Biochimica et Biophysica Acta, 776 (1984) 197–208
Elsevier

BBA 72218

EFFECTS OF $ZnCl_2$ ON MEMBRANE INTERACTIONS IN MYELIN OF NORMAL AND SHIVERER MICE

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(Received March 12th, 1984)

Key words: Myelin; Membrane structure; Metal cations; Zn^{2+}; Shiverer mutant; Myelin basic protein; X-ray diffraction; Myelin P_0 glycoprotein; (Mouse)

X-ray diffraction was used to record the effects of metal cations on the structure of peripheral nerve myelin. Acidic saline (pH 5.0) either with or without added metal cations caused myelin to swell by 10–20 Å from its native period of 178 Å. The X-ray patterns usually showed broad reflections, and higher orders were either weak or unobserved. With added $ZnCl_2$, however, the swollen myelin gave diffraction patterns that retained sharp reflections to approx. 15 Å spacing. Alkaline saline (pH 9.7) containing $ZnCl_2$ produced a reduction of the myelin period by approx. 5 Å which was at least twice as much as that produced by other metals. To examine the underlying chemical basis for these unique interactions of Zn^{2+} with myelin, we carried out parallel X-ray experiments on sciatic nerve from the shiverer mutant mouse, which lacks the major myelin basic proteins. Shiverer myelin responded like normal myelin to $ZnCl_2$ in acidic saline; however, in alkaline saline shiverer myelin showed broadened X-ray reflections which indicated disordering of the regularity of the membrane arrays, and additional reflections were recorded which indicated lipid phase separation. This breakdown may come about by the binding of Zn^{2+} to negatively-charged lipids which could be more exposed due to the absence of myelin basic proteins. Electron density profiles were calculated on the assumption that, except for changes in their packing, the myelin membranes were minimally altered in structure. For both normal and shiverer myelins, treatments under acidic conditions resulted in swelling at the extracellular apposition and a slight narrowing of the cytoplasmic space. This swelling is likely due to adsorption of protons and divalent cations. Interaction between Zn^{2+} and myelin P_0 glycoprotein could preserve an ordered arrangement of the apposed membrane surfaces. Alkaline saline containing $ZnCl_2$ produced compaction at the cytoplasmic apposition in both normal and shiverer myelins possibly through interactions with a portion of P_0 glycoprotein which extends into the cytoplasmic space between membranes.

Introduction

The packing of membranes in the multilamellar myelin sheath is determined by a balance of attractive and repulsive forces, and by molecular contact between components that protrude into the space between the membranes (for reviews, see Refs. 1 and 2). These forces and interactions depend ultimately on the arrangement of lipids and proteins in the individual membrane. The localization of specific myelin lipids (cholesterol [3–5], phosphatidylethanolamine plasmalogen [6], and galactosyl ceramide [7]) and proteins (basic protein [8–10], proteolipid protein [8,11–13], P_0 glyco-

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protein [13–15]) have been deduced from chemical labeling, electron microscopy, and X-ray diffraction. To obtain further information on the organization of components in lamellar myelin, we are examining the interactions of metal cations with myelin and with myelin components.

Among the metal salts tested for their effects on intact myelin, we found $ZnCl_2$ preserved an ordered packing of swollen membranes under acidic conditions. Under alkaline conditions $ZnCl_2$ reversed swelling at the cytoplasmic apposition and in addition caused compaction of membranes. To determine whether Zn^{2+}-myelin interactions might depend on myelin basic protein, we repeated some of the experiments using sciatic nerve from the shiverer mutant mouse which lacks the major myelin basic proteins [9,16,17]. In the peripheral nervous system, the myelin appears to be present in normal amounts and to have a normal ultrastructure despite the absence of basic proteins [9]. We found that shiverer myelin responds like normal myelin to $ZnCl_2$-saline treatment under acidic conditions. Under alkaline conditions $ZnCl_2$ disrupted the regularity of the membrane arrays in shiverer and produced a lipid phase separation. Our findings suggest that the binding of Zn^{2+} to P_0 glycoprotein may underlie Zn^{2+}-myelin interactions at sites both in the extracellular and cytoplasmic spaces.

Materials and Methods

Specimens. Sciatic nerves were dissected from mature mice, either normal ones (+/+) of the strain C57BL/6J, or mutant shiverers (shi/shi) on a hybrid background. The nerves were tied off at both ends, and incubated at room temperature in the test solutions which included: saline (154 mM NaCl buffered by 10 mM cacodylate at pH 7.3); 1–20 mM $ZnCl_2$ in saline buffered with 10 mM acetate at pH 4.0 and 5.0, 10 mM citric acid at pH 5.0, 10 mM cacodylate at pH 6.0, or 10 mM sodium bicarbonate/ammonium hydroxide at pH 9.7 or 10.6; and 1 or 10 mM chloride salts of other metals (including calcium, copper, cadmium, and barium) or uranyl acetate in saline buffered with 10 mM citric acid or sodium acetate at pH 5.0, or adjusted to pH 9.7 with ammonium hydroxide. In preliminary studies we found that all the metal salts at concentrations of 1 or 10 mM were soluble at pH 5.0 and pH 9.7. During the incubations the pH was monitored periodically and found to be constant to within 0.1 pH unit. To examine the reversibility of structural modifications, test solutions were replaced with saline. The treated nerves were mounted in thin-wall quartz capillaries containing solution and were examined at room temperature by X-ray diffraction. Experiments were carried out in duplicate or triplicate.

X-ray diffraction. Diffraction experiments were carried out using nickel-filtered and single-mirror focused CuK_α radiation from a fine-line source on a Rigaku generator (Rigaku/USA, Inc., Danvers, MA.) operated at 40 kV, 20 mA. The diffraction patterns were recorded with Kodak No-Screen or Direct Exposure, or CEA Reflex-25 (CEA America Corp., Greenwich, CT.) X-ray films. Short exposures of one hour were usually recorded before and after long exposures in order to detect structural changes in the specimen during the long exposure. The specimen-to-film distance (208 mm) was calibrated from the (010) and (020) reflections at 34.09 Å and 17.17 Å spacing from cholesterol powder (Sigma). These spacings were determined from the known reflections of sodium chloride at 3.258 Å and 2.821 Å [18], and aluminium powder at 2.338 Å [19].

