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CRYSTALLINE PATTERNS OF MYELIN LIPIDS VISUALIZED BY FREEZE FRACTURE

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Summary

Freeze fracture of rat optic nerve reveals smooth, particle-free regions on the lammellar fracture faces of myelin when prepared by standard procedures. When the fixed, glycerin-impregnated tissue is incubated at 6°C for two or more days, crystalline patterns indicative of a phase transition can be seen in the particle-free regions. The crystalline patterns can be destroyed by subsequent incubation at 37°C and are not seen when the initial incubation is at room temperature or 37°C. Butylated hydroxytoluene has no effect on the formation of the crystalline patterns. The time course of the formation of the crystalline patterns suggest that the rate-limiting step in the process is not the phase transition itself. We propose that the lipids associated with the particles in vivo are involved in the formation of the crystalline patterns.

Introduction

It is now well established that bilayers of lipids forming membranes can undergo phase transitions and phase separations (for reviews see refs. 1—3). Freeze fracture electron microscopy has been able to directly visualize some phase transitions in artificial and reconstituted membranes [4—9]. Similar studies on intact biological membranes have demonstrated morphological changes which correlate with phase transition temperatures determined calorimetrically [10—19]. In most of these cases, creation of particle-free regions constitute the changes; in one study, a banding pattern was observed which might be interpreted as a visual indication of order within the particle-free regions [10]. In artificial membranes, on the other hand, crystalline patterns have been observed through freeze fracture electron microscopy; these clearly

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indicate order and can be taken as prima facie evidence of a phase transition. To date there have been no reports of such crystalline patterns in biological membranes.

In previous reports from this laboratory, the fracture faces of myelin have been shown to contain particles intercalated within the hydrophobic matrix of the membrane [20]. Under normal preparative procedures, the particles become aggregated, creating particle-free regions which propagate radially in the myelin lamellae. This membrane reorganization is difficult to avoid except by freezing the tissue immediately upon removal from the animal.

In the present communication, we report a temperature-dependent (thermotropic) membrane reorganization which occurs over a 2-day period. This reorganization can be seen through freeze fracture techniques as crystalline patterns within the particle-free regions. Although phase transitions have been reported in myelin [21,22] the thermal properties of this transition suggest that the reorganization presented here is markedly different from those previously described. Preliminary reports of some of the work described here have appeared elsewhere [23,24].

Materials and Methods

Optic nerves were dissected from anesthetized rats (Lewis, 16—20 weeks old) into phosphate-buffered saline containing 2% glutaraldehyde, fixed and glycerin impregnated as described elsewhere [20]. After impregnation, samples were either frozen directly or incubated in phosphate-buffered saline with 25% glycerin and 1% glutaraldehyde for various times and temperatures (see Results). In some experiments, the incubation media contained 0.1% butylated hydroxytoluene or 0.02% digitonin (Sigma, 80% pure).

In the experiments involving "melting" of the tissue, initial incubation was done for 66—68 h at 6° C followed by incubation in 15-ml plastic test tubes in a 37° C bath for 30 or 60 min.

Freezing was accomplished by quenching the specimen mounted on cardboard disks in liquid freon. Freeze fracture was done at —120° C to —125° C on a Balzers 510 freeze fracture device equipped with an electron gun for platinum carbon evaporation. Replicas were cleaned and observed with an Hitachi HU 12 electron microscope.

Results

Myelin which has been fixed with aldehydes and impregnated with 25% glycerin has particle-free regions on the lamellar fracture faces. These regions are surrounded by particle-rich areas and are propagated across many myelin lamellae [20]. Within the limits of resolution of the freeze fracture technique, there is no fine structure in the particle-free regions; the surface is smooth. The contour is sometimes flat, but more often in the larger particle-free regions, is somewhat convex or concave (see Fig. 1).

