in Ringer's	12, +12 h	192	No EM
Air dried: rehydrated: 20% DMSO/Ringer's	12, +12, +2 h	124*, 110	---, 117
Air dried: rehydrated: 20% glycerol/Ringer's	12, +12, +2 h	191	---, ---
33 mM CaCl₂/8% DMSO/Ringer's	5 h	178*, 126	190, 145
33 mM CaCl₂/Ringer's at 37°C [+20% glycerol]	3.5 h	178*, 126	185, 141
33 mM CaCl₂/Ringer's [+20% glycerol]	18 h	175*, 126*	193, 130
10 mM lanthanum nitrate/Ringer's [+20% glycerol]	3.5 h	175**, 161*, 128	197, 130
20 mM tetracaine/Ringer's	3.5 h	238, 126	---, 113
20 mM tetracaine/20% glycerol/Ringer's	3 h	183, 126*	201, 130
2.5% Glutaraldehyde/cacodylate	30 min	178	180, ---
0.6 M NaCl/Ringer's: 2.5% glutaraldehyde/0.6 M NaCl/Ringer's	3 h, +2 h	178*, 125	No EM
0.6 M NaCl/Ringer's: 2.5% glutaraldehyde; 20% glycerol/H₂O	3, +2, +3 h	178	184, ---

The x-ray diffraction measurements are given from a single pattern for each experimental condition. The uncertainty in the repeat period measurement of the ordered arrays observed in a given specimen is <1%. (Among similarly treated nerves, the period of uncompacted arrays may vary by ≥5%, but the period for compacted arrays is relatively invariant.) The relative amount of membrane lipid in the compacted and uncompacted arrays is indicated by the relative intensity of their diffraction patterns. The asterisk (*) indicates the more intense diffraction pattern. Where both patterns have similar intensity, both are marked with an (*), and when three ordered arrays are evident, the most intense pattern is marked double asterisk (**) and the next (*).

The repeat periods of cross-fractured membrane arrays were measured from optical diffraction patterns taken from selected areas of electron microscope negatives. The uncertainty of these measurements is in the range of 5–10% and can be attributed to distortions resulting from the freezing and replicating procedures, obliqueness of the cross fracture, and variations in magnification of the microscope. The first column for the electron microscopic (EM) measurements is the repeat period measured for particle-containing arrays and the second column is the repeat period measured for particle-free arrays. The dagger (‡) indicates the absence of particle-free arrays. The dashes (---) indicate that micrographs of cross fractures of the specified arrays were not suitable for quantitative measurement. The addition of glycerol to the freeze-fracture specimen is noted in brackets.

gradually as a function of salt concentration. The extent of compaction increases with increasing salt concentration, showing the same sort of concentration dependence as with non-electrolytes. At 1.7 M NaCl, about half of the surface is smooth membrane, whereas 5.0 M NaCl is similar to the more extreme dehydrating conditions.

The specific cations, calcium, lanthanum, and tetracaine, have an all-or-nothing effect. Both the compacted repeat period of 126 Å and the extent of compaction at equilibrium are independent of concentration above a threshold of 5–10 mM (23). Smooth membrane comprises somewhat more than half of the surface, and the particle segregation is never so extensive as it is in extremely dehydrating conditions.

Kinetics

X-ray diffraction shows that the structural changes induced by most conditions that lower the water activity are complete after 2–3 h. This long time is due to permeability barriers in the tissue (such as the perineurium), because dissected single fibers observed with a light microscope are transformed in minutes. Because glycerol and glucose cause a transient compaction, specimens were studied at different intervals to sample both the compaction and its relaxation. With glycerol, the reexpansion to a normal repeat period is complete by 3 h (20),

whereas, with glucose, reversal of the compaction takes nearly 24 h. Water leaves the structure more rapidly than these agents penetrate it, causing a temporary dehydration. Both glycerol and glucose appear to interact like water with the membrane components. Once inside the sheath, these hydrophilic reagents must substitute for the water because the particles are again evenly dispersed in the normally arrayed membrane layers. The compacting action of calcium is slow, not reaching completion at room temperature for 18–24 h (23). Tetracaine, in contrast, acts quickly, inducing compaction and extensive particle segregation after only 10 min. The neutral tetracaine molecule, which is in equilibrium with the protonated form, readily penetrates membrane hydrocarbon layers that are permeability barriers for ions.

Reversibility

Particle segregation induced by glutaraldehyde and osmium tetroxide fixation is, as expected, irreversible. Treatment with di- and trivalent cations and tetracaine also leads to irreversible compaction and particle segregation, but limited reversal is observed if the reagent is removed during the early stages of action. Glucose and glycerol induce a transient particle segregation that reverses over time. With most other dehydrating treatments, the particles can be redispersed as the compaction

is reversed by increasing the water activity (Fig. 4 a and b). Only after extreme conditions (such as air drying, 24 h at 0.90 water vapor activity, or treatment with 50% acetone) is particle segregation irreversible (Fig. 4 c and d). In thin sections from these extreme treatments, as well as from protracted calcium treatments, the main period and intraperiod lines become indistinguishable in many of the compacted membrane arrays.