The spacings of diffraction spectra were measured directly off the X-ray films viewed at 6× magnification. The absorbances of the films were determined on an Optronics Photoscan P-1000 microdensitometer (Optronics International, Inc., Chelmsford, MA.) using a 25 μm raster. This raster size was sufficiently small to prevent systematic errors caused by the Wooster effect [20]. For each film, the densitometer scans were first averaged over the central one mm of the line-focused reflections, and then the left and right sides of the pattern (on either side of the central beam stop) were averaged together. This method increased the signal-to-noise level and eliminated experimental errors caused by displacement of the specimen and film plane from the focal circle. The integrated intensity $I(h)$ for each order h was obtained by summing the densities under each peak after subtracting the background which was determined by a polynomial fit (up to the fourth order) to the intensity on either side of the Bragg

reflection. Since the height of the X-ray beam was long enough to adequately average the mosaic spread due to disorientation of the nerve fibers, then the structure amplitudes $|F(h)| = (hI(h))^{1/2}$. Scaling of the structure amplitudes was based on setting the sum of the squares of the structure amplitudes, divided by the repeat period d, equal to a constant [21]. Errors in the structure amplitudes were evaluated as previously described [9].

Interpretation of the diffraction patterns. Since the repeating unit in myelin is centrosymmetric, each structure amplitude $|F(h)|$ has a phase angle of 0 or π, which corresponds to a positive or negative value. Phases for the structure amplitudes were assigned assuming that treatment resulted mostly in changes in membrane packing and minimally in changes in membrane bilayer structure [6].

Calculation of electron density profiles. The relative electron density $\rho(r)$ at a position r in the myelin membrane was calculated using a one-dimensional Fourier synthesis:

$$\rho(r) = 2/d \sum_{h=1}^{h_{max}} \pm |F(h)| \cos(2\pi hr/d),$$

where the phases of the structure amplitudes were assigned according to arguments presented above. The uncertainty in the electron density profile was calculated from the errors in the structure amplitudes [9]. To compare low-resolution profiles, which were calculated from diffraction data to approx. 30 Å spacing, we calculated the center-to-center separations of bilayers across their cytoplasmic and external appositions ($2u$ and $d - 2u$, respectively). To compare the high-resolution profiles, calculated from data to approx. 15 Å spacing, we measured the distance from polar headgroup peak across the cytoplasmic or extracellular gap to the corresponding peak in the neighboring bilayer (cyt and ext, resp.), and the peak-to-peak separation of headgroup peaks within the bilayer (lpg).

Results and Interpretation

(a) Ordered swelling at pH 5 in presence of $ZnCl_2$

The expansion of sciatic nerve myelin from the native 178 Å periodicity to about 185–200 Å was usually observed after approx. 2 h or longer treatment at pH 5.0 in acetate or citric acid buffered saline either with or without the addition of chloride salts of metals. Swollen patterns were also observed after 24 h treatment in acidic saline without buffer. Such swelling has been shown to be reversible to the native state when the nerve is returned to saline at neutral pH [27]. The diffraction patterns (Fig. 1), with one exception, always showed broad reflections which are characteristic of disordered membrane arrays. Only treatment with $ZnCl_2$-saline resulted in swollen myelin that still gave a diffraction pattern (Fig. 1d) which had

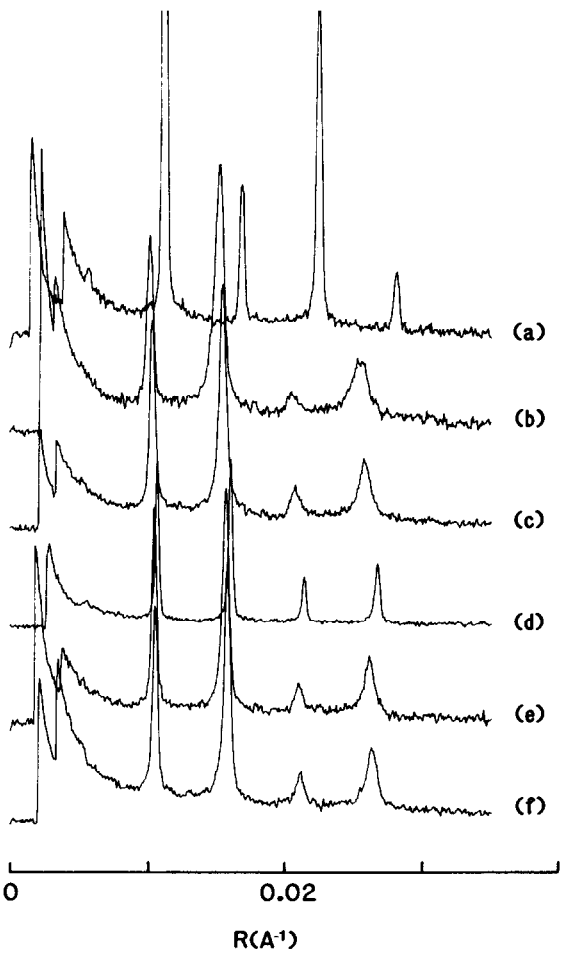

Fig. 1. Densitometric scans of X-ray patterns from mouse sciatic nerves treated with metal salts (10 mM) in 10 mM acetate-buffered saline (154 mM NaCl) solution at pH 5.0 for 2 h. (a) Control nerve at pH 7.3, $d = 178$ Å; (b) Control nerve at pH 5.0, $d = 195$ Å; (c) $CaCl_2$, $d = 192$ Å; (d) $ZnCl_2$, $d = 187$ Å; (e) $CdCl_2$, $d = 191$ Å; (f) uranyl acetate (22 h treatment), $d = 190$ Å.

reflections as sharp as those from native myelin (Fig. 1a).

