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Alteration of 5′-Nucleotidase and Na^+,K^+-ATPase in Central and Peripheral Nervous Tissue from Dysmyelinating Mutants (jimpy, quaking, Trembler, shiverer, and mld). Comparison with CNPase in the Developing Sciatic Nerve from Trembler

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Abstract: 5′Nucleotidase and Na^+,K^+-ATPase are very probably myelin-associated enzymes, although not specific for this membrane. Thus, it is important to determine their activity in dysmyelinating mutants in either CNS (quaking, jimpy, shiverer, and mld) or PNS (Trembler). *CNS:* The activity of 5′nucleotidase was lower in mouse than in rat (10.5 and 28.0 nmol/min/mg protein in brain, respectively). In mouse myelin, the activity was 30 nmol/min/mg protein (and 72 in rat myelin). In mutants, the brain activity was very close to normal. In contrast, ATPase, the activity of which was higher in myelin as compared with forebrain homogenate, presented a reduced activity in various 21-day-old and adult mutants, except Trembler. It was normal in 8-day-old quaking and in cerebella from mutants. *PNS:* ATPase was lower than in brain and reduced in most mutants, this being expected for Trembler and quaking but not for shiverer and mld. 5′-Nucleotidase activity was higher compared with that in brain homogenate (relatively stable between 10-day postnatal and adult). It was affected in the mutants; in Trembler it was nearly normal in young animals but increased during development. Thus in Trembler, two different myelin-related enzymes and a myelin-specific enzyme (CNPase) presented different developmental patterns: ATPase was always reduced, 5′-nucleotidase was normal, and CNPase was slightly below normal in young (68% of the control value); CNPase activity declined during development but 5′-nucleotidase increased (42% and 190% of the control in 60-day-old animals). It is necessary to consider these results in parallel with alterations in the PNS because of Schwann cell abnormalities. Thus, determination of these two enzymes will provide a useful tool to study myelination and myelin assembly under both normal and pathological conditions. **Key Words:** 5′-Nucleotidase—Na^+,K^+-ATPase—Myelin—Mutant mouse—CNPase. **Bourre J. M. et al.** Alteration of 5′-nucleotidase and Na^+,K^+-ATPase in central and peripheral nervous tissue from dysmyelinating mutants (jimpy, quaking, Trembler, shiverer, and mld). Comparison with CNPase in the developing sciatic nerve from Trembler. *J. Neurochem.* **38**, 643–649 (1982).

Although not specific for myelin, 5′nucleotidase is a myelin-associated enzyme (Cammer et al., 1980). It is possible that Na^+,K^+-ATPase follows the same pattern, as it has been shown that its specific activity is important in myelin (Adam-Vizi et al., 1979). Thus, useful information could be provided by determination of their activity in the nervous system from dysmyelinating mutants such as

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quaking, jimpy, shiverer, and *mld* for the CNS, and Trembler for the PNS.

Naido (1962) gave histochemical evidence for the possibility of a locus of 5′-nucleotidase in myelin. Later, subcellular fractionation methods yielded data consistent with the presence of 5′-nucleotidase in myelin: Banik and Davison (1969) showed that this enzyme increased during myelin-ation and that the crude myelin fraction from adult rat brain had a specific activity that was only slightly lower than that of the homogenate. Pilcher and Jones (1970) observed substantial enrichment of 5′-nucleotidase in a crude myelin fraction compared with the homogenate of mouse cerebellum, and Israel and Frachon-Mastour (1970) found that 28.1% of the activity of this enzyme in rat brain was recovered in a crude myelin fraction. Recently, biochemical measurements of the enzyme in purified myelin led to the conclusion that 5′-nucleotidase was actually intrinsic to the myelin in rat (Cammer et al., 1980), in agreement with data obtained by immunochemical methods (Pilcher and Jones, 1970; Vercelli-Retta et al., 1976; Bernstein et al. 1978; Kreutzberg et al., 1978).

Considerable evidence has accumulated to implicate Na^+,K^+-ATPase in the transport of Na^+ and K^+ across the cell membrane. The activity of this ATPase is high in membrane preparations, the highest activity being in membrane from nervous tissue (Skou, 1965). The enzymatic activity is present in glial cells (Kimmelberg et al., 1978); it is highly distributed in synaptosomes (Gilbert et al., 1975; Kissane and Hawrylewitz, 1978; Wu and Phillis, 1978). Moreover, when comparing the enzymatic activity in various brain subcellular particles, Adam-Vizi et al. (1979) found that specific activity was highest in synaptosomes and myelin. Recently, in the optic goldfish nerve, Schwartz et al. (1981) found that the enzyme was located in the node of Ranvier; however, labeling was also seen along the outer myelin margins. Thus, Na^+,K^+-ATPase is possibly a myelin-associated enzyme (Reiss et al., 1979).