While this image is consistently seen in samples which have been frozen immediately after impregnation or frozen after being stored at 6° C for 18 h after impregnation, a dramatically different picture emerges when the sample

Fig. 1. The fracture face of myelin quenched in liquid freon without incubation at 6°C. Particle-free regions have no detectable fine structure (magnification 32 800X).

is incubated at 6°C for over 44 h. In this case most of the particle-free regions are no longer smooth, but demonstrate patterns suggestive of crystalline structure with retention of the lamellar arrangement of the membranes (see Figs. 2

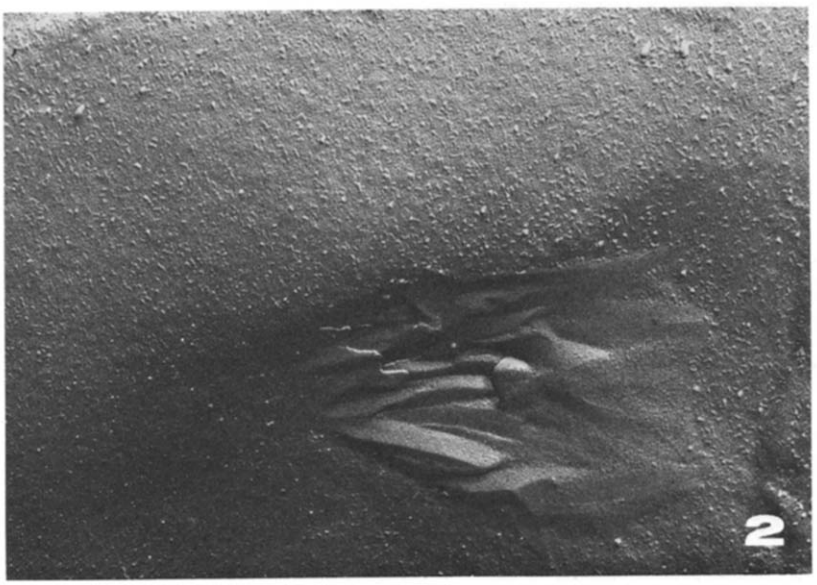

Fig. 2. Myelin quenched in liquid freon after a 2-day incubation at 6°C. Particle-free region contains a crystalline structure. In the center of the particle-free region, the fracture plan has revealed a portion of an adjacent membrane which is also particle free and crystalline (magnification 41 600X).

and 3). Often the fracture plane will stay within a single membrane as it passes through such a crystalline region indicating that the basic lamellar structure of the membranes has not been altered. Occasionally the fracture plane jumps to an adjacent membrane within the region revealing basically the same contours and crystalline patterns on neighboring lamellae (Fig. 2). Visualization of the pattern is dependent upon the local angle of shadow. While all samples prepared in this manner demonstrated crystalline patterns, the size and orientation of the planar surfaces varied considerably from sample to sample (compare Figs. 2 and 3).

The time course of formation of the crystalline patterns was investigated by incubation of the impregnated tissue for 18, 44 and 68 h at 6°C. In the first 18 h, no crystalline patterns can be seen through freeze fracture. By 44 h, up to

Fig. 3. Myelin prepared as in Fig. 2. There are two particle-free regions which contain crystalline patterns. Also, in the particle-rich region, there is some suggestion of crystalline structure (magnification 36 800X).

Fig. 4. Myelin prepared as in Fig. 2. Clusters of densely aggregated particles are sometimes seen in myelin incubated at 6°C for two or more days. At the top of this micrograph is a portion of the radial component (magnification 60 300×).

70% of the favorably shadowed particle-free regions contain these structures *. Further incubation at 6°C for a third day only results in boundaries between the faces of the crystalline patterns being more pronounced.

Butylated hydroxytoluene has been used in lipid biochemistry as an antioxidant for membrane lipids (e.g. see ref. 25). Incubation in the presence of butylated hydroxytoluene has no effect on the formation of the crystalline patterns.

In addition to the crystalline patterns seen in the particle-free regions of myelin, there are alterations in the particle distributions seen on some of the myelin membranes. These are less common than the crystalline patterns and consist of small (0.1–0.5 μm) regions in which particles are densely aggregated (see Fig. 4). These were often seen in replicas which contained crystalline patterns.

