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Surprising thermal transition in fish myelin

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A new structural transition in nerve myelin has been discovered by means of X-ray diffraction of excised teleost nerves in physiological saline. The reversible transition is between two structures, designated AS and AL, with repeating distances (*d* spacings) differing by 25–35 Å. When the temperature of bream spinal cord is lowered from room temperature to 4°C, much but not all of the AS (short spacing) myelin changes into AL (long spacing) myelin. The change is reversed when the temperature is raised back to 22°C, and it occurs a second time when the temperature is lowered again to 4°C. The myelin in bream optic nerve undergoes a similar thermal transition, but the myelin in brachial plexus does not. The thermal transition does not involve the liquid crystal-to-gel transition observed in lipids and natural membranes. When a specimen is kept at constant temperature, there is a gradual conversion from AS to AL myelin which is not thermally reversible, suggesting the existence of two distinct subclasses of AL. Similarly, two subclasses are indicated for AS myelin since part of it does not transform thermally. The observations reported here may have significance for the evolutionary development of myelin.

Nerve myelin consists of a multilayer of specialized membranes encircling a nerve axon [1]. A non-myelinated axon must have several hundred times the cross-sectional area in order to conduct as rapidly as the larger myelinated axons [2]. The myelin sheath therefore permits astonishing economies of space, biogenic material and metabolic energy in the complex nervous system [1].

The X-ray diffraction patterns of various myelinated nerves from a number of terrestrial vertebrates fall into two classes, corresponding to the central (CNS) and peripheral (PNS) nervous systems [3–11]. Any one nerve generally gives a series of evenly spaced X-ray reflections, indicating a single repeating distance in the myelin multilayer. The repeating distance in PNS myelin (170 to 185 Å depending on species) invariably is greater than in CNS myelin (150–165 Å). In addition, although the reflection intensities always favor even over odd orders, there are characteristic differences in detail between the intensity distributions from CNS and PNS myelins; these differences relate to different spaces between the lipid bilayers in the respective myelins [7,12]. The two myelin classes are here designated TS (terrestrial short spacing, CNS) and TL (terrestrial long spacing, PNS). No intermediate class has been found, even at the junctional zone between the CNS and PNS [10].

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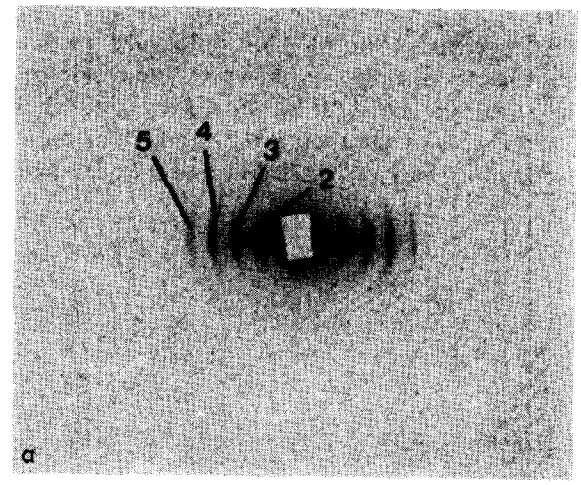
Given these generalities for terrestrial vertebrate myelin, it was surprising to discover in how many ways myelins of teleost fishes differ. First, the diffraction patterns of freshly excised CNS and PNS myelinated nerves of teleosts are nearly identical to one another [4–6,11,13], indicating similar membrane structures. Second, the diffraction pattern of a given nerve can show two series of reflections [5,6,11]; a predominant series indicating a repeating distance of approx. 150 Å, here designated AS (aquatic short spacing, found in CNS and in PNS), and a weaker series indicating a repeating distance of approx. 180 Å, denoted AL (aquatic long spacing, also CNS and PNS). Third, the AL series can intensify with time after dissection, indicating that myelin with the original AS structure gradually converts to the AL structure, even to the point that AL predominates [5,11,14]. Fourth, the intensity distribution in the AS series of reflections from teleost nerves generally is not the same as in either series from terrestrial vertebrates [4–6,11,13,15]. In particular, orders 3 and 4 have comparable intensity in the AS series (Fig. 1) whereas order 3 is much weaker than order 4 in both the TS and TL series. As in the case of TS vs. TL myelin, the different intensity distribution for AS vs. either of the T series is due primarily to different interbilayer spaces [12]. Despite these clear differences, the AS and AL repeating distances do fall within the ranges for TS and TL, respectively, and the AL intensities (Fig. 2, inset at lower right) resemble the TL series.