The aim of this study was to (1) confirm that 5′-nucleotidase and Na^+,K^+-ATPase are myelin-associated enzymes in CNS myelin from mouse, as previously shown in rat; (2) determine their eventual presence in PNS; (3) evaluate their activity in some dysmyelinating mutants during postnatal development in both CNS and PNS; (4) compare their developmental changes in PNS with CNPase so as to provide some information on the involvement of these enzymes in different steps of myelin formation and assembly.

MATERIALS AND METHODS

Animals

All the amimals were bred in our laboratory. Trembler mice (*Tr*/+) and their controls (+/+) were of the B6-CBA strain, as well as jimpy mice (*jp*/*Y*) and their controls (+/*Y*). Quaking (+*qk*/+*qk*) were marked on repulsion with *T* (controls are short tails, *T*+/+*qk*). Shiverer (*shi*/*shi*) and controls (+/*shi* ?) were C3H-SWV. Mld (*mld*/*mld*) and controls (+/*mld* ?) were of the MDB-DT strain.

Mice were killed by decapitation at different ages. Fresh brains and eventually frozen sciatic nerves were used (when tissue was frozen before use, ATPase was increased in brain but not in sciatic nerve. 5′-Nucleotidase and CNPase remained unchanged after freezing any tissue).

Tissue fractionation

Myelin was prepared according to the method of Norton and Poduslo (1973). It was routinely prepared in the laboratory (Bourre et al., 1978); the purity was checked by electron microscopy (Baumann et al., 1973) lipid analysis (Baumann et al., 1973; Bourre et al., 1977) and protein electrophoresis (Jacque et al., 1972).

Protein measurements

Protein concentrations in brain homogenates and myelin suspensions were determined as described by Lowry et al. (1951).

5′-Nucleotidase

Forebrains were homogenized in 0.32 M-saccharose (15 ml/g fresh weight) and sciatic nerve in 0.32 M-saccharose 1% Triton X-100 (50 ml/g fresh weight). Neither 0.01–5% Triton (from Sigma) nor Sarkosyl inhibited enzymatic activity in homogenate and in myelin in CNS as well as in PNS, in agreement with Arch and Newsholme (1978), but in contrast with the results of Cammer et al. (1980). 5′-Nucleotidase was assayed by measuring the inorganic phosphate liberated from 5′-AMP. The reaction was started by adding proteins (0.1, 0.05, and 0.05 mg, respectively to brain homogenate, myelin, and sciatic nerve) in a reaction mixture (0.2 ml) 100 mM-Tris-HCl, pH 7.5; 12.5 mM-5′-AMP, and 5 mM-$MgCl_2$. The tubes were incubated at 37°C for 20 min, in triplicate for each time point. Under these conditions, enzymatic activity was linear with time and protein concentration. For determination of K_m and V_{max}, 5′-AMP concentration was between 0.02 and 2 mM. Excess 5′-AMP did not inhibit the activity, and the 12.5 mM concentration used in the standard assay provided a value very close to V_{max}. The reaction was stopped by adding 0.3 ml of 2.5 M-H_2SO_4. Phosphate was measured as described by Lindberg and Ernster (1956) with the minor modifications of Cammer et al. (1980). Specific activity of the enzyme was defined as nmol P_i produced/mg protein/min.

Na^+,K^+-ATPase

The tissue was placed in a predrilled homogenizing vessel and homogenized in 10 ml/g of cold twice-distilled water with Potter type homogenizer (1500 r.p.m., 10 strokes; some sciatic nerves were frozen before use). The ATPase activity of 200–500 μg of homogenate protein was estimated in a basic incubation medium containing 40 mM-Tris-HCl, (pH 7.4; 6 mM-$MgCl_2$; 20 mM-KCl, 150 mM-NaCl; the inhibitor ouabain eventually was added (0.7 mM) (Abdel-Latif et al., 1970).