To test the effect of temperature on the formation of the crystalline patterns, samples were incubated for 2 days at 37, 22 and 6°C. Crystalline patterns were present in the 6°C sample but were entirely absent in the incubations at higher temperatures. Crystalline patterns present in tissue incubated at 6°C for 3 days can be "melted" by incubation at 37°C for 1 h. There is a considerable reduction in the pattern of the faces within 30 min at 37°C (see Fig. 5).

Since digitonin has been used as a membrane stabilizer for thin section electron microscopic preparation of myelin [26], an attempt was made to inhibit the formation of the crystalline patterns through stabilization of membranes with this drug. Incubation for 2 days in the presence of digitonin resulted in massive changes in the morphology of the tissue (Fig. 6). Tubules, presumably of digitonin · cholesterol complexes [27] formed within the exterior lamellae of the sheath and were seen in great numbers in the extracellular space. Often partial, hemicylindrical structures seemed to be continuous with the myelin lamellae creating a scalloped texture in the fractured surface (Fig. 6). Replicas where the fracture plane passed through the more internal membranes of the

* It should be noted that faults in an ordered structure represent the only visual clues for the crystalline patterns; a properly oriented, perfectly formed crystalline structure would not be distinguishable from the particle-free regions seen without incubation in the cold.

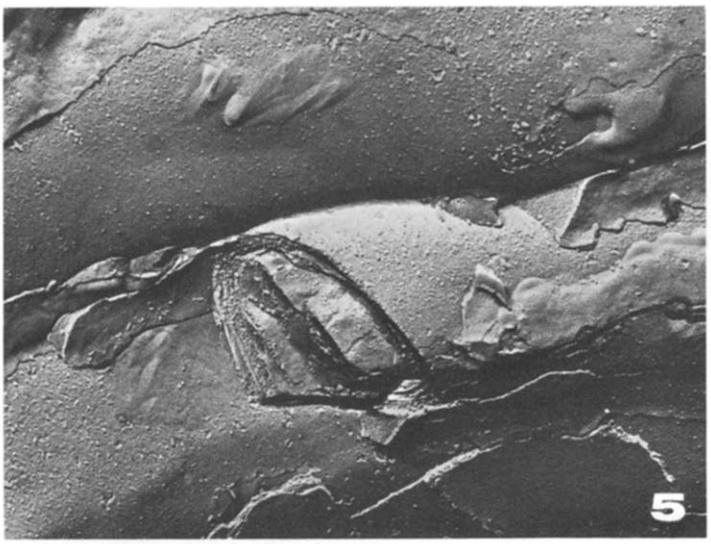

Fig. 5. Myelin which has been "melted" by incubation for 30 min at 37°C after incubation at 6°C for 3 days. This micrograph demonstrates one particle-free region with a readily observable crystalline pattern, one with a faint pattern and two particle-free regions with no detectable pattern (magnification 47 700×).

Fig. 6. Myelin incubated in the presence of digitonin. Digitonin-chloresterol tubules disrupt much of the myelin. At the top of this micrograph are a number of partially formed tubules (hemitubules) which retain the lamellar structure of myelin while in the central region tubules which have become detached from the membranes can be seen (magnification 66 000×).

myelin rarely demonstrated tubules, and the particle-free regions contained few crystalline regions. Often there were particles on the tubular structures, although it appears that most of the particles are excluded by the tubules.

Discussion

This is the first report of visualization of crystalline patterns in biological membranes with electron microscopy. Such visualization has not been possible through thin section, not only because usual preparative procedures involve extraction of many of the lipids during dehydration of the specimen, but also because the section thickness may obscure changes as small as those we have described. There have been many freeze fracture descriptions of phase transitions and co-crystallizations in simple, artificial membrane systems (e.g. ref. 4; for a review see ref. 2). Reconstituted membranes quenched in liquid freon from below their phase transition temperature can demonstrate crystalline patterns which exclude membrane particles [6,9]. In biological membrane systems, it has been adequately shown that there are reorganizations of particles on the fracture face which would necessarily imply at least passive reorganization of lipid (e.g. ref. 20). Some studies have been able to demonstrate that particle reorganizations (i.e. creation of particle-free regions) are correlated with phase transitions within the lipid bilayer of prokaryotic biological membranes (e.g. refs. 9, 12, 13 and 17). In none of these studies of biological membranes, however, was it possible to see patterns, similar to the patterns described here, indicative of a crystalline structure within particle-free regions of membrane (but cf. ref. 10). This is likely to be due to the heterogeneity of the lipids in even "simple" biological membranes.