Terrestrial vertebrate myelin has a remarkably stable structure [9], undergoing no obvious change when kept in saline for many weeks (unpublished observation of Chandross, R.J. and Bear, R.S.) or when exposed at 4°C [16]. The observations below show that this is not the case for the lower, aquatic vertebrates. We discovered that the temperature during X-ray exposure is a key determinant of structure in fish myelins, and that the temperature history of an excised specimen also influences the structure. Our findings raise questions as to whether AL myelin occurs in vivo under appropriate conditions and, if so, how it might function.

Specimens of bream (*Lepomis macrochirus*) spinal cord, optic nerve and brachial plexus were excised immediately after sacrificing the fish. Nerve specimens were sealed with physiological saline (120 mM NaCl, 2.5 mM KCl, 3 mM $CaCl_2$, 2 mM $NaHCO_3$, 5 mM glucose (pH 7.3)) in thin-wall glass capillaries to prevent drying during X-ray exposure; previous work established that the added saline does not affect the diffraction pattern [5,11]. Specimens were exposed in a Franks-type point-focusing small-angle diffraction camera [16] using X-rays predominantly of wavelength 1.542 Å (Cu K_α). Temperature of the specimen chamber was controlled by a thermostated circulating bath. Diffraction patterns were recorded on a stack of films [16].

The patterns included the usual concentric pairs of arcs (Fig. 1). A single myelinated axon, held straight, would give rise to diffraction spots restricted to a line at right angles to the long direction of the axon (reviewed in Ref. 12). A number of myelinated axons contribute to a pattern, however, and the arcing therefore indicates that these are not all parallel to one another. Repeating distances (d spacings) were computed from the diffraction angles of the arcs using Bragg's law [12]. Given sufficient exposure, two diffuse rings were visible at larger distances from the center (Fig. 3); the inner ring relates to the packing of the lipid chains while the outer ring arises from the water in the specimen.

In order to quantitate the proportions of AS and AL myelin, radial densitometer tracings of the patterns were made using a scanning densitometer as in Ref. 16. Tracings were corrected for the arcing and the Lorentz effect as well [16]; the latter accounts for decreasing amounts of myelin contributing to succeeding diffraction orders. Areas of the peaks in the corrected tracings were measured above a diffuse background (dashed line in Figs. 1 and 2); the intensity below this line has been attributed to disordered layering of the myelin membranes [16]. In mixed patterns, i.e. AS plus AL, the combined areas of two overlapping orders (2 with 2′, 3 with 4′, 4 with 5′, 5 with 6′) were divided up according to visual inspection of the films. Because they were very intense, orders 2 and/or 2′ in Figs. 1 and 2 were truncated by the densitometer [16]; applying the correction for arcing and Lorentz effect then reduced the truncated peaks relative to orders of greater index. Areas of peaks 2 and 2′ therefore were measured



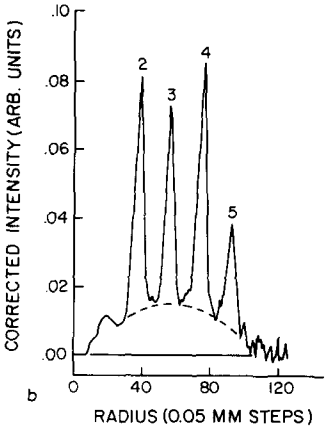


Fig. 1. (a) Small-angle X-ray diffraction pattern of bream brachial plexus at 22°C. The numbered reflections are orders of a 155 Å spacing, i.e., AS series. 3 h exposure begun 8 h after dissecting the fish. The specimen-to-film distance was $s = 9.25$ cm. (b) Corrected radial densitometer tracing of (a), obtained as described in Ref. 16. Note that the height of order 2 has been reduced as the result of an experimental artifact (see text). Although order 3 in (a) looks as intense as order 4, the correction makes peak 4 somewhat higher than peak 3.

on underlying films and scaled to the tracings here [16].

Because of its simplicity, we begin with the diffraction pattern from freshly excised bream brachial plexus at room temperature, Fig. 1a. This pattern includes four strong, equally spaced arcs to each side of center. The arcs, termed diffraction orders, are indexed according to their distance from the center. A single d spacing, $d = 155$ Å, accounts for all the arcs. The pattern is an example of the AS class from a PNS specimen.