According to the theory of lattice disorder in paracrystalline materials (such as myelin [22]), there is a linear relation between the square of the integral widths of Bragg peaks and the fourth power of the their orders (reviewed in Ref. 23). The ordinate intercept is inversely related to the width of the coherent diffracting domain; and the slope is proportional to the relative fluctuation in the width of the repeating unit. Our measurements of relative peak widths in the X-ray patterns from myelin treated at pH 5.0 in saline with metal salts (Fig. 2) showed that in unswollen, native myelin (pH 7.3) and in swollen, $ZnCl_2$-treated myelin (pH 5.0) the diffracting domains were larger and there was less fluctuation in the width of the membrane pair than in myelin treated with the other metals.

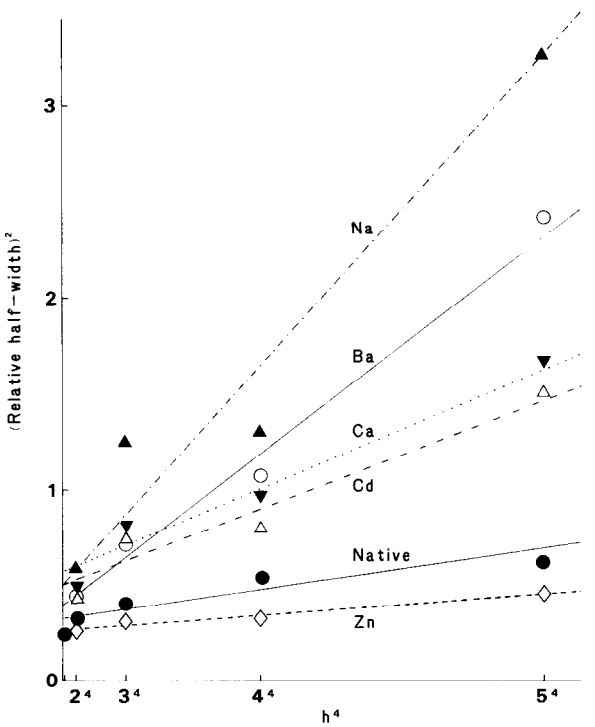

Fig. 2. Sharpness of X-ray reflections recorded from normal nerves swollen at pH 5.0 in saline containing different divalent cations. Ordinate: square of the relative half-width. Abscissa: fourth power of the Bragg order, h. The straight lines were visually fit to the data points. The ordinate intercept is inversely related to the width of the coherent length, and the slope is proportional to the relative fluctuation in the periodicity [23]. The control nerves were in 154 mM NaCl at pH 7.3 (10 mM cacodylate buffer) or pH 5.0 (Na: 10 mM acetate buffer; 2 h). The other nerves were treated for 2 h in 139 mM NaCl (10 mM acetate buffer, pH 5.0) with 10 mM added chloride salts of different divalent metals (Ca, Zn, Cd, Ba).

(b) Sites of swelling and packing disorder in swollen myelin

The relative intensity of Bragg orders 1–5 in control nerve at neutral pH was $2 > 4 > 3 > 5 > 1$. In the partially swollen myelin, the relative intensity of these orders was $3 \gtrsim 2 > 5 \gtrsim 4 > 1$. This change in the distribution of diffracted intensity and the observed increase in period suggested that the myelin membranes swelled apart at their extracellular surfaces while coming closer together at their cytoplasmic apposition [24].

To determine the sites of membrane disorder in myelin swollen under acidic conditions with or without metal salts, we calculated the center-to-center distances of bilayers across their cytoplasmic and extracellular appositions. A plot of these distances as a function of the relative lattice disorder (Fig. 3) reveals that the period and extracellular packing distance increased in parallel to and linearly with increasing disorder, while the cytoplasmic packing distance remained constant. This suggests that disorder in the partially swollen

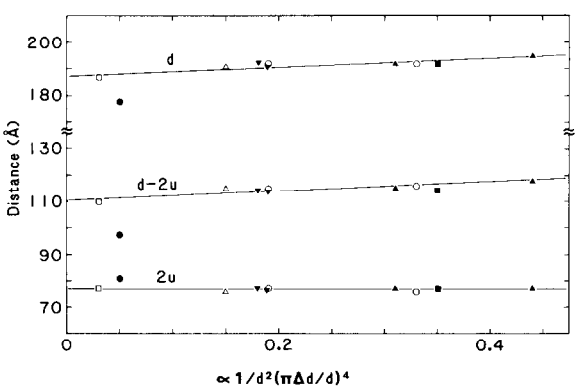

Fig. 3. Dependence of the periods and separation of the membranes at cytoplasmic and extracellular apposition on the relative fluctuation of the period in normal nerve in different metal solution (cited in Fig. 2). d, periodicity; $2u$, separation of the membranes at the cytoplasmic apposition; $d-2u$, separation of the membranes at the exteracellular apposition. Abscissa, reading of slope of the curves in Fig. 2. Symbols: ▲, Na^+; ○, Ba^{2+}; ▼, Ca^{2+}; △, Cd^{2+}, □, Zn^{2+}; ■, Mg^{2+}; ●, native at pH 7.3.

membrane arrays depends primarily on intermembrane contacts at the extracellular apposition.

(c) Effects of $ZnCl_2$ at other pH

Nerves incubated in $ZnCl_2$-saline at different pH generally showed the coexistence of two myelin membrane arrays having different repeat periods. The predominant phase had a period of 186–191 Å, and the additional phase had one of slightly greater than 200 Å at pH 6.0 or 10.6 and a period of 169 Å at pH 4.0. A single structure having a 189 Å period was obtained from nerve treated at pH 5.0 in citric acid buffer (see above), and another single structure having a 173 Å period was obtained at pH 9.7. Control nerve treated at pH 9.7 in the absence of Zn^{2+} gave a myelin period of 180 Å which reverted to its native value when the nerve was returned to saline at neutral pH. Long X-ray exposures from the nerves at pH 9.7 showed sharp reflections to approx. 15 Å spacing (Fig. 4b–d).