Compaction of Central Nervous System Myelin

Observations made on optic nerve and brain white matter
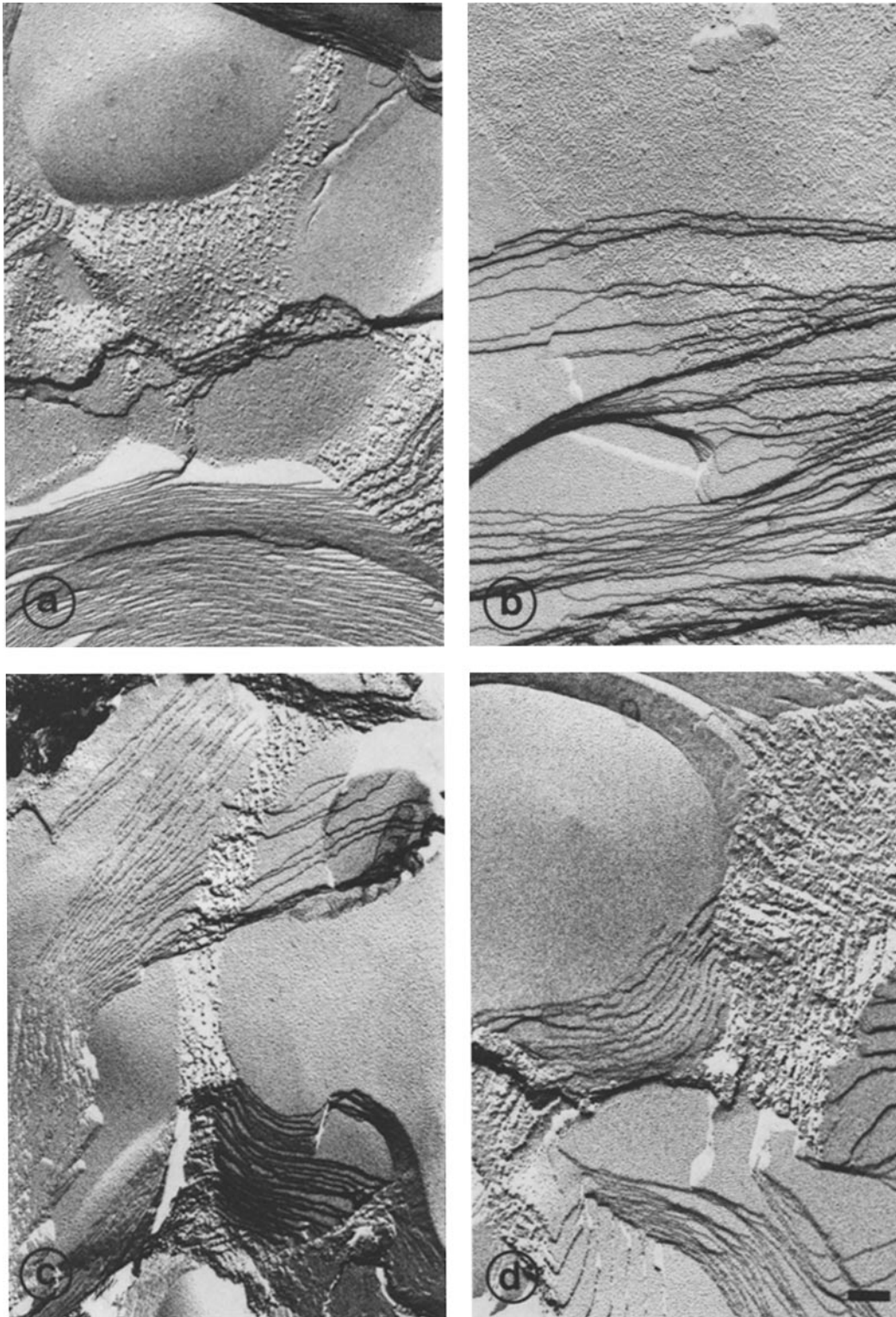
FIGURE 4   Segregation of particles can be reversed in some cases. Particle-rich domains at very low water activity are reduced to narrow bands extending radially across the sheath, and the particles are large and irregular. (a) Treated with 45% DMSO for 8 h. (b) Treated with 50% DMSO for 24 h, followed by 15% DMSO for 2 h. The myelin has resumed the appearance typical of low concentration DMSO treatments (cf. Figs. 2 a and 7 a). (c) Dehydrated by vapor diffusion at 0.90 water activity for 24 h. (d) Dehydrated by vapor diffusion at 0.90 water activity for 24 h, followed by rehydration at 0.99 water activity for 24 h. Under these conditions the segregation of particles into tightly packed zones is not reversed. Bar, 0.1 μm. × 61,000.

(Fig. 5), though not so extensive as those on sciatic nerve, indicate that central nervous system (CNS) tissue is affected in the same way as the peripheral nervous system (PNS). In fresh, untreated tissue, particles are evenly dispersed on the membrane faces, except for those particles that make up the long chains of the radial component (6, 34). Globular, 80–100-Å particles are seen more frequently than in PNS myelin. Brief exposure to glycerol induces membrane compaction and particle segregation, which reverses when equilibrium is reached after 2–3 h of treatment. DMSO-induced compaction reaches completion after 1–2 h, and the proportion of compacted membrane increases with DMSO concentration in the range of 10–50% (16). Because of the small number of lamellae involved in CNS myelin, the shape of the compacted domains is not so clear as in PNS myelin. Smooth patches are not always completely bounded by rough-surfaced membrane but may fuse together, leaving an angular domain of particle-rich membrane (Fig. 5 b).

P and E Fracture-face Identification

The repeating unit in myelin consists of a pair of membranes that are inverted with respect to each other, which means that the fracture faces alternate, EF to PF, across the sheath. Although all fracture faces in the smooth membrane arrays appear identical, the rough membranes show a clear alternation of layers. The layer with a higher density of particles exhibits a heterogeneous population ranging from low ridgelike projections to large globular particles of 80–100-Å Diam. The large particles are never seen on the alternate particle-sparse layer which is only slightly rougher than the smooth membrane fracture faces in compacted myelin.