Ladbrooke et al. [21] Schummer et al. [22] and Viret [28] were unable to find phase transitions in central and peripheral mammalian myelin in their hydrated form. Ladbrooke and coworkers [21] were able to obtain such transitions when the hydration of myelin fell below 20% water. These measurements were done with thermal analysis at scanning rates on the order of 10°C/min. The formation of crystalline regions occurs over a period of 2 days and seems to bear no relation to the two transition temperatures (both above 35°C) found in those studies.

In view of the long time required to produce the crystalline patterns, an initial interpretation of the phenomenon is that the lipids are undergoing decomposition in the lengthy incubation period. The most likely types of decomposition are breakdown by enzymes and autolytic oxidations. The prior fixation and incubation of the tissue in the presence of glutaraldehyde and at 6°C renders the first possibility unlikely, although there are enzymes which are active after chemical modification (e.g. see refs. 29—31). The second possibility was tested by incubation in the presence of an antioxidant, butylated hydroxytoluene. Butylated hydroxytoluene has no effect on the formation of the crystalline patterns. Thus, it is not likely that the crystalline patterns are created by lipid decomposition products.

Lateral diffusion coefficients of most lipids in artificial membranes and sarcoplasmic reticulum are on the order of 10^{-8} cm^2/s, while those for steroids are about an order of magnitude higher [32—36]. An effective diffusion coefficient

consistent with the time course of the formation of the crystalline patterns is in the order of 10^{-13} cm^2/s. There are at least three reasons why this value could be this small: (A) the temperature at which the tissue is incubated is below the phase transition temperature of many liquids which make up the crystalline patterns; (B) there are interactions across the lamellae which prevent lateral mobility of membrane particles [20] these may also hinder lateral mobility of the lipids; (C) there are many particles in myelin membranes, and when these are aggregated they may form an effective barrier against lateral diffusion of the appropriate lipids into the particle-free regions. A and possibly B may be involved in the particle-free regions; in the particle-rich regions, C may play an especially significant role.

The location of cholesterol in fixed, impregnated myelin is not known. Pinto da Silva and Miller [20] have suggested that the segregation of particle-rich and particle-free regions represents neurokeratin formation. Adams (ref. 37, Fig. 9) and Adams and Davison (ref. 38, Fig. 91) have shown that neurokeratin networks are quite visible in myelin which has been stained for cholesterol with perchloric acid napthoquinone. The size and disposition of the densely staining regions suggests that it is the particle-free regions which contain significantly more cholesterol than the particle-rich regions. This staining pattern could simply reflect an increased concentration of lipid in the particle-free regions. Alternatively, it could indicate that considerable quantities of specific lipids are associated with the particles * (see also refs. 41, 42). Demel, London and their coworkers [43,44] have shown that in artificial systems there are preferred lipids associated with the proteolipid and myelin basic protein. However, in these systems, the lipid being tested was a monolayer; proteins which may normally be in the center of the lipid bilayer or pass through more than one membrane [20] may not have the same lipid specificities as they would in situ (Miller, R.G., in preparation). Thus, as identification of particle specificities for lipids seems premature at present.