The reaction was started after a 5-min temperature equilibration by addition of 4 mM-ATP (Tris salt). The incubation was carried out in a shaking bath at 37°C for 15 min. The reaction was stopped by addition of 1 ml of ice-cold 10% w/v TCA. The tubes were placed in ice for 20 min. The precipitated protein was centrifuged and the

supernatant was analyzed for inorganic phosphate by the Fiske-Subbarow assay (1925). All assays of ATPase were carried out in triplicate. Appropriate blanks were made from a preparation of zero time incubation.

The difference between total Mg^{2+},Na^+,K^+-ATPase and Mg^{2+}-ATPase (measured in the presence of ouabain) was considered to be Na^+,K^+-ATPase activity. Specific activity of ATPases were defined as nmol of P_i produced/mg protein/min.

CNPase

The sciatic nerves from controls and mutants were homogenized in 0.2 M-Tris-HCl, pH 7.5; 1% Triton X-100 (100 ml/g fresh weight). CNPase was measured according to the method of Sogin (1976), using 2',3'-cyclic NADP as a substrate.

Enzymatic assays

For enzymatic assays (5'-nucleotidase, ATPase, and CNPase) one experiment was performed on three separate series of one or two animals (measurements in duplicate) at least three experiments were performed. Thus a minimum of nine animals of each type was used, and the values are mean values from at least 18 measurements (so as to have valid statistical measurements). The total number of animal used, controls, and mutants was approximately 300.

RESULTS AND DISCUSSION

5'-Nucleotidase and Na^+,K^+-ATPase in normal central and peripheral nervous tissues

Brain. As shown in Table 1, both enzymes are associated with myelin. Brain myelin was enriched two- to threefold in 5'-nucleotidase as compared with homogenate: 30 and 10.5 nmol/min/mg protein in mouse, respectively. For rat brain myelin, the activity was 72 nmol/min/mg protein; and 28 nmol/min/mg protein in the homogenate. Thus, the activity of the enzyme (V_{max}) was lower in mouse brain then in rat brain: 1600 and 4800 nmol/min/g fresh weight, respectively; these values were somewhat higher if compared with previously reported results of Arch and Newsholme (1978). K_m values were also higher in rat brain than in mouse: 290 and 170 μM, respectively.

Taking into account the amount of protein/brain (Table 2), 16% of the 5'-nucleotidase in brain homogenate was obtained in isolated myelin. At least 40% of the myelin was lost during isolation (Norton and Poduslo, 1973). It is likely that myelin accounted for as much as 30% of the 5'-nucleotidase in mouse brain, as previously shown in rat by Cammer et al. (1980).

In good agreement with Adam-Vizi et al. (1979) in rat, mouse brain myelin was enriched two- to threefold in Na^+,K^+-ATPase as compared with homogenate: 232 and 95.5 nmol/min/mg protein. Thus, 12% of the Na^+,K^+-ATPase in brain homogenate was obtained in isolated myelin. As some myelin was lost during isolation, myelin accounted for approximatively 25% of the Na^+,K^+-ATPase in mouse brain. The reduced amount of ATPase originally found in myelin by Norton and Poduslo (1973) is in contrast with our results as well as with those of Reiss et al. (1980) and Adam-Vizi et al. (1979). The reason for this difference is unclear.

Peripheral nervous tissue. In peripheral both Na-K ATPase and 5'-nucleotidase are present (Table 1) as compared with brain homogenate, 5'-nucleotidase was higher, but Na^+,K^+-ATPase was lower.

In brain (Table 2) as well as in sciatic nerve (not shown here), in both young and adult animals, there were no significant differences among strains.

5'-nucleotidase and Na^+,K^+-ATPase in dysmyelinating nervous tissue

5'Nucleotidase. As shown in Table 2, all mutants analyzed presented normal enzymatic activities. As total proteins per brain were only slightly altered in the mutants, the total 5'-nucleotidase activities per brain were altered by only a few percentage points

TABLE 1. *5'-Nucleotidase and Na^+,K^+-ATPase in CNS, PNS, and myelin*

	Homogenate		Myelin	
	Rat	Mice	Rat	Mice
CNS				
5'-Nucleotidase (SA)	28.0	10.5	72.0	30.0
Total activity per brain	—	661	—	95
ATPase (SA)	—	95.5	—	232
Total activity per brain	—	5430	—	656
PNS				
5'-Nucleotidase (SA)	—	16	—	—
ATPase (SA)	—	14	—	—

5'-Nucleotidase and Na^+,K^+-ATPase in CNS and PNS. Specific activities are expressed as nmol/mg protein/min; total activities as nmol/min/brain. Mean values from at least six measurements (seven 60 day-old animals used for one myelin preparation, three preparations measured in duplicate). SA, specific activity. The values for mouse brain homogenates are the mean values from the different controls in Table 2.