We have used swollen myelin to identify which of these faces is the EF and which the PF. When sciatic nerves are swollen in <0.1 M salt solutions, the membranes separate in pairs, the separation occurring at the external boundary (31). When hypoionically swollen sciatic myelin is freeze-fractured, it is clear that the particle-dense layer is immediately adjacent to the swollen external boundary and is therefore the EF face of one membrane, whereas the particle-scarce face is the PF face on the protoplasmic side of the second membrane in the pair (Fig. 6).

Domain Boundaries

The junction between smooth and rough membrane is marked on the E fracture face by a fringe of elongated particles oriented perpendicular to the border (Fig. 7 a). Border particles are never found at the boundary on the P fracture face. These particles are seen after all the conditions we studied, except for treatment with sodium chloride (Fig. 7 b) or chemical fixation (Fig. 3 a). If treatment with sodium chloride is followed by DMSO, border particles appear. Glutaraldehyde inhibits their appearance even when applied in the presence of DMSO (Fig. 7 c) or polyethylene glycol or followed by glycerol. In CNS myelin, border particles are less obvious, but they have been observed.

Comparison of Bilayer Fracture Faces

In most cases, the particle-free fracture faces of compacted membrane appear very smooth, as do fractures from untreated myelin lipid bilayers (Fig. 8 a and b). DMSO, however, usually causes widely spaced low protuberances to appear on these faces (Fig. 8 c). These dome-shaped bumps increase in size as
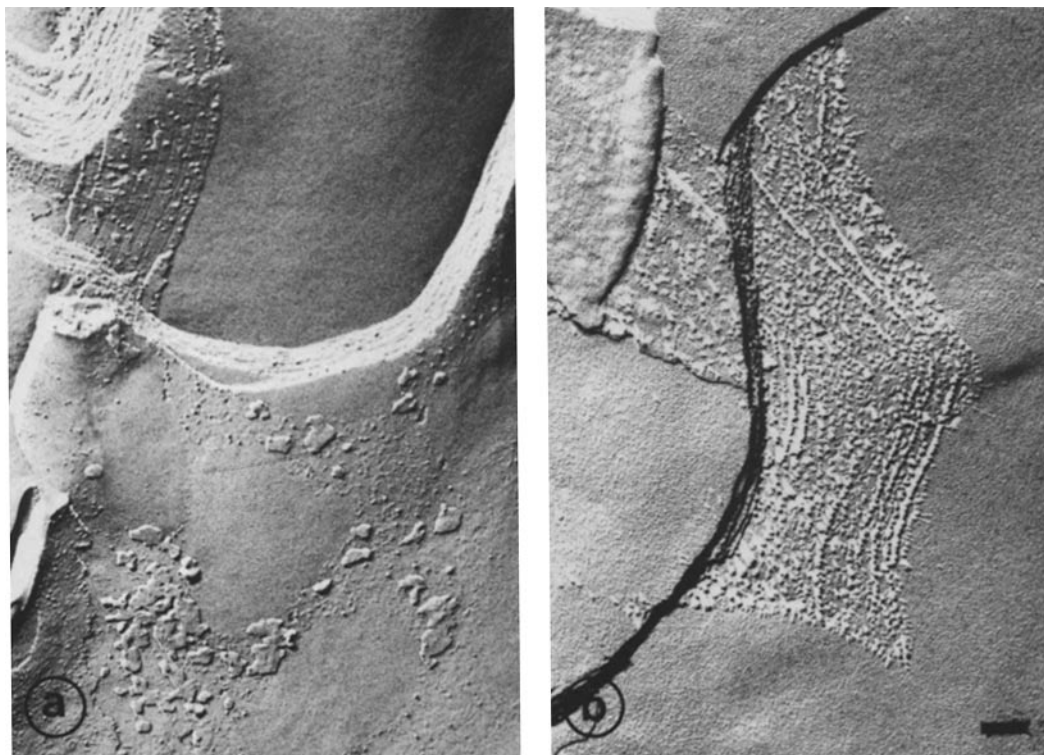

FIGURE 5 Particle segregation occurs in the CNS much as it does in the peripheral nervous system. The particle-rich areas generally include the radial component, seen in normal CNS myelin, which consists of long chains of particles that are in register between successive layers. (a) Brain white matter treated with 20% glycerol for 20 min. (b) Optic nerve treated with 20% DMSO for 2 h. Bar, 0.1 μm. × 61,000.
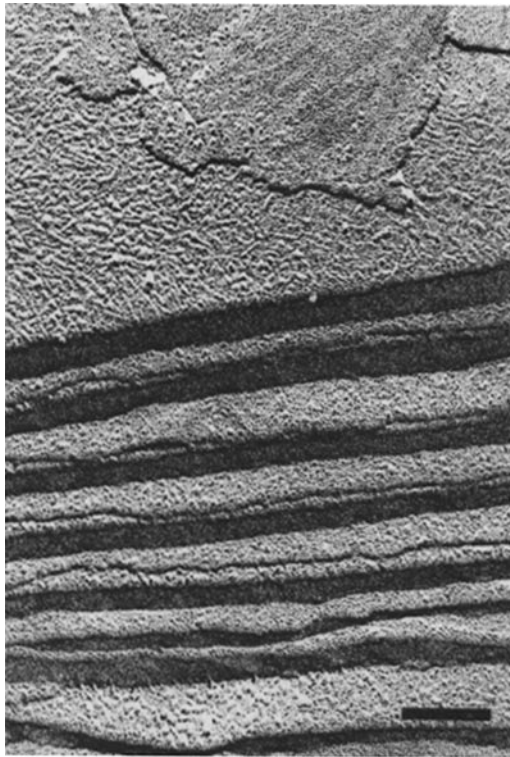
FIGURE 6 The EF of peripheral nerve myelin has a higher density of particles than the PF. In hypoionic solutions, membrane pairs separate at their external surfaces. Solvent layers are seen as large steps in this oblique fracture of sciatic nerve swollen for 24 h in 20% glycerin/water solution. The particle-rich fracture face is adjacent to the external solvent layer, whereas the more particle-poor face of the upper membrane in these pairs is on the cytoplasmic side. Bar, 0.1 μm. × 125,000.

