X-RAY DIFFRACTION STUDY OF THE KINETICS OF MYELIN LATTICE SWELLING

EFFECT OF DIVALENT CATIONS

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ABSTRACT The time-course of myelin lattice swelling and its reversal in dissected peripheral nerves was determined by small-angle x-ray diffraction using a position-sensitive proportional detector. The process of swelling can take place either in several hours or in <1 h depending on pretreatment of the nerves. The reversal of swelling was always completed within 1 h. The rapid structural transitions involved the disordering of membrane pairs as indicated by the transient appearance of a continuous intensity distribution similar to the membrane pair transform for myelin. The slow transitions involved the gradual replacement of the discrete reflections from the native structure by the reflections from the swollen lattice. Myelin membrane arrays reformed in normal Ringer's solution were much more stable to subsequent swelling than arrays reformed in Ca^{+2} and Mg^{+2}-free Ringer's. These results suggest that these ions participate in stabilizing the interactions between the external surfaces of adjacent membrane pairs.

INTRODUCTION

The nerve myelin sheath is an array of membrane pairs derived by the spiral infolding of Schwann or glial cell membranes around the axon (Geren, 1954; Maturana, 1960). Peripheral nerve myelin has been shown to undergo reversible swelling of the lattice under different conditions in vitro. Finean and Millington (1957) using x-ray diffraction first recorded a larger than normal period for frog sciatic nerves immersed 24 h in hypotonic Ringer solutions. Robertson (1958), in an electron microscope study, showed that the increase in repeat period for peripheral nerve could be accounted for by the separation of the membrane pairs at their outer surfaces. Finean and Burge (1963), and more recently, Lalitha and Worthington (1975) using small-angle x-ray diffraction, and McIntosh and Robertson (1976) using electron microscopy, reported that optic nerves also can swell in hypotonic solutions. Swollen myelin membrane arrays have also been detected by electron microscopy in brain tissue in experimental edema (Hirano et al., 1966) and in Wallerian degeneration (Glimstedt and

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Wohlfart, 1960; Williams and Hall, 1971). In dissected peripheral nerves the lattice swelling can be reversed by treating with >10 mM NaCl or KCl, or 1 mM $CaCl_2$ (Worthington and Blaurock, 1969*a*).

From the low-angle x-ray diffraction patterns of isolated sciatic nerves we have measured the time-course of myelin lattice swelling and its reversal, and the effect of divalent cations on these transitions. We find that the swelling in water can take place either in minutes or after several hours depending on pretreatment of the nerves, whereas the reversal of the swelling in Ringer's is always very rapid. We also find that divalent cations at physiological concentration in Ringer's will stabilize the array of membrane pairs obtained after reversal of swelling as measured by its subsequent response to distilled water.

METHODS

The kinetics of myelin swelling and recompaction was measured in experiments on 36 sciatic nerves from toads (*Bufo marinus*). For each experiment, a freshly dissected nerve was desheathed by removal of the epineurium, divided into two segments of similar length, and immersed at room temperature (23°C) in normal Ringer's solution (101.8 mM NaCl, 2.7 mM KCl, 2.1 mM $MgCl_2$, 1.9 mM $CaCl_2$, 2.0 mM $NaHCO_3$, and 0.36 mM $NaHPO_4$). The two pieces of nerve were tied off and mounted in a perfusion cell with compartments for a treated specimen and its control. The flow rate of perfusates was ~1 ml/min. During an experiment x-ray diffraction data was collected alternately from a treated nerve and its control.

The x-ray source was an Elliott GX6 rotating-anode generator (Marconi-Elliott Avionic Systems, Ltd., Borehamwood, Herts, England) with a copper target, operated at 33 KV, 26 mA. Linear collimation was achieved using a bent, nickel-coated optical flat. The diffraction patterns were recorded at room temperature with a linear position-sensitive proportional detector, 50 mm effective length and 10 mm aperture width (Gabriel and Dupont, 1972). The distance from the specimen to the detector was 192 mm. The use of a position-sensitive detector to monitor rapid structural changes in myelin has been previously demonstrated (Kirschner, 1974).