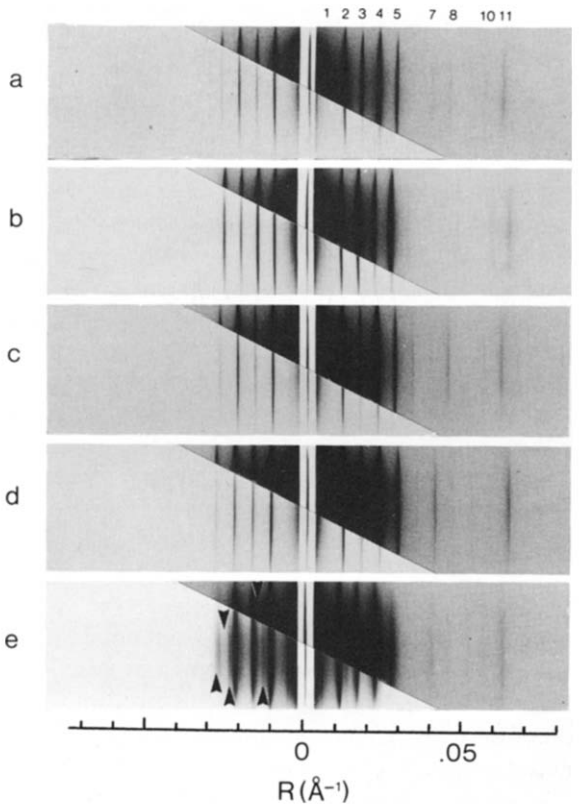

Fig. 4. X-ray diffraction patterns from mouse sciatic nerves. The diffraction orders h for the pattern of native myelin at neutral pH are numbered along the top and the reciprocal lattice coordinate R ($= 2 \sin \theta/\lambda$) is indicated along the bottom. (a) Control myelin in cacodylate buffered saline at pH 7.3, $d = 178$ Å. (b) 10 mM $ZnCl_2$-saline at pH 5.0, citric acid buffer, $d = 189$ Å. (c) Control myelin in bicarbonate buffered saline at pH 9.7, $d = 180$ Å. (d) 10 mM $ZnCl_2$-saline at pH 9.7, bicarbonate buffer, $d = 173$ Å. (e) Same conditions as (d), but shiverer mouse sciatic nerve, $d = 179$ Å. Top films are shown with underfilms or underexposed films in order to display more clearly the weak high-order and strong low-order intensities. Structure amplitudes measured from these patterns are listed in Table I.

(d) Reversibility of $ZnCl_2$ treatments

Both the periodicity and ordering of the swollen structure obtained after $ZnCl_2$-saline at pH 5.0 were preserved after washing out with saline at pH 5.0, 6.0, or 7.3. Ordinarily, treatment with saline at the acid pH would give a partially swollen structure which was disordered (Fig. 1b). Thus, $ZnCl_2$ at pH 5.0 seemed to stabilize an ordered packing of the swollen membrane arrays even after removing excess Zn^{2+}. In contrast, the 173 Å-period structure obtained after $ZnCl_2$-saline at pH 9.7 was not preserved when the nerve was returned to saline at pH 5.0, 7.3, or 9.7. At pH 9.7 the myelin period reverted to a native value, while at the neutral and acid pH, the myelin swelled to approx. 190 Å.

(e) $ZnCl_2$ treatment of shiverer sciatic nerve

The diffraction patterns from shiverer sciatic nerves treated identically as the normal nerves showed similar effects in response to saline at pH 5.0 either with or without 10 mM $ZnCl_2$. That is, the myelin swelled and the reflections were broad in the absence of Zn^{2+} but sharp in its presence. With saline at pH 9.7 shiverer gave a similar pattern as normal myelin. In contrast, treatment with $ZnCl_2$-saline at pH 9.7 produced a different effect in shiverer. While sharp X-ray reflections from a slightly compacted structure were observed from normal myelin, broadened reflections from a near native period structure were observed from shiverer. In addition, shiverer gave extra reflections at spacings of about 74, 64, 41, 39 and 34 Å (Fig. 4e, arrowheads).

(f) Membrane packing and structure

Electron density profiles for normal and shiverer

TABLE I

STRUCTURE FACTORS FOR PRINCIPAL DIFFRACTION PATTERNS

d, periodicity; h, diffraction order; $F(h)$, structure factor; $\delta F(h)$, uncertainty in $F(h)$; R_i, measured uncertainty for each data set calculated from $\Sigma_h \delta F(h)/\Sigma_h |F(h)| \times 100\%$; R_{ij}, calculated from comparison of native structure factors with modified structure amplitudes,

$$= \sum_h ||F_0(h)| - |F_c(h)|| / \sum_h |F_0(h)| \times 100\%,$$

where $F_0(h)$ are measured amplitudes from the modified pattern, and $F_c(h)$ are structure factors calculated by repositioning native membranes in modified unit cell (see Ref. 6 for details).