It was hoped that the incubation in presence of digitonin would stabilize the membrane lipids to the extent that crystalline patterns would be inhibited. Such an effect was, indeed, seen on the interior membranes of the few remaining intact myelin sheaths. Most of the tissue, however, was disrupted by digitonin tubules (see ref. 45). The existence of any intact sheaths may reflect a lack of complete penetration of the drug. Intact myelin lamellae contain partially formed hemitubules which may represent the initial step in the disruption of the membranes by budding of tubules. The hemitubules are seen only in the outer few lamellae of the myelin in the sample. It is unclear whether the outer lamellae of those seen in these preparations are the original outer lamellae; it is possible that the lamellae are successively peeled away by the formation of new tubules. Tubules of this sort have been previously described in thin sections of leukocytes [46], and a variety of other tissues [45]. Napolitano and Scallen [26] have used digitonin as an additive to aldehyde fixatives for myelin and report an increased retention of cholesterol and an enhanced preservation of

* This distinction is especially significant in light of the role that cholesterol has in broadening temperatures of phase transitions in membranes (see ref. 39 and 40). The high initial concentration of cholesterol within the particle-free regions may contribute to the apparent lack of phase transitions as measured by calorimetric studies.

the myelin lamellae; no mention was made of tubules in this report.

There are many states of myelin distinguishable on the basis of their X-ray diffraction patterns [47—50]. Particularly interesting are the states which require several days to enter. Worthington and McIntosh [50] have described "condensed I" and "separate I" states, both of which require several days for formation (cf. ref. 21). Since none of the states described were achieved through the same procedures which we have used for freeze fracture, at this time it is difficult to determine the relationship between the present findings and the X-ray diffraction data.

While it is likely, that the crystalline patterns represent a phase separation and transition (see ref. 33) of lipids endogenous to the myelin, it is not at all clear that they represent a reorganization of the lipids which were originally in the particle-free regions. The lengthy time necessary for the initial cyrstallization suggests that the rate-limiting step in the creation of the crystalline patterns could be the dissociation of appropriate lipids from the particle-rich regions rather than diffusion in the particle-free regions (but cf. ref. 51). Once a lipid has been incorporated into the crystal, there would be a tendency for it to remain there as long as the temperature was below the phase transition temperature of the crystallizing lipids. Since there is no evidence of creation of the crystalline patterns with incubation at room temperature or at 37°C, the phase transition temperature(s) must be between 6 and 21°C.

One model of formation of the crystalline regions which would account for the time required for their formation is that the lipids which undergo phase transition are associated with the membrane particles in situ [41,52,53]. At temperatures below their phase transition temperature, these lipids may be more stable in their crystalline form than in association with the particles. Thus, the time course for the formation of the crystalline patterns may be a closer reflection of the dissociability of the lipids from the particles than a reflection of their mobilities in the membrane. The patches of densely aggregated particles which were seen on some of the replicas support this mechanism. However, no explanation has been found for the occurrence of these patches on some membranes and their absence on others. No evidence has been obtained to indicate whether these are radially propagated.

The generality of the crystallization effect is not known at this time. The possibility that crystalline patterns may be obtainable from other membranes under appropriate conditions is especially interesting. In preliminary experiments with peripheral nerve (rat sciatic nerve), the effect is erratically demonstrable. The source of the variability in this tissue is unknown at present (Miller, R.G. work in progress). Experiments without the cryoprotectent, glycerin, have been difficult to interpret due to freezing artifacts.

In conclusion, freeze fracture electron microscopy demonstrates crystalline patterns on the particle-free fracture faces of myelin which has been incubated in the cold for two or more days. We feel that the crystalline patterns represent a phase transition of some of the lipids in myelin. Since calorimetric studies of phase transitions typically use sweep speeds of 1—10°C/min, (but cf. ref. 51), the phase transition described here is probably different from those previously described for myelin [21,22]. One interpretation of these data is that the particles in myelin have bound, non-sterol, lipid which dissociate from the particles

and diffuses into the particle-free regions where it crystallizes into the patterns observed.

Nageotte [54] and Ramon y Cajal [55] felt that neurokeratin formation is an artifact. These results support Nageotte's contention that although it is an artifact study of neurokeratin formation can reveal much about the interactions of the elements in myelin (see ref. 56). Coupled with X-ray diffraction, spectroscopic, and separation techniques, it may be possible with this system to determine what protein-protein and protein-lipid specificities exist in myelin in vivo.

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