The computer-stored diffraction patterns were displayed on a cathode ray tube and photographed onto 35-mm film. Periodicity and intensity measurements were made from print enlargements of the films. The periods measured from the positions of the reflections were accurate to within 1%. Intensities of the reflections were measured from peak heights after background substraction.

Three kinds of x-ray diffraction patterns were identified which correspond to native, subnormal, and swollen myelin (Worthington and Blaurock, 1969*a*). The first five diffraction orders from native and subnormal myelin were clearly resolved after 5 min counting time using the detector. For swollen myelin, however, it was necessary to sum in the computer several consecutive 5-min patterns (see Fig. 1 *d*) to obtain adequate resolution of the x-ray patterns for measurements. The repeat periods d ranged from 170–173 Å for the native, 200–252 Å for the swollen, and 166–169 Å for the subnormal. Despite the variability in the lattice dimensions, the sequence of changes in the small-angle x-ray diffraction patterns was completely reproducible from one nerve to another under similar conditions.

RESULTS

Swelling of Sciatic Nerve in Distilled Water

Sequential x-ray diffraction patterns were recorded alternately from a pair of specimens from the same sciatic nerve during perfusion with distilled water. No significant changes in the x-ray patterns were detected during the 1st h after onset of the perfusion (Fig. 1 *a*); but starting from this time the intensity of the native reflections decreased linearly to 0 over the next 6–9 h, with the exact time-course depending on the specimen. When the total intensity of

the native reflections was ~¼ its initial value (between 4 and 7 h), a new set of reflections from a 238-Å-period structure became visible (Fig. 1 *b*). The intensity of these reflections increased as those of the native pattern continued to decrease, but the repeat periods remained constant at 238 and 171 Å, respectively. At ~5 h the intensity of the 3rd-order reflection from the swollen myelin was similar to that of the 2nd order from the native myelin. (Fig. 1 *c*). By