A pattern from freshly excised bream spinal cord also showed four prominent pairs of arcs. The intensities of the arcs were similar to those from the brachial plexus, order for order, and the d spacing was nearly the same, $d = 153$ Å. Thus the initial pattern is another example of the AS class, but this time from a CNS specimen. A radial tracing of the subsequent pattern from spinal cord (Fig. 2, inset at upper left) includes both the AS series (unprimed indices) and a second, weaker series of equally spaced peaks (primed indices) that are not related to the AS series. All of the additional peaks are accounted for by a second d spacing, $d = 181$ Å, and therefore this is an example of the AL series from a CNS specimen. The AL series slowly intensifies at room temperature, indicating a gradual conversion of myelin from the AS to the AL structure. Other teleost fishes [5,6,11,14] and even an invertebrate crustacean, shrimp (Blaurock, A.E., unpublished data), can exhibit AS and AL patterns. Although the PNS myelin of skate, an elasmobranch, initially gives a pattern more nearly resembling TL than AS, this myelin also converts to a spacing larger by approx. 30 Å (Refs. 13,17; Blaurock, A.E., unpublished data).

In seeking to understand the relation between AS and AL myelins, we first tested whether the proportions of myelin belonging to the AS and AL classes in teleost nerves are sensitive to environmental temperature. Acclimation of two different fishes (bream, goldfish) to different temperatures ($T_a = 4$, 16 or 23°C) for three weeks or more had no obvious effect, i.e., the AS series always predominated in patterns from freshly dissected specimens exposed at T_a.

We then tested the influence of the temperature of the excised specimens (T_e) on the myelin diffraction pattern. An exposure of the bream spinal cord after lowering T_e to 4°C, begun two days after dissection, shows a marked increase in the intensities of the AL series, inset at lower left in Fig. 2. At the same time, the AS series becomes less intense. The intensity of order 4′, relative to orders 3 and 4 on either side of it, is a good indicator of the change. Thus cooling causes order

4′ to increase greatly relative to orders 3 and 4, indicating a major change in the proportions of myelin having the two *d* spacings (cf. insets in Fig. 2 at upper and lower left). No intermediate *d* spacings are observed.

The extent of the change has been estimated in terms of the percentage of the total intensity (AS + AL) occurring in the AS reflections in the densitometer tracings. As shown by the crosses in Fig. 2, myelin having the AS structure is estimated at about 80% of the total myelin in bream spinal cord kept for half a day in saline and then exposed