h	$F(h)$				
	pH 7.3 +/+	pH 5.0, Zn^{2+} +/+	pH 9.7 +/+	pH 9.7, Zn^{2+} +/+	pH 9.7, Zn^{2+} shi/shi
d (Å):	178	189	180	173	179
1	-0.14 ± 0.01	-0.11 ± 0.01	-0.17 ± 0.01	-0.06 ± 0.01	-0.21 ± 0.02
2	1.40 ± 0.06	1.06 ± 0.02	1.28 ± 0.03	1.34 ± 0.04	1.39 ± 0.02
3	0.83 ± 0.04	1.36 ± 0.02	0.74 ± 0.03	0.94 ± 0.02	1.04 ± 0.05
4	-1.42 ± 0.05	-0.91 ± 0.02	-1.52 ± 0.06	-1.21 ± 0.03	-1.44 ± 0.07
5	-0.66 ± 0.03	-1.26 ± 0.02	-0.72 ± 0.02	-0.79 ± 0.03	-0.48 ± 0.08
6	0.08 ± 0.01	-0.09 ± 0.01	0.19 ± 0.02	-0.08 ± 0.01	
7	-0.12 ± 0.02	-0.09 ± 0.01	-0.06 ± 0.02	-0.33 ± 0.02	
8	0.18 ± 0.02	0.0 ± 0.05	0.30 ± 0.02	0.07 ± 0.02	
9	0.0 ± 0.07	0.14 ± 0.02	0.0 ± 0.04	-0.11 ± 0.02	
10	0.20 ± 0.03	-0.08 ± 0.01	0.15 ± 0.02	0.0 ± 0.05	
11	0.39 ± 0.04	0.20 ± 0.02	0.41 ± 0.03	0.53 ± 0.02	
12	-	0.41 ± 0.03	-0.16 ± 0.03	-	
R_i (%)	7.0	4.2	5.8	5.0	5.3
R_{ij} (%)	< 0.1	10.0	12.7	14.7	10.1

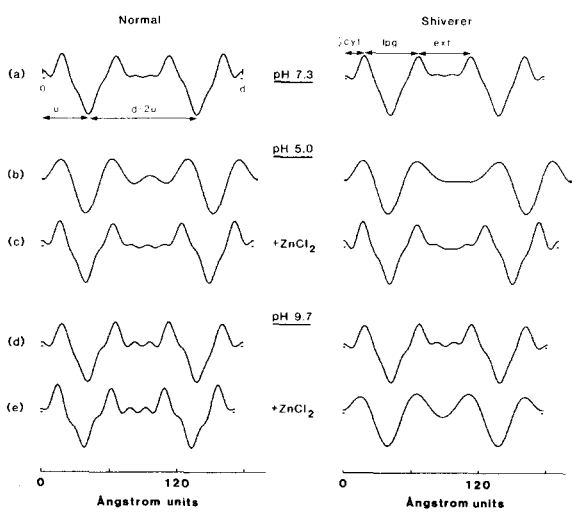

Fig. 5. Electron density profiles calculated from the X-ray patterns shown in Fig. 4 and from additional patterns in Ref. 49. The profiles, which have been offset in the vertical direction for clarity, each show a single membrane pair, with membranes apposed at their extracellular (ext) boundaries. cyt, cytoplasmic boundaries. Features of the profiles from which dimensional parameters were measured are indicated in the uppermost profiles, and the measurements are listed in Table II. (a) Native myelins at pH 7.3. (b) pH 5.0, no added $ZnCl_2$. (c) pH 5.0, with $ZnCl_2$. (d) pH 9.7, no added $ZnCl_2$. (e) pH 9.7, with $ZnCl_2$. (The shiverer nerve at neutral pH was in phosphate-buffered saline. The other shiverer nerves were treated the same as the corresponding normal nerve.)

myelin (Fig. 5) were calculated from the X-ray diffraction data. Table I lists the measured structure amplitudes with phases assigned as described under Materials and Methods. The profiles show alterations in myelin membrane packing and in electron density levels which occurred as a result of treatment with saline at pH 5.0 with or without $ZnCl_2$, or with saline at pH 9.7 with or without

TABLE II

MEASUREMENTS OF THE ELECTRON DENSITY PROFILES (Å)

d, periodicity; $2u$, separation of the membranes at the cytoplasmic apposition which minimizes the R_{ij} value (see Table I and Ref. 6); $d-2u$, separation of the membranes at the extracellular apposition; cyt, cytoplasmic space; lpg, distance between head polar headgroups; ext, extracellular space.

Sample	d	$2u$	$d-2u$	cyt	lpg	ext
+/+						
pH 7.3	178.1	81.2	96.9	33.6	48.8	47.0
pH 5.0 [a]	192.4	78.0	114.4	33.8	45.6	67.5
pH 5.0, Zn^{2+}	188.7	79.0	109.7	32.3	48.1	60.3
pH 9.7	180.0	83.0	97.0	36.9	47.6	47.9
pH 9.7, Zn^{2+}	173.3	76.4	96.9	30.2	48.5	46.2
shi/shi						
pH 7.3	177.7	82.4	95.3	34.0	47.8	48.2
pH 5.0 [a]	202.6	80.4	122.2	33.1	48.3	73.0
pH 5.0, Zn^{2+}	191.8	82.0	109.8	34.1	48.5	60.8
pH 9.7	182.3	85.8	96.5	37.9	48.9	46.7
pH 9.7, Zn^{2+} [a]	178.6	79.4	99.2	32.4	49.8	46.6

[a] denotes values from low resolution profiles (diffraction data approx. 30 Å spacing); all other values are from high resolution profiles.

$ZnCl_2$. Table II lists the dimensional parameters (d, $d-2u$, etc.) for the profiles.

(1) Acidic saline. Electron density profiles for both types of myelin were of low resolution (Fig. 5b) corresponding to the diffraction data which extended only to approx. 35 Å spacing. The separation of the membranes decreased by 2–3 Å at the cytoplasmic apposition ($2u$) while increasing by around 20 Å at the extracellular apposition ($d-2u$). The extent of extracellular swelling in shiverer was more than in normal myelin, and the wider space was accompanied by a flatter electron density level at the extracellular boundary.

(2) Acidic $ZnCl_2$. The period reversed by 3 Å for normal and 11 Å for shiverer myelin, due mainly to shrinkage at the extracellular apposition (ext; Fig. 5c).