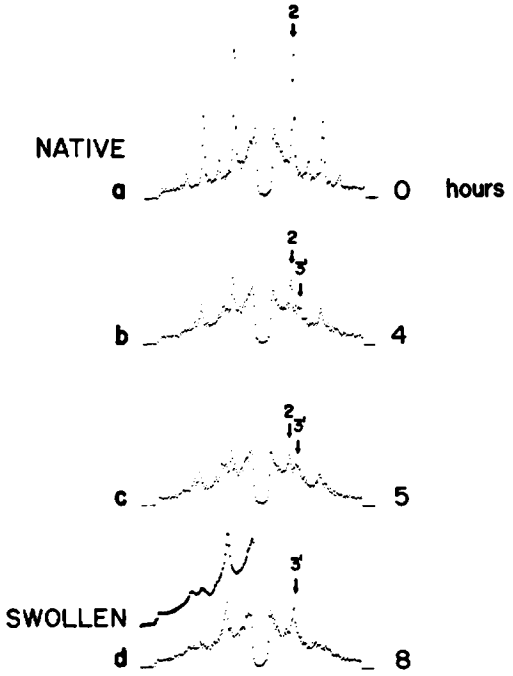

FIGURE 1

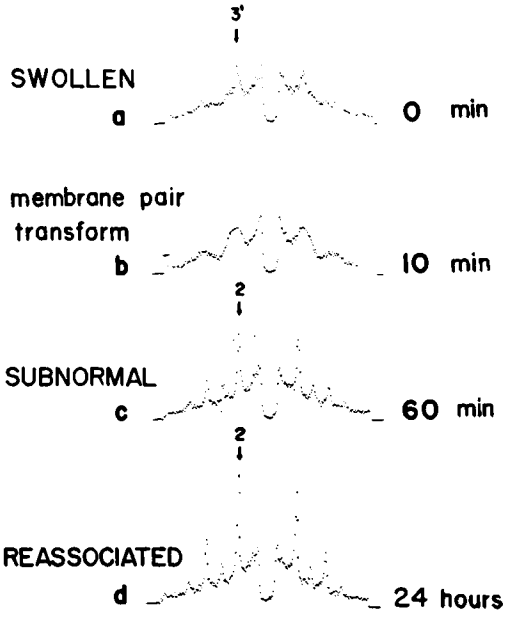

FIGURE 2

FIGURE 1 The kinetics of myelin swelling in distilled water. The times indicated in this and subsequent figures represent the elapsed time at the onset of each 5-min data collection period. To aid in identifying and distinguishing the diffraction spectra in these figures (1–4), the second-order reflection from the native and recompacted structures and the third-order reflection from the swollen lattices are indicated. Although the exact time dependence for the structural transitions was somewhat specimen dependent, the relative differences were completely reproducible. (*a*) Native pattern from myelin in normal Ringer's solution. Bragg reflections 1–5 from a 171-Å period lattice are observed. (*b–d*) Progressive replacement of native pattern by reflections from swollen myelin. At 5 h the intensity of the 3rd-order reflection from the swollen lattice is similar to that of the 2nd order from the native. (*d*) Swollen pattern from myelin at equilibrium in distilled water. The addition of 6 consecutive 5-min spectra is shown in the darker upper trace. Bragg reflections 2–6 from a 238-Å period lattice are observed.

FIGURE 2 The reversal of swelling in normal Ringer's solution. (*a*) Swollen pattern from myelin at equilibrium in distilled water. Bragg reflections 2–6 from a 238-Å period lattice are observed. (*b*) By the end of 10 min, after replacing the distilled water with Ringer's, the discrete reflections from the swollen pattern have been completely replaced by a continuous intensity distribution similar to the membrane pair transform for peripheral nerve myelin (Finean and Burge, 1963; Moody, 1963). (*c*) Subnormal pattern. Bragg reflections 1–5 from a 168-Å period lattice are detected. The slightly reduced period and enhanced third-order intensity with decreased fourth order are characteristic of this structure (Worthington and Blaurock, 1969*a*), and have been interpreted as indicating a reduction in the distance between cytoplasmic surfaces of the apposed membranes (Blaurock, 1971). (*d*) The recompacted membrane pairs after prolonged perfusion (24 h) with Ringer's have recovered both the 171-Å periodicity as well as the relative intensity distribution of native myelin.

~7–10 h after the initial perfusion with distilled water, the diffraction pattern became stable (Fig. 1 *d*). The kinetics of the swelling and the final diffraction patterns were indistinguishable for the two identically treated nerve specimens.

Reversal of Swelling in Ringer's Solution

Within the first 10 min after perfusing with Ringer's (either with or without divalent cations), the relatively sharp x-ray reflections from the swollen myelin (Fig. 2 *a*) were replaced by a continuous intensity distribution characterized by broad maxima at spacings of 74 and 41 Å (Fig. 2 *b*). At 25 min, sharp reflections from a 168-Å-period structure first became visible. The line widths of these reflections were nearly as sharp as those in the 171-Å-period native pattern. As the intensity of these new, discrete reflections increased, the intensity of the broad maxima gradually diminished. This diffraction pattern, detected after treatment of swollen myelin with saline solution (Fig. 2 *c*), has been previously designated as the subnormal pattern (Worthington and Blaurock, 1969*a*). After continued perfusion with Ringer's, the myelin eventually recovered both its original, native periodicity of 171 Å as well as its relative intensity distribution (Fig. 2 *d*, at 24 h) (Finean and Millington, 1957). Except for the subnormal period structure, no discrete intermediate states between the 238 Å period swollen and 171 Å period native equilibrium structures were detected.