the DMSO concentration increases. Similar bumps are found when myelin lipids are exposed to DMSO or very rarely in untreated lipids (Fig. 8 d).

Treatment with 15% acetone at 0°C and 50% acetone at room temperature produces smooth, particle-free compacted membrane arrays like those produced by other compacting treatments; but in myelin treated with acetone at a concentration of 50 or 75% at 0°C, the particle-free areas have a mazelike mosaic appearance (Fig. 8 e). Lipid multilayers treated with 50% acetone at 0°C have the same mosaic appearance as the compacted myelin membranes (Fig. 8 f).

Swelling Particle-containing Arrays after Specific Cation Compaction

Treatment with calcium and tetracaine can lead to separation of the myelin membrane pairs at the cytoplasmic boundary. In thin sections fixed only with OsO₄, membrane pairs in the particle-enriched domains are often split at the main period line (see Fig. 1 b in reference 23), but prefixation with glutaraldehyde prevents this separation. Freeze-fracture observations show that when unfixed nerve is compacted with 20 mM tetracaine in Ringer's solution and then swollen in aqueous glycerol while maintaining the tetracaine concentration, smooth membrane layers remain compacted, whereas the particle-rich layers swell in pairs with the separation occurring at the cytoplasmic boundary (Fig. 9). This is in contrast to myelin swollen in aqueous glycerol without tetracaine, in which the membrane pairs separate at the external boundary (Fig. 6).

Swelling Particle-free Arrays after Fixation

When particle segregation is fixed with glutaraldehyde and the nerve placed in an aqueous glycerol swelling solution, the particle-rich areas seen in freeze-fracture maintain the normal periodicity, whereas the smooth membranes swell apart in a
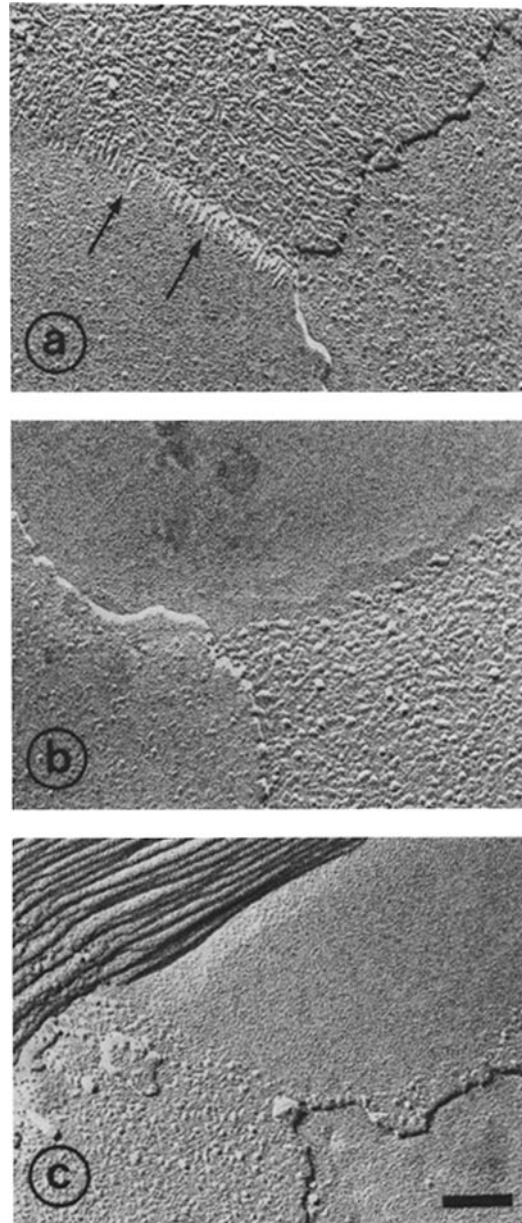