(3) Alkaline saline. Electron density profiles (Fig. 5d) compared to those at neutral pH showed that the cytoplasmic space (cyt) became approx. 3 Å wider in both normal and shiverer myelins. In normal myelin, the small peak in the extracellular space and the headgroup region on this side became more prominent. This change in density was the same in the presence of $ZnCl_2$ (Fig. 5e).

(4) Alkaline $ZnCl_2$. Comparison of the profiles (Fig. 5e) with those from myelin treated with alkaline saline in the absence of $ZnCl_2$ shows that in normal myelin there is a shrinkage of approx. 7 Å in the width of the cytoplasmic gap (cyt) and one of approx. 2 Å in the width of the extracellular gap (ext). Also, the electron density levels of the headgroup peak and of the shoulder in the cytoplasmic half of the bilayer increased, presumably due to redistribution of material upon narrowing of the cytoplasmic space. The limited resolution of the shiverer diffraction pattern (Fig. 4e) precluded calculating a high resolution profile; however, at low resolution the packing distance across the cytoplasmic apposition decreased like normal, whereas that across the extracellular apposition increased by several Ångstroms.

(g) Packing of membranes after divalent cation treatments at pH 9.7

To determine whether the appreciable narrowing of the cytoplasmic space in both normal and shiverer myelins after treatment with 10 mM $ZnCl_2$-saline at pH 9.7 is unique to Zn^{2+}, we tested the effects of other metals. X-ray diffraction patterns were recorded from normal and shiverer nerves which had been treated for 6 or 24 h or more at pH 9.7 with saline solutions containing 1 mM chloride salts of various metals. The center-to-center packing of membranes across their cyto-

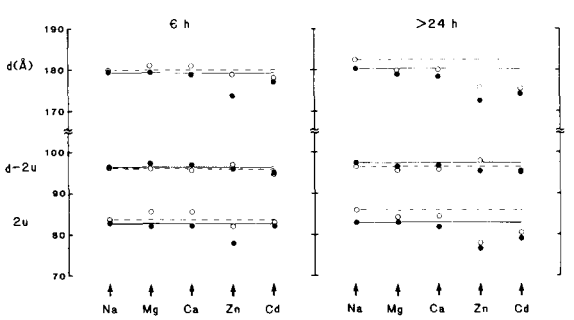

Fig. 6. Packing of myelin membranes in normal (●) and shiverer (○) nerves after treatments of 6 h or 24–38 h with 1 mM metal chloride salts in saline at pH 9.7. d, repeat period; $d-2u$, packing distance at the extracellular apposition; $2u$ packing distance at the cytoplasmic apposition.

plasmic and extracellular appositions was calculated (Fig. 6).

Treatment for 6 h with $ZnCl_2$ produced no net change in period of the shiverer myelin, but reduced the normal period by 5 Å. In the shiverer, a slight increase in the extracellular separation of membranes was offset by a corresponding decrease in the cytoplasmic separation. In the normal, the decrease in period was wholly accounted for by a shrinkage of the cytoplasmic separation. After longer treatment with $ZnCl_2$, the period decreased in shiverer about as much as it did in normal (approx. 6–8 Å), mostly due to compaction of the cytoplasmic space. Other metal salts also modified the periodicity and packing of myelin, but none did to the same extent or in the same way as did $ZnCl_2$ (see Fig. 6).

Discussion

Our studies on the effects of metal cations on peripheral nerve myelin demonstrate that $ZnCl_2$ modifies the interactions between membranes in a unique way. Comparisons between normal and shiverer myelins suggest that the P_0 glycoprotein is likely involved in these altered interactions at the extracellular as well as at the cytoplasmic apposition.

(a) Zn^{2+} preserves regularity of membrane packing in swollen myelin

When myelin swells in hypotonic media [25,26] or in acidic saline [27;and this study], the X-ray reflections become broader and discrete higher orders are not observed, indicating disordering of the membrane packing. We found that the addition of divalent metal cations to acidic saline tended to reduce the amount of swelling and to sharpen the X-ray reflections. The addition of Zn^{2+}, in particular, resulted in reflections which were at least as sharp as the native ones (recorded at pH 7.3) and which were visible to 15 Å spacing. The presence of sharp, higher orders indicates that in the partially swollen myelin the regularity of the membrane packing is maintained with Zn^{2+}. The relation between the relative disorder and the period or distances in the membrane pair ($2u$, $d-2u$) indicates that membrane arrays in native myelin (at pH 7.3) should be more ordered than they are. Perhaps steric hindrance prevents a more ordered close-packing of the membranes.

The swelling of membranes at their extracellular apposition at acid pH in the absence of added metal cations can be accounted for by the adsorption of protons which would increase the electrostatic repulsion between membrane surfaces. Since this swelling was reversible (Ref. 27; and this study) it is unlikely that the acidic conditions produced a chemical modification. Added metal cations seemed to accelerate the repulsion, yet the magnitude of the separation was less with these cations, suggesting a more specific binding. Such specificity is supported by the preservation of the regularity of membrane packing after the washout of excess Zn^{2+}, probably due to the retention of these cations in the arrays.

The relatively low atomic number of Zn^{2+} does not allow us to localize it as an electron dense label in the membrane density profile, so we must infer its position and interaction from previous studies on other systems. Zn^{2+} has been reported to interact with lipids in model bilayers; e.g., it will associate with phosphatidylcholine [28] with which it can form a 1 : 1 double salt [29], it complexes with phosphatidic acid [30], and it binds to phosphatidylserine about twice as strongly as does Ca^{2+} [31]. Interaction with lipids, however, does not easily explain how Zn^{2+} could modulate the close contacts between myelin membranes, contacts that occur in the extracellular space about 20 Å or more from the lipid headgroups in swollen myelin.