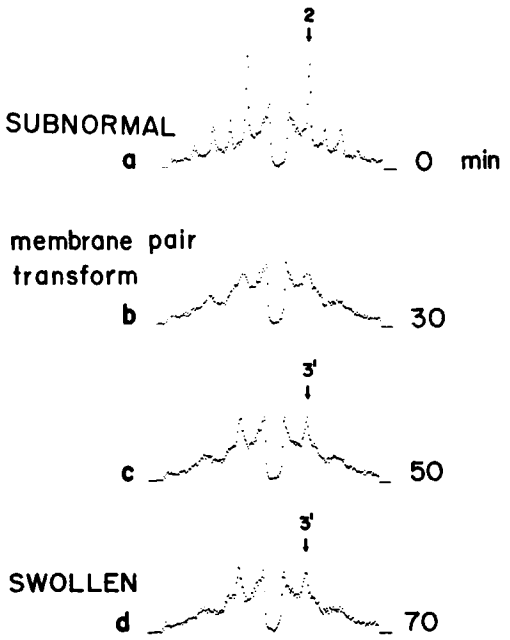

FIGURE 3 Swelling of the subnormal period myelin. (*a*) Pattern from subnormal period myelin after 60 min in Ringer's solution with divalent cations. (*b*) By the end of 30 min after replacing the Ringer's with distilled water, discrete reflections from the subnormal pattern have been replaced by the continuous intensity distribution similar to the membrane pair transform of myelin. (*c*) The 3rd-order reflection of the swollen lattice is clearly visible at 50 min. (*d*) Pattern from swollen myelin at equilibrium in distilled water.

Stability of Membrane Pairs in Recompacted Myelin with Subnormal Period

The stability of the recompacted membrane pairs in the subnormal and native period structures that had been treated with Ringer's either with or without divalent cations was examined by observing their response to subsequent treatment with distilled water.

During the first 30 min after perfusing with distilled water, the intensity of the discrete x-ray reflections from the 168-Å-period structure (Fig. 3 *a*) decreased to a level below detectability, and was replaced by broad intensity maxima (Fig. 3 *b*) similar to those observed in the pattern obtained from swollen myelin perfused with Ringer's (Fig. 2 *b*). At 50 min the 3rd order reflection of the newly forming swollen lattice became clearly visible. By 60 min the swelling of subnormal myelin was completed (Fig. 3 *c* and 3 *d*), in contrast to the 7–10 h required to swell the original native structure; but the final x-ray patterns obtained after swelling these nerves were indistinguishable (compare Fig. 1 *d* with 3 *d*). This rapid swelling occurred whether the subnormal lattice had been formed in the presence or absence of divalent cations in the Ringer's.