FIGURE 7 The boundary between smooth and particle-rich membrane is usually marked on the EF by characteristic elongated border particles (arrows in a). These are seen in all conditions we have studied, except for myelin that has been treated with hypertonic saline (b) or fixed with glutaraldehyde (c) or osmium tetroxide. The difference in particle density between the EF and PF is clear in all the particle-enriched domains, whereas alternate faces of the smooth membranes look alike. (a) Treated with 15% DMSO for 2 h. Border particles can also be seen in the particle-rich faces shown in Figs. 3 c and d, 4 b, 5, and 8 a and c. (b) Treated with 5.0 M NaCl for 2 h. Fig. 3 b also shows the lack of border particles at the boundary in a nerve treated with hypertonic saline. (c) Treated with 20% DMSO for 2 h, then fixed with 2.5% glutaraldehyde for 30 min before being glycerinated. Fig. 3 a shows lack of border particles in a glutaraldehyde-fixed specimen that was frozen without cryoprotection. Bar, 0.1 μm. × 96,000.
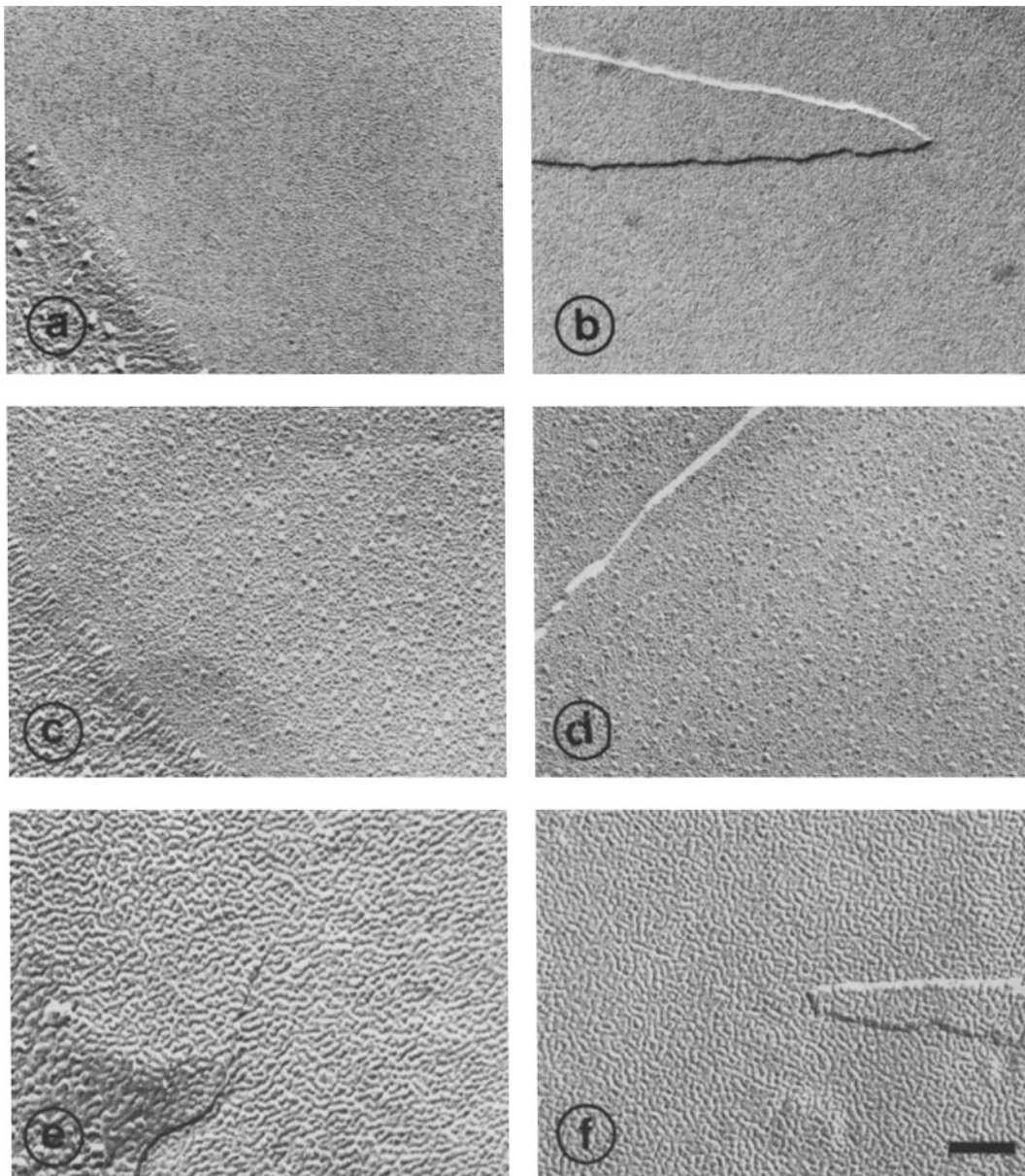
FIGURE 8 Particle-free fracture faces in compacted myelin (left column) are not always smooth, but the variant morphologies can be duplicated with purified myelin lipid multilayers (right column). In the pictures of freeze-fractured compacted myelin, a portion of particle-rich membrane is shown in the lower left corner. (a) Intact myelin treated with 20% DMF for 2 h. (b) Myelin lipid multilayers, untreated. (c) Intact myelin treated with 15% DMSO for 2 h. The small dome-shaped bumps on the smooth face are frequently seen in DMSO-treated nerves. (d) Myelin lipid multilayers, untreated. A region with small bumps. (e) Intact myelin treated with 50% acetone at 0°C for 4 h. (f) Myelin lipid multilayers treated as in e. Bar, 0.1 μm. × 96,000.

disordered array that gives no detectable diffraction (Fig. 10). Interactions of particle-free arrays have also been examined after particles are irreversibly segregated by air-drying. Air-dried nerve, rehydrated in Ringer's solution, was immersed in either DMSO or glycerol for 2 h. In both cases, extreme particle segregation was seen by freeze-fracture. By x-ray diffraction, the DMSO-treated nerve had the expected compacted repeat period of 126 Å. After equilibration in Ringer's solution with or without glycerol, however, the spacing was ~190 Å. Although this is close to the native repeat period, the freeze-fracture pictures of extreme particle segregation show that the diffraction comes from swelling of the smooth layers.