Another possibility is interaction with protein. For example, the stabilization of lysosomal membranes [32] or of plasma membranes by Zn^{2+} during their isolation [33] may be due to an interaction between Zn^{2+} and cytoskeletal protein [34,35]. A corresponding interaction between Zn^{2+} and a myelin protein may underlie the Zn^{2+}-induced regularity of membrane packing in partially swollen myelin. Myelin basic proteins are unlikely to be involved since normal myelin and the basic protein-deficient shiverer myelin respond similarly in acidic $ZnCl_2$-saline, and since the major basic proteins are localized in the cytoplasmic and not in the extracellular half of the membrane [8–10]. Rather, binding sites for Zn^{2+} may be present on the P_0 glycoprotein which pro-

jects into the extracellular space between myelin membranes in peripheral nerve [14,36,37]. Possible sites on P_0 which are negative at pH 5 include: the β-carboxyl of aspartic acid, with pK_a 3.0–4.7, and the γ-carboxyl of glutamic acid, with pK_a approx. 4.4 (pK_a values from Ref. 38). These amino acids together comprise nearly 20 mol% of the groups in P_0 [39].

(b) $ZnCl_2$ reveals reduced stability of shiverer peripheral myelin

In alkaline saline containing $ZnCl_2$, shiverer myelin was less stable than normal myelin. The broad reflections of the predominant lamellar phase in shiverer indicate disarray of the membrane packing. The extra reflections, which do not index on a single fundamental repeat, are consistent with a separation of lipid phases from the myelin membranes. The spacings of these reflections (74, 64, 41, 39 and 34 Å) are similar to or in the range of those recorded from the separate lamellar phases of the lipids which are present in nerve [40], e.g., phosphatidylcholine, 69 Å; cerebroside, 70 Å; sphingomyelin, 78 Å; sulfatide, 65 Å; dehydrated phosphatidylserine or phosphatidylethanolamine, 44 Å; and cholesterol, 34 Å.

Phase separation in the shiverer could come about by the binding of Zn^{2+} to lipids which become negatively-charged at alkaline pH. For example, phosphatidylserine and phosphatidylethanolamine in myelin could lose some of the protons from their primary amino groups [41]. These lipids may be more exposed because of the absent basic proteins.

In addition to the phase separation after treatment with alkaline saline + $ZnCl_2$, the packing of myelin in the predominant phase of shiverer was different than in normal myelin. The greater cytoplasmic shrinkage with short treatment in normal myelin compared to shiverer may be due to interactions between Zn^{2+} and myelin basic proteins, whereas the comparable cytoplasmic shrinkage obtained after long treatment may come from a second site of Zn^{2+} interaction, for example, on the P_0 glycoprotein, which would be present in both normal and shiverer myelins. Groups on the basic proteins and on P_0 which could react at alkaline pH [38] with Zn^{2+} are listed with their amounts in mol% [39]:

	Basic protein	P_0 glycoprotein
Histidine (imidazolium, pK_a 5.6–7)	6	2.5
Tyrosine (phenolic-OH, pK_a 9.8–10.4)	2	1.6
Lysine (ϵ-amino, pK_a 9.4–10.6)	7	10.6
Cysteine (–SH, pK_a 8–9)	0	5.1

(c) What modulates interactions between extracellular surfaces of myelin membranes?

In physiological solution the lipid headgroups in the extracellular half of the bilayer are separated by about 47 Å across the external space. The membranes cannot come together more closely unless the intrinsic membrane protein is laterally displaced [13,14,42]. Under hypotonic conditions the myelin membranes become separated at the extracellular boundaries by 85–180 Å or more [26,43]. The extent of maximum swelling, which is constrained by the connective tissue surrounding the individual myelinated fibres, is indefinite in the absence of such constraints [44]. The connective tissue does not seem to affect myelin swelling until a period of approx. 240 Å or more is attained [44]. Considering the great width of the intervening fluid layers between the membrane pairs, it is very unlikely that any molecular contacts remain between the extracellular surfaces in hypotonically-swollen myelin.

In partially-swollen myelin in which the period is approx. 190–200 Å, however, the separation between the extracellular surfaces of membranes is about 60–70 Å (Fig. 5 in Ref. 27, and this study). Since this swelling is limited, molecular contacts between the apposed surfaces are probably still present. The fact that the swelling is observed in both normal and shiverer myelin suggests that the contacts are likely between molecules of P_0 glycoprotein which is the major protein present in both types of myelin [9] and is believed to project into the spaces between the extracellular surfaces of the membranes [14,36,37]. Under the conditions for obtaining moderate swelling, P_0 may undergo a conformational change which allows continued contact between these molecules across the widened extracellular gap.

The major source of disorder in the partially-swollen membrane arrays is due to variation in the

width of the extracellular space. The amount of variation, or the relative lattice disorder, depends on the cations present. Zn^{2+} has a charge density greater than that of any of the other cations examined [45]. Therefore, Zn^{2+} which binds to groups at the membrane surface may be particularly effective in ordering the hydration water around the interacting molecules at these apposed surfaces. This may prevent the usual disordering of the swollen membrane arrays seen under acidic conditions.

Curiously, the extent of extracellular swelling in the basic protein-deficient shiverer myelin was consistently greater than in normal myelin. This was observed after treatment with acidic saline (no $ZnCl_2$) or with alkaline saline containing $ZnCl_2$. Since the major basic proteins are localized to the cytoplasmic side of the membrane [8–10], our finding might be due to a difference between shiverer and normal in the charge density of their extracellular surfaces. The chemical basis of this more subtle difference between shiverer and normal myelins remains to be determined. An alternative explanation is that the localization of P_0 glycoprotein in the bilayer and its conformation in the extracellular half may depend on an interaction with basic protein in the cytoplasmic half. The absence of basic protein from shiverer myelin may result in a potential for altering P_0-dependent membrane contacts at the extracellular apposition.

(d) What modulates interactions between cytoplasmic surfaces?