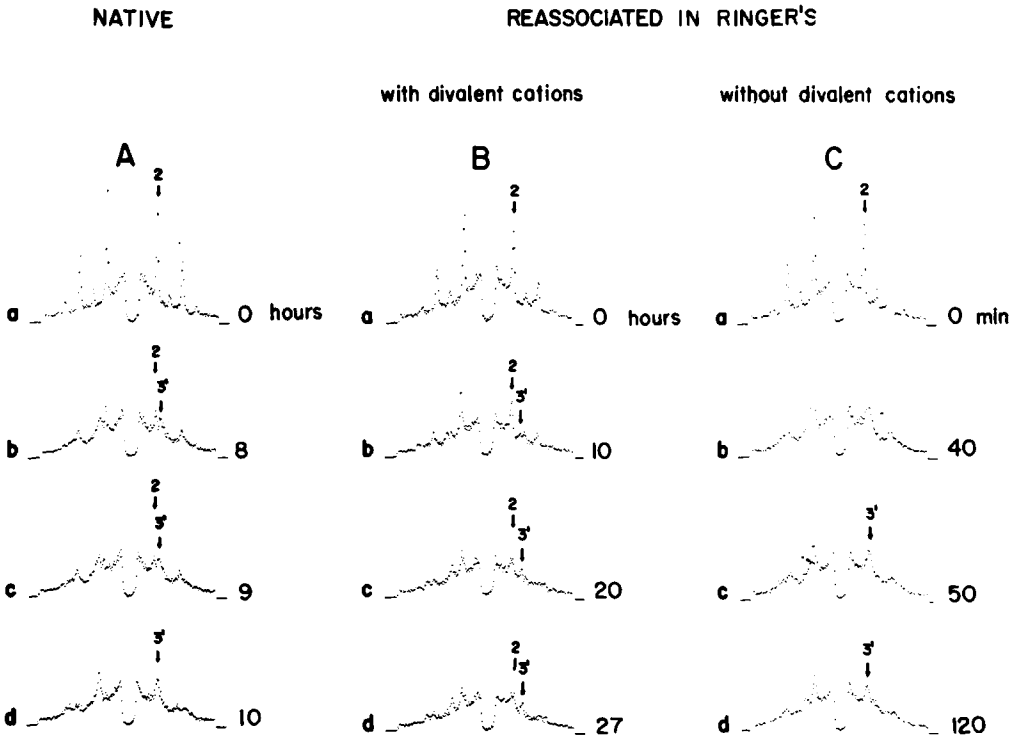

FIGURE 4 Kinetics of swelling in distilled water of (A) native myelin compared to that of recompacted membranes recovered from swollen myelin after 24-h treatment with (B) normal Ringer's (with divalent cations) and with (C) Ringer's without divalent cations. The three initial spectra at zero time (*a*) are indistinguishable, but the subsequent swelling kinetics are completely different. The recompacted membrane arrays after 24 h in Ringer's with divalent cations (B, *d*) are even more stable than the native (A, *d*); complete formation of the swollen lattice was not observed even after 27 h. In contrast, the swelling of the recompacted membrane arrays after 24 h in Ringer's without divalent cations was completed within 2 h (C, *d*) and closely resembled swelling of the subnormal structure (Fig. 3).

Stability of Membrane Pairs in Recompacted Myelin with Native Period: Effect of Divalent Cations

The time to swell the native period structure recompacted after 24 h or more in Ringer's solution depended on whether divalent cations had been present in the solution used to reverse the swelling. During the 1st h after perfusing with distilled water the total intensity of the reflections from the membrane arrays that had been recompacted in the presence of divalent cations decreased by ~⅓; but another 20 h elapsed as the intensities decreased linearly by another ⅓. During this gradual reduction in intensity a new set of reflections from a 200-Å swollen structure appeared at ~10–15 h (Fig. 4 B, *b*), and their gradual increase in intensity mirrored the gradual decrease in intensity of the native reflections. After ~22 h treatment with distilled water, no further changes in the diffraction pattern were seen; and the native and swollen patterns had comparable intensity (Fig. 4 B, *c–d*).

In contrast, the intensity of the reflections from the native period structure that had been recompacted in the absence of divalent cations decreased to below the level of detectability within 40 min after perfusion with distilled water. At this time, the x-ray diffraction patterns (Fig. 4 C, *b*) were dominated by continuous, broad intensity maxima from which discrete reflections of a 248-Å period swollen structure appeared (Fig. 4 C, *c*). The swelling was completed by ~2 h (Fig. 4 C, *d*) after which no additional changes in the diffraction pattern were observed.

DISCUSSION

The property of swelling in ordered biological systems has been used previously for x-ray phase determination: for example, in proteins (Bragg and Perutz, 1952) and in myelin (Finean and Burge, 1963; Moody, 1963; Blaurock, 1971). In the current study (summarized in Fig. 5) the property of swelling in peripheral nerve myelin was used to examine the possible role of the divalent cations Ca^{+2} and Mg^{+2} in stabilizing the myelin sheath against swelling.

What Factors Determine the Kinetics of Myelin Swelling?