DISCUSSION

Comparison of Compacting Treatments

Treatments that we have used to compact myelin can be subdivided into the following categories. (a) Lowered water vapor pressure: the tissue is dehydrated by evaporation leading to concentration of nonvolatile solutes in the cytosol. (b) Hypertonic solutions of hydrophilic polymers (PEG in Ringer's solution): the tissue is osmotically dehydrated without changing the concentration of diffusible salts. (c) Hypertonic solutions of permeant nonelectrolytes in Ringer's solution: (i) inducing an equilibrium compaction (DMSO, DMF, acetone); and (ii)
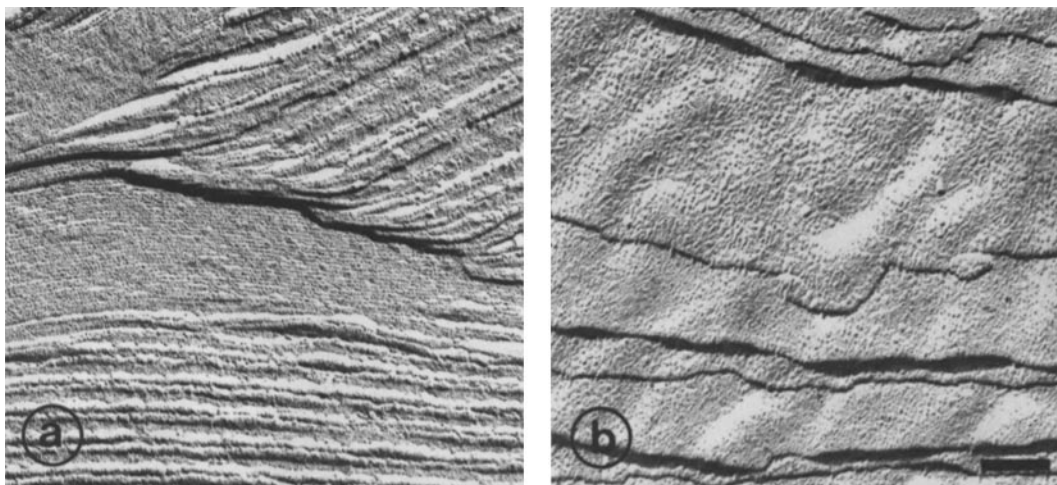
FIGURE 9 Tetracaine can induce a separation at the cytoplasmic boundary in the particle containing domains. (a) Treated with 20 mM tetracaine in buffered Ringer's solution for 3.5 h to induce compaction and then immersed in 20% glycerol plus 20 mM tetracaine and no other salt for 2 h to allow swelling. Compacted membrane domains seen in cross fracture remain closely packed with a period of ~125 Å, whereas particle-rich membrane swells apart in pairs to a period of ~395 Å. (b) Treated as in a. An oblique view of the swollen particle-rich area shows that with the addition of tetracaine the separation of the membrane pair takes place at the surface opposite to that of nerve swollen in just hypoionic glycerol (Fig. 6). Therefore, the swelling observed here results from separation of the cytoplasmic surfaces. Bar, 0.1 μm. × 96,000.

inducing transient compaction (glycerol, glucose). For both subcategories, the water appears to leave the tissue more rapidly than the reagent enters it. In category ii, as its activity increases inside the tissue, the reagent can substitute for water in stabilizing a normal period structure; but category i reagents do not act like water, although they penetrate the tissue. (d) Hypertonic solutions of monovalent salts (NaCl in Ringer's solution): salt induces an osmotic dehydration similar to that induced by the nonelectrolytes, and, in addition, dampens electrostatic interactions. (e) Strongly binding cationic reagents (Ca^{++}, La^{+++}, tetracaine—with or without monovalent salt to maintain normal ionic strength): compaction results from specific binding properties. All alkali-earth cations act like calcium (23), but all cationic anesthetics do not act like tetracaine.[1]

With all of the treatments we have examined, the occurrence of a compacted myelin phase detected by x-ray diffraction from intact nerves is correlated with lateral particle segregation away from compacted membrane arrays into the contiguous normal period arrays. Compacted membrane arrays are always particle free. We have shown that particle segregation can be fixed (chemically or after extreme dehydration) and the compaction of the particle-free membranes relaxed; we have not seen compaction following standard glutaraldehyde fixation, although particle segregation has been observed (28). Thus, particle segregation may exist in the absence of compaction, but compaction always leads to particle segregation.

Compacted membrane domains produced by different treatments have a similar three-dimensional shape with the boundary between compacted and particle-enriched arrays staggered in a stepwise fashion from layer to layer. In the particle-enriched layers, the EF face is more particle-dense than the PF face, whereas in the compacted layers no intramembrane particles are seen on either face.

Morphologically, compaction by specific cation binding cannot be distinguished from compaction to the same extent induced by hypertonic nonelectrolytes. The all-or-nothing, rel-

atively irreversible compacting action of specific cations is, however, dynamically different from the graded, reversible effects of dehydration by nonelectrolytes. Compaction by sodium chloride differs morphologically in the lack of border particles. Like dehydration by hypertonic nonelectrolytes, sodium chloride compaction is reversible and, with respect to the extent of compaction, is graded. Like compaction by specific cations, however, the repeat period of the sodium chloride-compacted phase is independent of reagent concentration.