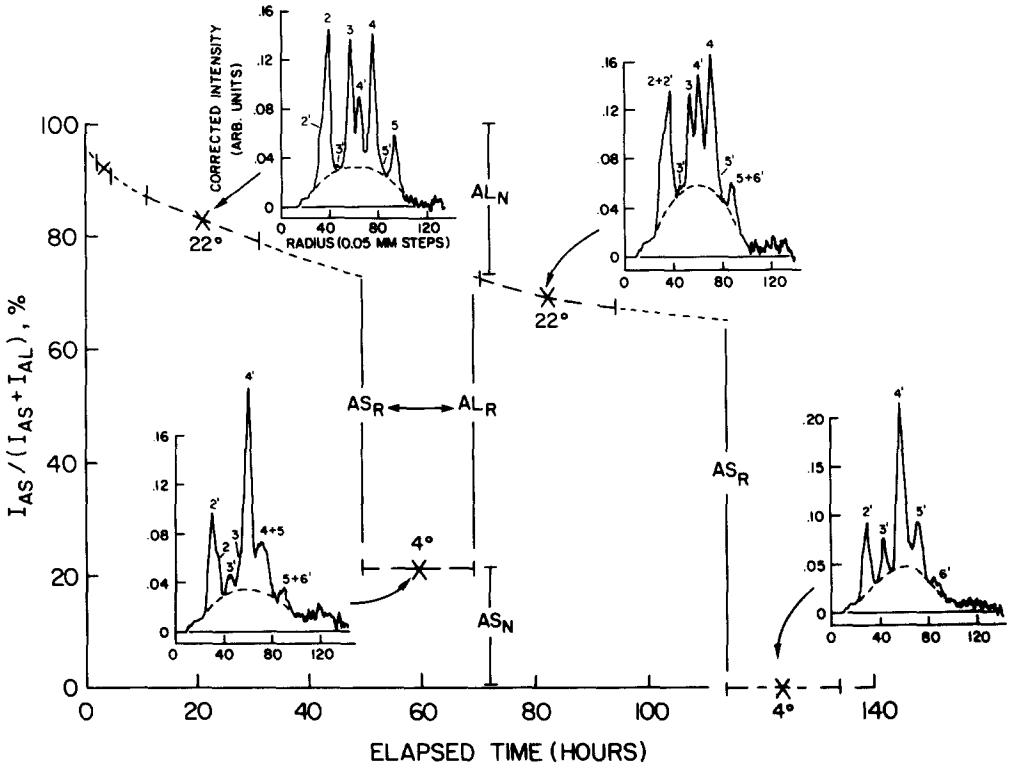


Fig. 2. Proportions of AS and AL myelins in bream spinal cord as a function of temperature and elapsed time. For each X-ray exposure, a cross (×) indicates the AS intensity (%) as measured on the adjacent corrected densitometer tracing; numerical values are given in Table I. Each cross is plotted at the mid-time of the exposure, and the short vertical bars to either side indicate beginning and ending times of the exposure. The amount of AS (or AL) myelin is proportional to the total AS (or AL) intensity [30]. $s = 8.85$ cm except as noted. The first pattern, recorded shortly after dissection, was similar to Fig. 1 and therefore is not shown. Corrected tracings of four subsequent patterns are displayed. Note that orders 2 and/or 2′ have been reduced by an experimental artifact (see text); areas of these peaks were measured on tracings of underlying films and then scaled to the tracings here [16].

Upper left inset: Radial tracing of second exposure of bream spinal cord at 22°C. The primed reflections of the AL series ($d = 181$ Å) are much less intense than the unprimed reflections of the AS series ($d = 153$ Å), e.g., order 4′ is less intense than neighboring orders 3 and 4. 20 h exposure. $s = 9.25$ cm.

Lower left inset: Exposure of same specimen at 4°C. Order 4′ ($d = 181$ Å) has become much more intense than orders 3 and 4. 20 h exposure.

Upper right inset: Exposure of the same specimen at 22°C again. Order 4′ ($d = 181$ Å) is about as intense as orders 3 and 4. 26 h exposure.

Lower right inset: Exposure of same specimen at 4°C again. The primed reflections are orders of a 188 Å spacing, i.e., AL series. The AS reflections are not visible. 20 h exposure.

The dashed line through the crosses at 22°C has the general form found for bream, goldfish [14] and carp spinal cord [11] kept at all times at 22°C; similarly for the 4°C lines. The 22°C line separates the AL_N subclass (above the line) from the other three subclasses, and the 4°C line similarly separates the AS_N subclass (below the line) from the other subclasses. AS_R and AL_R are revealed by alternating between 22°C and 4°C.

for a day at room temperature, but only about 20% of the total is AS myelin after subsequently cooling to 4°C.

Fig. 2 also shows that the change is largely reversible. Thus after raising T_e from 4°C back to 22°C, order 4′ decreases relative to orders 3 and 4 (cf. insets in Fig. 2 at lower left and upper right). Measurement shows that about 70% of the spinal cord myelin now has the original AS structure. Lowering T_e to 4°C for a second time transforms virtually all of the myelin to the AL structure; the AS reflections are undetectable in this case (inset in Fig. 2 at lower right). Thus about half of the bream spinal-cord myelin can be cycled between two structural states having d spacings differing by approx. 30 Å.

As shown in Table I, bream optic-nerve myelin can be similarly cycled. About 85% of the myelin had the AS structure at room temperature, while lowering T_e to 4°C reduced the AS myelin to about 30% of the total. Raising T_e to 16°C restored much of the myelin to the original AS structure. Thus about half of the myelin in optic nerve, which is a CNS tract, also undergoes a reversible thermal transition.

Unlike spinal cord and optic nerve, the thermal transition did not occur in bream brachial plexus myelin when T_e was lowered and raised again (Table I). Thus the AS pattern in Fig. 1 also is representative of exposures at room temperature after one week in saline and, with minor changes of d spacing and intensities, at $T_e = 4$°C. The slowness of the conversion from AS to AL in brachial plexus kept at constant temperature, and the failure of the thermal transition to occur at all, are the chief respects in which this PNS specimen differed from bream CNS specimens in our diffraction studies.