TABLE 2. *5′-Nucleotidase and Na⁺,K⁺-ATPase in dysmyelinating mice brains*

	Specific activities (nmol/mg protein/min)								Total activities (nmol/min/brain)							
	5′-Nucleotidase				Na⁺,K⁺-ATPase				5′-Nucleotidase				Na⁺,K⁺-ATPase			
	15 days		60 days		21 days		60 days		15 days		60 days		15 days		60 days	
T+/+qk	9.9	NS	10.8	NS	79	*c*	79	*c*	396	NS	628	*a*	3160	*c*	4819	*c*
+qk/+qk	10.0		9.3		66		67		380		511		2508		3685	
+/shi?	10.2	NS	11.1	NS	66	*b*	83	*a*	408	NS	677	NS	2640	*b*	5063	*a*
shi/shi	13.5		11.6		55		74		513		696		2090		4440	
+/mld?	9.6	NS	11.0	NS	74	*c*	104	*c*	384	NS	671	NS	2960	*a*	6344	*c*
mld/mld	10.0		10.6		59		86		470		614		2673		4988	
+/Y	9.3	NS	—		67	*b*	—		399	NS	—		2680	*b*	—	
jp/Y	9.2		—		50				395				2150			
+/+	9.7	NS	11.0	NS	66	NS	90	NS	388	NS	671	NS	2640	NS	5490	NS
Tr/+	10.0		9.3		58		85		370		585		2146		5355	

Specific activities are expressed as nmol/mg protein/min. Total activities are expressed as nmol/min/brain. For the number of animals and experiments see Materials and Methods.

[a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$, Significant difference from control. NS, Not significant.

in one mutant; they were reduced by 10% in the adult quaking ($P < 0.05$).

This was unexpected as most mutants (quaking, jimpy, shiverer, mld) present considerable reduction of myelin; only Trembler presents a slight increase in brain myelin quantity (Bourre et al., 1980). This absence of correlation between myelination and 5′-nucleotidase activity was very probably due to the presence of the enzyme in various brain subcellular particles (Israel and Franchon-Mastour, 1970), especially in plasma membranes of astrocytes and oligodendrocytes (Kreutzberg et al., 1978).

Na⁺,K⁺-ATPase. As shown in Table 2, in both young and adult animals, there was no difference among the various strains. Trembler was not affected. But in all the hypomyelinated mutants there were very significant reductions in enzymatic activity; however, the reduction was the same in all mutants. This was probably not due to a common inhibitor: after mixing aliquots of quaking and control homogenate, no inhibition could be demonstrated. Increased activity in controls as compared with mutants was not due to some detergent effect of myelin: addition of increasing amounts of boiled myelin (to destroy its ATPase activity) to quaking brain homogenate did not change the activity. Normal Na⁺,K⁺-ATPase was found in the 8-day-old quaking (not yet tremoring): 13 and 16 nmol/min/mg protein in control and quaking.

In contrast with forebrain, cerebellum presented a normal activity in adult mutants (controls: 89.5 nmol/min/mg protein; 87.7 for Trembler; 93.0 for quaking; 93.5 for shiverer; and 86.3 for mld).

5′-Nucleotidase and Na⁺,K⁺-ATPase compared with CNPase in the PNS of the mutants (Table 3). Na⁺,K⁺-ATPase was reduced in all dysmyelinating mutants, in both young and adult animals, Trembler being most affected. In contrast, 5′-nucleotidase was nearly normal in 15-day-old mutants, with the exception of Trembler. In 60-day-old animals, 5′-nucleotidase was generally increased; it was close to normal in mld. Thus, there were unexpected alterations in the enzymatic activities of two membrane-related enzymes in the mutants: one was reduced, the other was increased. Moreover, there were discrepancies between 5′-nucleotidase and Na⁺,K⁺-ATPase activity, biochemical composition, and morphological aspects in the mutants.

For example, in jimpy, under light (Sidman and Hayes, 1965) and electron microscopy (Herschkowitz et al., 1971), the PNS should appear normal; its biochemical composition is normal; thus, normal 5′-nucleotidase was expected. The reduced activity of Na⁺,K⁺-ATPase was unexplained and could eventually be related to the modifications observed in the membrane fluidity (Viret et al., 1979).