Morphology of Bilayer Fracture Faces

The corresponding appearance of fracture faces from smooth membrane arrays and of those from purified lipid multilayers indicates that we are seeing uninterrupted lipid hydrocarbon layers. Distinctive features on the compacted membrane fracture faces result from specific preparative treatments and are identically reproduced in the purified lipid multilayers. Small blisterlike bumps, seen in intact myelin only after treatment with DMSO, may not be a direct consequence of the presence of DMSO, because similar bumps are very occasionally found in untreated lipid multilayers (Fig. 8d). The maze-mosaic surface seen after 50% acetone treatment at 0°C is identical in particle-free myelin domains and isolated lipids (Figs. 8e and f). This appearance is dependent on both high acetone concentration (50 or 75%) and cooling (to 0°C for about 4 h) before freezing. About 30% of the cholesterol content is lost when nerves are treated with pure acetone for 12 h at 0°C, leaving other lipid components largely intact (8). Less cholesterol may have been extracted in our treatments that did not exceed 4 h for a maximum acetone concentration of 75%. Because treatment with 50% acetone at room temperature does not induce this mosaicked morphology, the effect results from some sort of structural reorganization induced by cooling during the acetone treatment which may not be a direct consequence of cholesterol extraction.

Regardless of how the distinctive effects of acetone and DMSO on the morphology of the freeze-fractured bilayer are produced (whether an artifact of freezing, or a result of lipid

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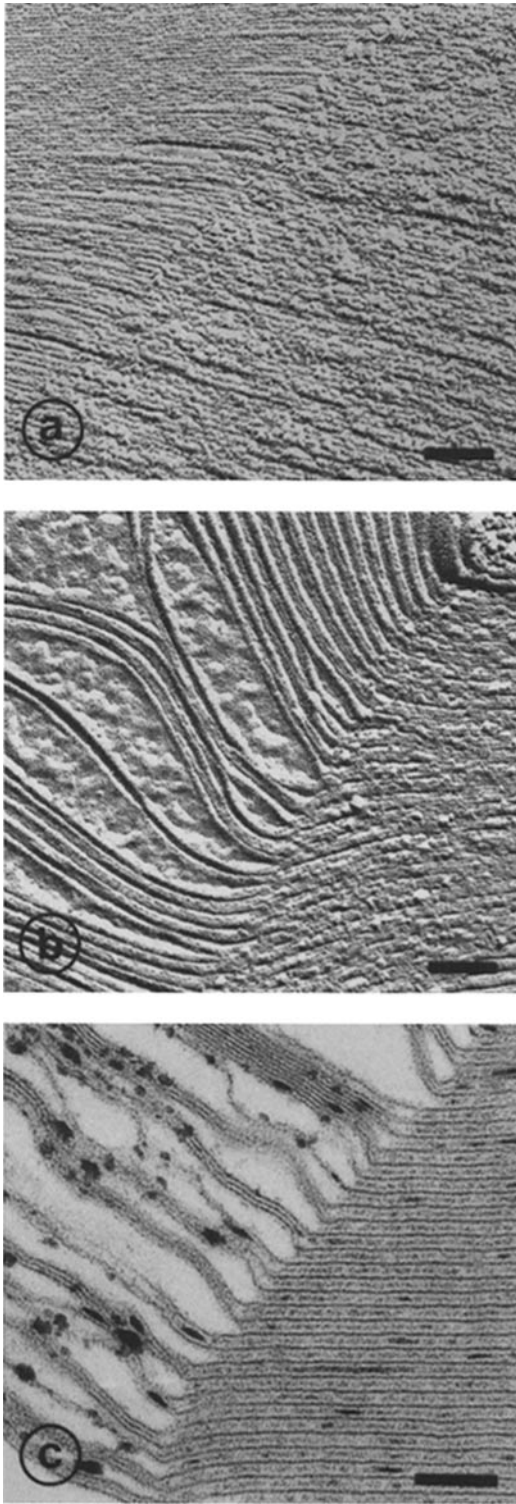[1] V. Melchior. Unpublished observations.

FIGURE 10 Glutaraldehyde fixes the particle-rich membrane domains but does not stabilize the separation of the particle-free layers. (a) Treated with 0.6 M NaCl in buffered Ringer's solution for 2 h before freezing. The compacted membrane array has a period of ~120 Å whereas the particle-rich domain has a slightly expanded period of ~205 Å. × 96,000. (b) Treated as in a, then fixed with glutaraldehyde. Subsequently, the nerve was swollen in 20% glycerol/water for 3.5 h and then frozen. The spacing of the rough membranes has been fixed at the 205-Å periodicity, whereas the membranes in the smooth region swell apart freely. × 96,000. (c) Treated as in a, then fixed in glutaraldehyde. Subsequently, the

solubilization, or contamination, or a phase change), the fact that the effect of each agent on compacted myelin and on myelin lipids is the same demonstrates the close similarity of the particle-free myelin membranes and the protein-free lipid layers. This similarity suggests that there is no intramembrane protein in the bilayer of the compacted arrays.

Composition of Particles

Proteins make up only 20–25% of the dry weight of purified myelin preparations (4, 26). Protein in intact myelin accounts for ~10% of the hydrated volume (9, 16, 33). Neutron diffraction measurements from rabbit sciatic nerve indicate that the amount of protein spanning the hydrocarbon layer is similar to that in the aqueous spaces between membrane surfaces (18). Thus, transmembrane and intermembrane proteins each occupy on the average ~10% of the cross-sectional area.