The results in Fig. 2 and Table I help to define the nature of the changes occurring in bream myelins (see also Addendum at end of report). We distinguish two kinds of structural change: a conversion from AS to AL, not thermally reversible, that occurs in CNS and, more slowly, in PNS myelin at room temperature; and a reversible thermal transition that occurs in CNS myelin. The two kinds of change together suggest the existence of four different subclasses of myelin. Two of them are subclasses that do not change with temperature, e.g. the 21% myelin retaining AS structure when the temperature of the spinal cord was lowered to 4°C for the first time, denoted AS_N; and the 31% myelin retaining AL structure after the temperature subsequently was raised back to 22°C, denoted AL_N. The other two are subclasses that transform reversibly into each other as the temperature is changed back and forth between 22°C and 4°C. These subclasses, denoted AS_R and AL_R, account for about half of the total myelin in spinal cord at elapsed times around 60 h (and for a similar proportion in optic nerve around 300 h; Table I). It follows that at 22°C, the total myelin would be the sum of $AS_N + AS_R + AL_N$; at 4°C the total would be $AS_N + AL_R + AL_N$.

We emphasize that we have not distinguished AS_R from AS_N on the basis of X-ray diffraction: only one AS-like d spacing is evident in any given pattern, and the intensity distribution in the AS pattern appears unchanged despite the presence of the two subclasses (AS_R and AS_N) in various proportions in our specimens. These observations point to similar structures for AS_R and AS_N; the

TABLE I

THERMAL TRANSITION IN BREAM MYELINS

I_{AS} and I_{AL}, total intensity of the reflections in the AS and AL series, respectively, measured on the corrected densitometer tracing.

Specimen	Time from dissection to start of exposure (h)	T_e (°C)	$\frac{I_{AS}}{(I_{AS}+I_{AL})}$ (%)
CNS			
Spinal cord	2	22	92
	11	22	83
	50	4	21
	70	22	69
	96	34	65
	114	4	0
Optic nerve	5	22	86
	312 [a]	4	33
	320	16	78
PNS			
Brachial plexus	8	22	> 99
	32	22	98
	144 [a]	4	90
	168	16	90
	192	34	90

[a] Specimen sealed in capillary after dissection and refrigerated until starting series of two (optic) or three (plexus) exposures.

same holds for AL_R and AL_N.

Although it conceivably could be a prerequisite, proteolysis cannot be the immediate cause of the AS_R to AL_R transition since the effects of proteolysis would not be reversible. As to the possibility that proteolysis might be the immediate cause of the nonreversible conversion of AS to AL_N, we note that proteolysis does not trigger a conversion in isolated rat CNS myelin [18]. The conversion process continues throughout the long exposures at 22°C [11,14], but the thermal transition appears to go rapidly to completion, as judged by the absence of AS reflections from the radial tracing in Fig. 2 at lower right. Finally, the radial sharpness of the X-ray reflections in both series in Fig. 2 indicates that a number of adjacent membranes in the myelin multilayers have converted or transformed, rather than a single membrane here and there doing so; however, the patterns do not indicate whether all the myelin along any given axon, or even within any given internode, changes at the same time.

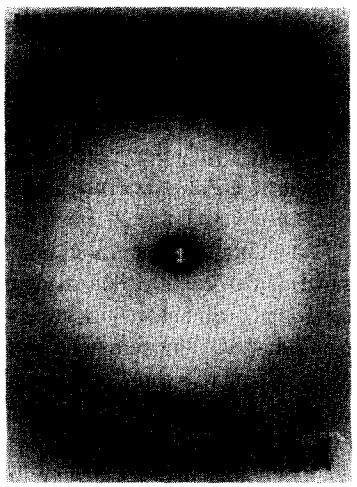


Fig. 3. Wide-angle pattern of bream spinal cord at 4°C. The inner diffuse ring, centered at a diffraction angle corresponding to a 4.6 Å Bragg spacing (arrow), arises from the myelin lipids. A degree of orientation of the 4.6 Å ring is apparent on the original film, as reported for various myelinated nerves [5,11,31]. Much the same ring was observed at 16°C and 29°C. The outer diffuse ring arises from the water in the spinal cord. The absence of a sharp ring with a somewhat larger diameter than that of the 4.6 Å ring indicates that the lipid gel-state structure [19–21] does not occur at 4°C. 8 h exposure begun 214 h after dissection. $s = 5.00$ cm.