The time-course for myelin swelling may be due not only to the intrinsic properties of the myelin sheath, but also could be affected by the perineurial permeability barrier that separates the perfusate from the myelinated fibers. In peripheral nerve, each fascicle of nerve fibers is enclosed by a connective tissue sheath, the perineurium. The collagen in the perineurium provides structural support and the perineurial cells with their tight junctions serve as a perifascicular diffusion barrier to macromolecular substances and to certain ionic species (reviewed by Thomas and Olsson, 1975). The perineurium does not, however, block the rapid exchange of water between the perfusate and the aqueous spaces in myelin (Kirschner et al., 1975). The connective tissue exerts a mechanical constraint on the extent to which myelin swells in water, as is demonstrated by the result that in collagenase-treated nerve, the myelin will swell indefinitely (Rand et al., 1979). In untreated nerve, in contrast, myelin only swells to periods up to 350 Å (Worthington and Blaurock, 1969*a*). In our experiments the myelin always swells to a finite period indicating that the mechanical constraint of the connective tissue remains intact. The diffusion barrier of the connective tissue, however, is probably broken down by the hypotonic shock which would cause lysis of the perineurial cells and an irreversible leakiness to ions. The observed rate at which myelin

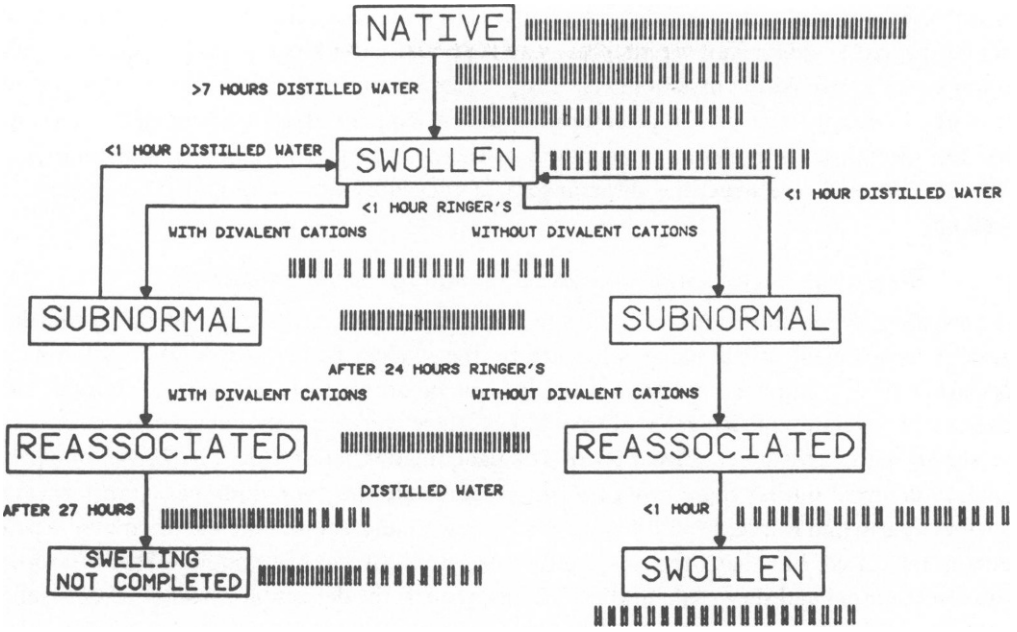

FIGURE 5 Kinetics, based on x-ray diffraction measurements, for the swelling of myelin membranes in distilled water, their reassociation in Ringer's solution with and without divalent cations, and their subsequent swelling in distilled water. Each treatment and its duration is indicated alongside the arrow linking the structural states before and after treatment. Membrane array formation during swelling and recompaction is indicated symbolically by the step-function models. The black bars represent the position of the centers of membrane bilayers that are closer together across their cytoplasmic than across their external boundaries (Finean and Burge, 1963; Moody, 1963; Worthington and Blaurock, 1969*b*; Blaurock, 1971; Finean and Millington, 1957; Robertson, 1958). In the native and reassociated structural states, this center-to-center membrane separation is ~78 Å for amphibian sciatic nerve myelin; in the swollen and subnormal states this cytoplasmic separation decreases by ~5 Å (Blaurock, 1971). The slow structural transitions during which membrane pair disordering was not detected is shown symbolically by the contiguity of distinct lattices. The rapid transitions during which the disordering of membrane pairs was detected is shown by randomly packed membrane pairs. Membrane arrays that recompacted in the presence and in the absence of Ca^{+2} and Mg^{+2} were structurally indistinguishable, but the rapid swelling in their absence demonstrates the stabilizing effect of divalent cations on the myelin membrane arrays.

swells, recompacts, and subsequently swells, would, therefore, be determined by the structural properties of the myelin sheath itself.