Shiverer and mld are alleles (Bourre et al., 1980). The PNS of each is normal in terms of morphology and both mutants have reduced amounts of basic protein in their PNS (Kirschner and Ganser, 1980; Ginalski et al., 1980). Their differing alterations of

TABLE 3. *5′-Nucleotidase and Na,⁺,K⁺-ATPase in sciatic nerve from dysmyelinating mutants*

	5′-Nucleotidase		Na⁺,K⁺-ATPase	
Age (days)	15	60	15	60
Control	16.2	17.2	14.1	14.6
Trembler	24.3[c]	32.9[c]	8.6[c]	7.0[c]
quaking	15.9 NS	20.6[a]	7.2[a]	8.3[b]
shiverer	12.9 NS	22.3[a]	3.9[b]	8.3[b]
mld	14.5 NS	15.4 NS	7.4[b]	7.7[b]
jimpy	16.1 NS	—	8.3[a]	—

Na⁺,K⁺-ATPase and 5′-nucleotidase in sciatic nerve from dysmyelinating mutants (nmol/mg protein/min).

[a] $P < 0.05$; [b] $P < 0.01$, [c] $P < 0.001$ are the level of significant differences from control. NS, Not significant.

5'-nucleotidase in 60-day-old animals were unexplained, as well as the CNPase reduction: 70% of the control value in adult mld (in agreement with Ginalski et al., 1980); 90% of the control in the shiverer (in agreement with Mikoshiba, 1980).

By electron microscopy the peripheral nerves of quaking reveal several unusual features in the pattern of myelination in addition to hypomyelination (Samorajki et al., 1970; Suzuki and Zagoren, 1976, 1977). Schwann cell transplantation has shown some evidence for a primary sheath cell disorder causing hypomyelination in these quaking mice (Aguayo et al., 1977*b*). Thus, in quaking, increased 5'-nucleotidase was unexpected. Reduced Na^+,K^+-ATPase could be eventually related to hypomyelination, as measured by CNPase reduction: 58% of the control in the adult (30% according to Matthieu et al., 1980).

In Trembler, the alterations were even more interesting (Table 5 and Fig. 1). During normal postnatal development, Na^+,K^+-ATPase, 5'-nucleotidase and CNPase specific activities slightly increased between 10 and 30 days, and slightly decreased from then on. In this mutant, the activities of these three enzymes were very close to normal in 10-day-old animals, but followed an entirely different developmental pattern: CNPase activity became more reduced when increasing the age of the animal, being less than fourfold reduced in the adult. The Na^+,K^+-ATPase was less than half the control value in adult Trembler. 5'-Nucleotidase specific activity increased during development, up to two times the control value in the adult. As CNPase is a myelin enzyme, its activity in Trembler PNS could be expected to be drastically reduced, as the amount of myelin is only a few percent of the control in Trembler (Matthieu et al., 1979). The only fourfold reduction means that this enzyme is also present in myelin-related membranes in the normal, and possibly in onion-bulb formations in Trembler (shown by electron microscopic studies by Ayers and Anderson, 1975 and Low, 1977). These formations could also contain some 5'-nucleotidase activity, as this enzyme was increased in Trembler. Reduced Na^+,K^+-ATPase suggests abnormal Schwann cell membranes.

As Trembler sciatic nerve contains higher amount of perineurium and much more collagen as compared with control (Ayers and Anderson, 1975), the alteration could be more important, as our results were based on total proteins, including collagen.

Conclusions

5'-nucleotidase and Na^+,K^+-ATPase are true myelin-associated enzymes, although not specific for this membrane. Their alterations in CNS and PNS of various dysmyelinating mutants raise questions related to their role in the membranes; the study could provide very interesting information about normal and pathological membrane function.