Because the protein extends uniformly across the membrane unit, it does not appear as discrete features in the electron density profile. The measured x-ray diffraction arises predominantly from the electron density contrast in the lipid bilayer, and the protein contributes little to the scattering density contrast of the membrane array (17). If the lipid:protein ratio were the same in both compacted and native-period myelin membranes, the diffracted intensities for each phase would be in proportion to their surface area. However, when myelin is compacted to the extent where the particle-enriched domain occupies 15–20% of the surface area, the x-ray diffracting power of this phase is <5% of the total, although the micrographs show that there is ordered stacking in these arrays with normal or slightly expanded repeat period. Therefore, the loss of diffracting power for x-rays must be due to a decrease in the lipid:protein ratio; and the transmembrane protein, which occupies ~10% of the total surface area, must be concentrated in the particle-enriched domains. Correspondingly, the diffracting power of the compacted phase is comparable to that of untreated myelin (16), indicating that these arrays contain most of the membrane lipid and must be relatively free of transmembrane protein. (X-ray diffraction shows that some protein remains associated with the lipid polar groups of the compacted membrane [20, 23].) Correlation of our x-ray diffraction and electron microscope observations provides a direct basis for identifying the intramembrane particles seen in freeze-fracture with the transmembrane protein.

Empty holes, seen where compacted arrays would be expected in thin sections of glutaraldehyde-fixed compacted myelin dehydrated with alcohol, indicate that the alcohol is extracting the particle-free membrane lipids; this is further evidence for the reduced protein content of the particle-free membrane arrays.

The particles that are laterally displaced from compacted membrane domains may correspond to a single class of myelin protein. These particles can plausibly be identified with at least the glycoprotein of peripheral nerve or the proteolipid protein of central-nerve myelin. The glycoprotein and the proteolipid proteins have amino acid compositions and solubility properties characteristic of intrinsic membrane proteins (4), and each protein constitutes about half the total found in the corresponding purified myelin preparation (26). Each particle, seen in freeze-fracture, is not necessarily a single protein molecule, but

nerve was swollen in water for 3 h before postfixation with 1% OsO₄ in water and embedding in Epon. The appearance in thin section is very similar to that seen in freeze-fracture. Bar, 0.1 μm. × 114,000.

may be an aggregate and include some lipid. In the particle-enriched layers of extremely dehydrated myelin, the large lumps are clearly aggregates.

Interactions of Transmembrane Proteins

The repeat period of particle-enriched layers is usually close to normal (~180 Å) or slightly expanded (Table I). (Extreme dehydration, as in preparation for thin sectioning (19), can lead to shrinkage by ~30 Å.) The transmembrane proteins appear to act like spacers that prevent the close approach of bilayer surfaces and serve to stabilize the normal period separation. Lateral mobility of the transmembrane protein is demonstrated by the reversibility of the particle segregation induced by most dehydrating treatments.

Our studies of myelin swollen in the presence of tetracaine or calcium and observations on membrane pair splitting in thin sections indicate alterations of the membrane protein interactions. Swelling at the cytoplasmic boundary in the particle-enriched domains at low ionic strength following tetracaine treatment (Fig. 9) could be due to electrostatic repulsion of positively charged surfaces resulting from the bound cations; furthermore, the absence of swelling at the external boundary normally observed at low ionic strength could resut from neutralization of the normal negative surface charge by the bound tetracaine. Because the particle-free membrane bilayers remain compacted by tetracaine at low ionic strength (under these conditions myelin lipids are flocculated [23]), the altered interactions in the particle-enriched layers can be attributed to changes in the charge distribution on the transmembrane protein due to tetracaine binding.

Interactions of Particle-free Membranes

After particles have been irreversibly segregated either by glutaraldehyde fixation (Fig. 10) or by extreme dehydration (Fig. 4), the particle-free membranes can move apart to maintain equilibrium with the solution in which the nerve is immersed. Interpretation of earlier observations that compaction following extreme dehydration could be reversed on rehydration (10, 22, 38) did not take into account the irreversible particle segregation that has now been recognized following these treatments. The swelling properties of the particle-free membrane arrays are very similar to those of multilayers containing acidic lipids (5, 27, 30) in which the equilibrium separation is determined by the balance of electrostatic repulsion and electrodynamic attraction. It is interesting that the equilibrium spacing of the particle-free membrane pairs in Ringer's solution is only 10–20 Å greater than that of normal myelin, indicating that both the lipid-lipid and protein-protein interactions favor about the same separation under physiological conditions.

Compacted myelin membranes are not identical with myelin lipid bilayers (23). The asymmetry of the compacted membrane may be stabilized by the small amount of proteins associated with the inner and outer polar layers. This structure is labile because, after prolonged compaction, the membrane becomes symmetric as seen both by electron microscopy and x-ray diffraction. The loss of asymmetry implies redistribution of the protein and lipid components in the compacted membranes.

Close Contact

Dispersions of total myelin lipids undergo rapid flocculation on treatment with DMSO, calcium, and tetracaine at levels similar to those that induce compaction of myelin membranes (23). This suggests that membrane bilayers are brought together by the same sort of forces that cause lipid flocculation (dehydration or charge neutralization). The structure and stability of compacted myelin predominantly reflect properties of the lipid bilayers and not the proteins. As the surfaces of facing bilayers are drawn together, the intramembrane particles appear to be squeezed out of the way. Exclusion of the protein particles is unlikely to be due to lateral phase segregation of different lipid classes, because the particle-free membranes in extensively compacted myelin contain almost all the myelin lipid in uniform-appearing bilayers.

Close apposition of membranes with particle-free fracture faces has been seen in a number of cell systems (13, 15, 21, 25, 29, 35, 36). Because the areas of close contact in these membranes are focal and not ordered in periodic arrays as in myelin, structural information is available only through microscopy. Close contact of membranes and the associated particle segregation seen in other systems are likely to involve the same sort of interactions between the membrane bilayers as we have characterized in myelin by correlation of electron microscope, x-ray diffraction, and physicochemical observations.

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