There is no obvious indication that the myelin lipids are the basis for the AS_R–AL_R transition. A sharp ring with a d spacing of 4.1–4.2 Å would indicate the gel-state structure in the lipids [19–21], but our patterns at 4°C, 16°C and 29°C all show the diffuse reflection at 4.6 Å that is characteristic of the more fluid, liquid-crystal structure [19–21], and there is no trace of a sharp ring. Fig. 3 shows the pattern at 4°C. In fact, a transition to the lipid gel state would not be expected to occur since the large proportion of cholesterol in fish myelin [22] would suppress the gel state [19,21,23].

In calculated electron-density profiles of the AS and AL structures, the principal difference is a swelling of the spaces between bilayers in going from AS to AL (Blaurock, A.E., unpublished data). For this reason, we have suspected that the AS to AL transition may involve chiefly a rearrangement of the myelin protein molecules. A rearrangement of the lipid molecules conceivably could be involved as well. Rand and Pangborn [23] have published evidence that a structural change occurs at approx. 12°C in a phosphatidylcholine/cholesterol bilayer. Since they observed no change in their 4.5 Å ring [23], a similar rearrangement of the lipid molecules might possibly have occurred in our specimens with no observable effect on the 4.6 Å ring.

Published results with carp [11] and preliminary results with goldfish (*Carassius auratus*) [14] are similar but not identical to those with bream. Inouye, in a very thorough study [11], found that the myelin in excised carp spinal cord, optic nerve and lateral line gradually develops a second, larger d spacing when these tissues are kept in saline for prolonged times at a constant temperature between 15°C and 33°C. The rate of conversion was greater, the higher the temperature. Our results with bream and goldfish [14] generally are in agreement with Inouye's findings; the temperatures studied ($\geq$ 15°C) may well be the reason that Inouye did not induce a thermal transition in carp myelin. Isolated goldfish brain myelin undergoes a thermal transition similar to that in bream spinal cord and optic nerve (Blaurock, A.E., Yale, J.L. and Roots, B.I., unpublished data).

Comparative biochemical, immunological and X-ray diffraction analyses all indicate that important changes occurred in the supramolecular

architecture of myelin when the terrestrial vertebrates evolved. As for several other teleost fishes [4–6,11,13], the diffraction patterns of freshly excised bream PNS and CNS specimens are nearly identical to one another. The diffraction data, together with the presence in both CNS and PNS myelins of a major family of glycoproteins, having similar molecular weights and amino-acid compositions and cross-reacting immunologically with one another [24–26], indicate nearly the same supramolecular architecture for both CNS and PNS myelins in teleost fishes. The same is not true of the terrestrial vertebrates: differences in X-ray spacing and intensity distribution, summarized at the beginning of this report, and different chemical compositions [27,28] indicate distinctive, stable architectures for the TS (CNS) and TL (PNS) myelins [12,29]. It is interesting that this distinction is anticipated in the elasmobranchs (Ref. 17; Blaurock, A.E., unpublished data).

In sum, the comparative studies promise to shed light, not only on the molecular structure of myelin, but also on the evolutionary development of this organelle that is so crucial to the functioning of complex nervous systems [29].

Addendum. The reversible thermal transition reported here held out the prospect of going through a similar reversible transition at constant temperature. This has now been done [32]. As noted by Inouye for carp [11], the gradual forward conversion from AS to AL myelin in excised goldfish spinal cord requires Ca in the physiological saline. A metabolic inhibitor, NaCN, accelerates the conversion in the presence of Ca, but there is no conversion in the absence of Ca, with or without NaCN [32]. After accelerating the conversion with NaCN, the reverse conversion from AL back to AS is brought about by treating with a Ca-free recovery medium lacking NaCN. This back conversion does not occur if NaCN is present in the recovery medium as well [32]. Regarding specificity among divalent cations, Ba and Sr cause only a trace of the AS myelin to convert to AL when they are substituted for Ca in the saline [32]. These results are consistent with the hypothesis that active metabolism is required to maintain the AS structure by preventing the accumulation of free Ca within compact myelin and/or the myelin-generating cells [32].

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