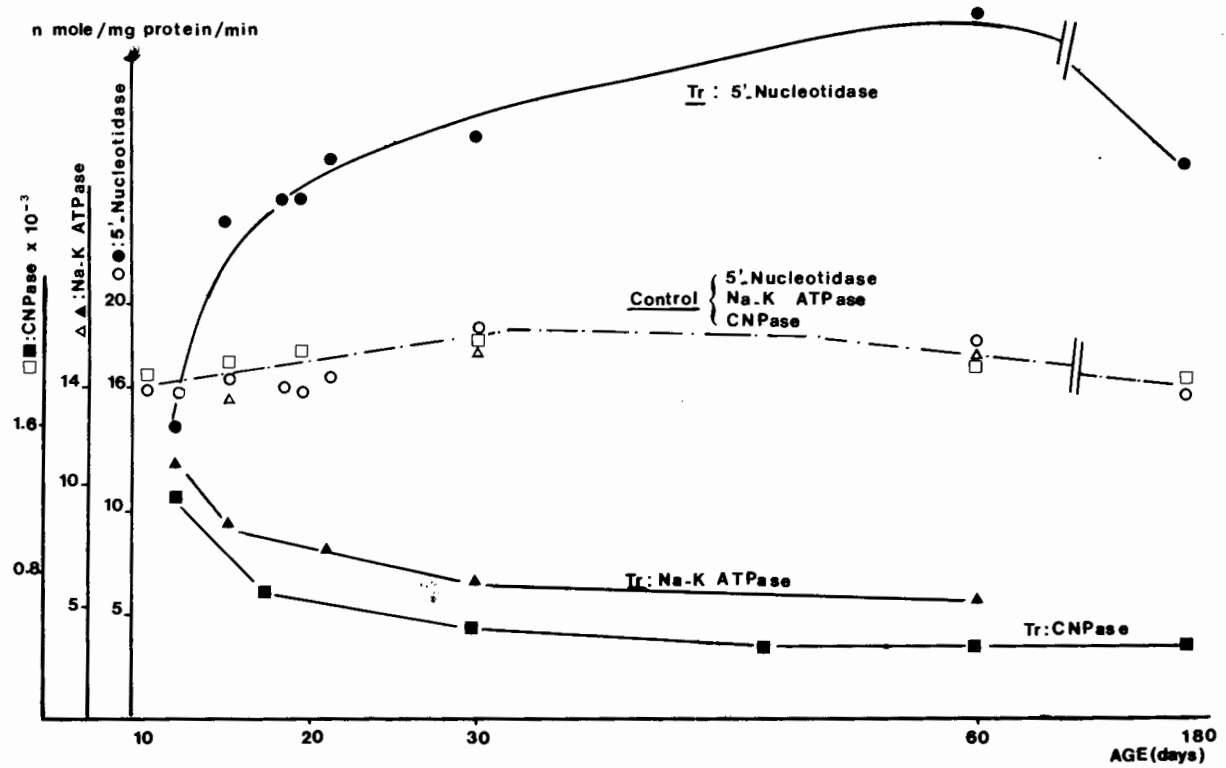

FIG. 1. 5'-Nucleotidase, Na^+,K^+-ATPase CNPase in the developing sciatic nerve from Trembler and control mice. The level of significance between mutant and control is at least < 0.01 (except for 5'-nucleotidase in 12-day-old animals).

In CNS, however, Na^+,K^+-ATPase was reduced to approximately the same extent in any mutant (except Trembler) and 5'-nucleotidase was not affected in the various mutants: in total brain homogenate measurements of these enzymes did not provide information about myelination (lower activities of Na^+,K^+-ATPase in CNS from mutants cannot be attributed to abnormality of myelin itself). Therefore it could be postulated that neurons and/or astroglia are eventually affected in the mutants. Note that activities were normal in the cerebellum. In PNS, the increased activity of 5'-nucleotidase and the reduced activity of Na^+,K^+-ATPase and CNPase raise questions about the membranes of the affected Schwann cells in Trembler. Thus the measurement of these two enzymes in various myelin-related membranes (Bourre et al., 1980) would be also a useful tool to study myelination and myelin assembly under normal and pathological conditions: for example, are these myelin-associated enzymes actually dissociated from myelin during ontogeny as shown by Waehneldt et al. (1979) for CNPase and carbonic anhydrase?

The location of these enzymes in the plasma membrane could be similar in brain membranes and other membranes. 5'-Nucleotidase is located on the external side of liver plasma membrane (Farquar et al., 1974; Little and Widnell, 1975). Thus it could be possibly present in the intraperiod line in myelin. Na^+,K^+-ATPase is located along the inner surface of red cells (Marchesi and Palade, 1967); the larger sub-unit is an intrinsic protein in the membrane from kidney outer medulla: ouabain binding is on the outer surface; the phosphorylation site is on the inside surface of the membrane (Chapman et al., 1979), thus possibly in the major dense line in myelin.

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