Within the myelin, ionic interactions between apposed external membrane surfaces as well as specialized intermembrane contacts could affect the time-course of myelin swelling and recompaction. The 1-h delay in the onset of the swelling process may be due to physical constraints that restrict the unwinding of the spirally wrapped sheath during swelling. Specialized intermembrane contacts, including mesaxonal and paranodal tight junctions, and paranodal axoglial junctions that are at the periphery of the myelin sheath (reviewed by Schnapp and Mugnaini, 1975) could constrain the unwinding during this initial period. The gradual formation of swollen myelin may be accounted for by the breaking of cationic bridges between apposed external membrane surfaces. The reversal of myelin swelling by salt solutions is likely due to the rapid efflux of water from between the membrane pairs. The ionic contacts between apposed membrane surfaces appear to be re-established only after

adequately long treatment with divalent cation-containing Ringer's; otherwise, the apposed membrane pairs swell apart immediately as with the subnormal period myelin or with recompacted native period myelin in Ca^{+2}, Mg^{+2}-free Ringer's. The fact that the subsequent swelling of recompacted myelin after equilibrium in Ringer's starts without delay suggests that the specialized intermembrane contacts at the periphery of myelin have not reformed, probably due to severe structural disarrangement caused by the initial hypotonic shock and swelling.

Structural Transitions during Myelin Swelling and Recompaction

The swelling of myelin membrane arrays in water may occur either over several hours with gradual replacement of the native structure by the swollen period arrays, or in <1 h with formation of a continuous scattering distribution before the swollen arrays appear. The reversal of swelling in Ringer's always takes place very rapidly, involving continuous scattering with subsequent formation of compact membrane arrays. The broad intensity maxima detected during these rapid transitions resemble the membrane pair transform for myelin (Finean and Burge, 1963; Moody, 1963), which indicates that the membranes of a pair remain associated, but that the pairs are disordered relative to one another. The difference between the rapid and slow structural transitions, then, is the detectable disordering of myelin membrane pairs that occurs in the fast process but not in the slow. The fast process resembles a crystal→liquid→crystal transition, whereas the slow is like a crystal→crystal transition.

Stabilization of Membrane Arrays by Divalent Cations

X-ray diffraction patterns to 34-Å spacing recorded from myelin membrane arrays recompacted during 24 h in normal Ringer's or in Ca^{+2} and Mg^{+2}-free Ringer's were identical to each other and to the native. Their subsequent swelling in distilled water, however, showed entirely different kinetics. The swelling of myelin that had recompacted in normal Ringer's (Fig. 4 B) took place over many hours as swollen arrays formed gradually from the native period arrays; this process was similar to the original swelling of native myelin (Fig. 4 A). In contrast, the membrane arrays that had recompacted in Ringer's free of divalent cations (Fig. 4 C) disordered within 40 min, and formed swollen arrays by ~2 h.

Therefore, physiological concentrations of calcium and magnesium ions in Ringer's must stabilize the recompacted arrays of membrane pairs against subsequent swelling, for in the absence of these cations, the membrane pairs begin to separate and disorder immediately. The membrane pairs in subnormal myelin also separate immediately when perfused with distilled water even though the membrane arrays have recompacted in the presence of divalent cations. This result suggests that the stabilization by divalent cations is gradual. Current experiments are in progress to examine the specificity of calcium and magnesium ions in myelin sheath stability and to test the notion that the time-course of myelin swelling is determined by the intrinsic properties of the myelin sheath itself.

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