Experimentally-induced alterations in the width of the cytoplasmic space between myelin membranes are small compared to those of the extracellular space. A number of different factors account for the changes which have been produced. In hypotonically-swollen myelin the separation between cytoplasmic surfaces decreases by approx. 5 Å and reverses to its native width when ions are restored [46]. Increased dipolar attraction at low ionic strength could account for this shrinkage [47]. Fixation with glutaraldehyde or osmium tetroxide also results in a 5–7 Å decrease in the cytoplasmic separation of membranes, probably due to crosslinking between molecular groups on the apposed surfaces [24]. In both these cases of cytoplasmic compaction there is an accompanying swelling at the extracellular apposition.

Other treatments result in swelling of the cytoplasmic space. Low resolution X-ray patterns from peripheral nerve incubated in hypertonic saline [25] indicate that the membranes separate at their cytoplasmic apposition by about 5 Å [46]. Cytoplasmic swelling has also been viewed in thin-section [42] and in freeze-fracture replicas [13] of nerves treated under certain conditions with $CaCl_2$ or tetracaine; electrostatic repulsion of positively charged surfaces resulting from bound cations likely accounts for this swelling. In shiverer myelin the cytoplasmic separation is typically greater by 1–2 Å than that found in normal myelin (see Fig. 1 in Ref. 9). The absence of myelin basic proteins from the cytoplasmic side of the membrane could enhance the negativity of the apposed surfaces resulting in greater electrostatic repulsion and a wider cytoplasmic space.

We now report that treatment of nerve with alkaline saline is also sufficient to produce swelling at the cytoplasmic apposition, probably due to electrostatic repulsion between negatively-charged membrane surfaces. Since this swelling was reversible it is unlikely that the alkaline conditions produced a chemical modification. In shiverer myelin the swelling at this apposition is greater than normal, probably due to enhanced electrostatic repulsion in the absence of basic protein. In normal myelin it is unlikely that basic protein at the cytoplasmic side could account for the cytoplasmic swelling; rather, P_0 might. This protein is believed to be transmembrane, and has the appropriate amino acid side chains which could become more negative under alkaline treatments. The reversal of swelling and compaction of the cytoplasmic space by Zn^{2+} suggests that there is a specific binding of this cation to such groups, probably on P_0, which may underlie molecular contacts at the cytoplasmic apposition.

(e) P_0 glycoprotein: transmembrane protein with contacts at both surfaces

X-ray and neutron diffraction studies indicate that the amount of protein spanning the hydrocarbon layer is similar to that in the aqueous spaces between membrane surfaces [48]. Results from freeze-fracture electron microscopy [12], and from correlated freeze-fracture and X-ray diffrac-

tion studies on normal [13,14,42] and on shiverer myelin [36] indicate that most likely it is P_0 which extends across the membrane bilayer since this is the major intrinsic protein in peripheral nerve myelin, accounting for about 60% of its total protein content [39]. A small electron dense shoulder in the extracellular space extends about 40 Å from the lipid headgroup region and likely comes from a hydrophilic portion of P_0 glycoprotein [37]. In myelin treated with alkaline saline the enhancement of this shoulder and the accompanying increase in the headgroup density could be due to a conformational change in P_0. The altered packing of membranes that we find at both cytoplasmic and extracellular appositions under acidic and alkaline conditions, with and without $ZnCl_2$, can be most easily explained by effects involving amino acid side chains on P_0 which become negatively or positively charged depending on pH. Alterations of charge-dependent interactions between P_0 and particular lipids or between molecules of P_0 could also contribute to the observed structural changes.

Conclusions

These studies on the effects of $ZnCl_2$ on myelin structure in normal and shiverer peripheral nerves demonstrate that Zn^{2+} modifies the interactions between myelin membranes in a unique way. Our findings suggest that molecular interactions between Zn^{2+} and specific proteins, particularly P_0 glycoprotein, and lipid components of myelin underlie these effects. Conclusions drawn from experiments on intact myelin will be tested by studies on the interaction of $ZnCl_2$ with model membranes.

(1) Zn^{2+} preserved the stacking regularity of membranes in acid-swollen myelin, possibly through interactions with portions of the P_0 glycoprotein (e.g., glutamic and aspartic acid residues) that extend into the extracellular space between membrane bilayers.

(2) Zn^{2+} induced a lipid phase separation in shiverer nerves in alkaline saline, most likely by binding to phosphatidylethanolamine and phosphatidylserine. These lipids become more negative in this pH range and may be inaccessible to Zn^{2+} in normal myelin where the major basic proteins are present.

(3) Zn^{2+}, but not other metals, at low concentration in alkaline saline caused cytoplasmic compaction in normal myelin, presumably due to interaction with myelin basic protein. At high concentration in alkaline saline, Zn^{2+} caused cytoplasmic compaction in both normal and shiverer myelins, perhaps through interactions with a portion of P_0 glycoprotein that extends into the cytoplasmic space between membranes. Possible reactive groups on these proteins at this pH include those on histidine, tyrosine, lysine and cysteine.

(4) Acidic saline or Zn^{2+} at high concentration in alkaline saline caused swelling at the extracellular apposition in shiverer but not in normal myelin. This may be accounted for by a chemically uncharacterized difference between the extracellular surfaces of normal and shiverer myelins. Alternatively, the absence of basic proteins from the cytoplasmic half of the bilayer may allow for a configurational change in the P_0 glycoprotein in the extracellular half.

Acknowledgement

We thank Mary Ann Hsu and Ton-Yun C. Fang for assistance with the X-ray film measurements; and Wolfgang G. Busse and William P. McIntosh for expert photographic services. The research was supported by NIH grants NS 20824 (D.A.K.) and NS 11237 (to Dr. R.L. Sidman) from the National Institute of Neurological and Communicative Disorders and Stroke. The work was carried out in facilities related to the Mental Retardation Research Center at Children's Hospital, Inc., and was supported by NIH Core Grant HD 06276. Preliminary reports of some of this work have been presented [49,50].

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