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Effect of lipid peroxidation on Na^+, K^+-ATPase, 5′-nucleotidase and CNPase in mouse brain myelin

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In the presence of H_2O_2, solutions of Fe^{2+} were applied to brain homogenate and isolated myelin from adult SWV control mice and the shiverer dysmyelinating mutant mouse as a source of a reactive oxygen species (Fenton reaction). Under these conditions, lipid peroxidation was initiated and measured as thiobarbituric acid-reactive oxidation products (TBAR). This was accompanied by 85% inhibition of myelin-associated Na^+,K^+-ATPase and 25% inhibition of 5′-nucleotidase. In contrast, CNPase activity was not altered. Studies on the shiverer mutant brain revealed that in spite of hypomyelination and prevalence of premature, myelin-like membranes in the homogenate, the myelin-related enzymes reacted as normal enzymes to peroxidation. Differences in the resistance of Na^+,K^+-ATPase to peroxidation in the brain homogenate and myelin suggest that the myelin enzyme is extremely sensitive to reactive oxygen toxicity.

Introduction

Central nervous system myelin could be a target for oxidative attack, resulting in the peroxidation of membrane lipids [6] or proteins [16]. Thus, myelin enzymes could be affected directly by free radicals or indirectly through a mechanism involving lipid peroxidation which alters membrane fluidity [6,11]. Moreover, the degradation products of lipid peroxidation (e.g., malondialdehyde) could inhibit enzymes [26].

Oxidative damage has been proposed to be a primary factor in the toxicity of several exogenous agents [9,11,30,32], in the ageing processes [13], and in post-ischemic neurological disorders [15]. Most of these pathological processes are partially directed against myelin and can produce demyelinating lesions in the CNS. Moreover, the involvement of abnormal oxidation of long-chain fatty acids in genetically determined disorders of myelin in human adrenoleukodystrophy was recently determined [21].

A number of biochemical studies have indicated that the myelin structure is changed [6] and myelin-specific structural proteins are aggregated [16] after peroxidation has been induced in myelin in vitro. However, little is known about the susceptibility of myelin functional proteins (enzymes) to reactive oxygen. In a previous study [9] we observed that CNPase (EC 3.1.4.37), an enzyme believed to be fairly myelin-specific, was resistant to peroxidation in vitro. In contrast, Na^+,K^+-ATPase (EC 3.6.1.3) and 5′-nucleotidase (EC 3.1.3.5), enzymes known to be present also in subcellular fractions other than myelin, seem to be strongly affected by peroxidizing conditions [1,27]. The myelin-associated proportion of their activity was recently shown to play an active role in the transport of material (adenosine and monovalent cations) in and out of the axon [20].

The aim of the present work was to evaluate whether in vitro membrane peroxidation affects myelin-related enzyme activities in homogenates and isolated myelin fractions. For this study we also used the shiverer dysmyelinated mutant mouse. This animal is known to possess, due to severe hypomyelination, myelin-enzyme activities preferentially associated with premyelin membranes [4]. This allowed us to investigate the problem of susceptibility of these immature myelin-like membranes to oxidative attack.

Abbreviations: TBAR, thiobarbituric acid-reactive oxidation purposes; BHT, 2,6-ditertbutyl-*p*-cresol; TcA, trichloroacetic acid.

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Materials and Methods

The homogenate and myelin were prepared from adult Swiss Vancouver (SWV) mouse brains of both sexes. Shiverer mutant mice (C3H SWV) were also used where indicated.

The animals were decapitated and brains were cooled in ice-cold buffer. The homogenate (10% w/v) was freshly prepared in 0.9% NaCl buffered with 10 mM Tris-HCl (pH 7.4). The myelin was prepared according to Norton and Poduslo [23]. The samples (stored at −70 °C) were suspended (unless otherwise indicated) in 10 mM Tris-HCl buffer (pH 7.4) at a concentration of about 1 mg protein/ml and sonicated for 1 min at 21 kc/s. All incubations were carried out at 37 °C in an air atmosphere. Solutions of $FeCl_2$ were prepared in degassed H_2O purged with nitrogen. All iron solutions were freshly prepared in H_2O adjusted to pH 7.0 and used immediately.

TBAR determination

The formation of thiobarbituric acid-reactive oxidation products (TBAR) during incubation was determined as described by Kogure et al. [15]. 2,6-Ditertbutyl-*p*-cresol (BHT) was used to prevent artifactual oxidation of fatty acids during assay procedures. To an 100 µl aliquot of homogenate or myelin suspension, 10 µl methanol, containing 1 µg of BHT, was added and the mixture was solubilized with 50 µl of 7% SDS. An 0.5% aqueous solution (200 µl) of thiobarbituric acid and 400 µl of 0.1 M HCl were added, mixed well and heated for 45 min in boiling water. After cooling, 1 ml of *n*-butanol was added and the entire mixture was centrifuged. The absorbance of the organic layer was measured at 530 nm. Quantitation was based on a molar absorption coefficient of $1.56 \cdot 10^5$.

Enzymatic assays

CNPase was assayed using adenosine 2′3′-cyclic monophosphate as substrate as described previously and measuring the rate of adenosine 2′-monophosphate formation [9]. The substrate and product were estimated spectrophotometrically at 260 nm after thin-layer chromatographic separation.

Na^+,K^+-ATPase activity was determined by measuring the amount of inorganic phosphate liberated by hydrolysis of ATP in the presence of cations and in the presence or absence of ouabain. The assay mixture (0.5 ml final volume) contained about 50 µg of protein in 0.05 M Tris buffer (pH 7.4), 20 mM KCl, 150 mM NaCl and 5 mM $MgCl_2$. The reaction was initiated by addition of ATP (vanadium-free Tris ATP, Sigma A 0270) to a final concentration of 4 mM and continued for 15 min. The reaction was stopped by addition of an equal volume of a trichloroacetic acid 10% solution (slight modification of the method of Bourre et al. [3]). The Na^+,K^+-ATPase activity represents the differences in the amount of P_i liberated in the presence or absence of 0.75 mM ouabain.

5′-Nucleotidase

The amount of phosphate ion liberated per unit time was determined using adenosine 5′-monophosphate (5′-AMP) as substrate. The assay mixture (0.5 ml final volume) contained about 200 µg of protein, 0.2 M Tris-HCl buffer (pH 7.5) and 10 mM $MgCl_2$. The reaction was started by the addition of 5′-AMP to a final concentration of 5 mM. After 15 min, the reaction was stopped by the addition of an equal volume of 10% TCA [3].

Determination of phosphorus

The TCA extracts were assayed, after centrifugation, following the procedure of Lin and Way [18]. Briefly, an aliquot was transferred to a test tube in an ice bath and an equal volume of $FeSO_4$-ammonium molybdate reagent was added. The reagent was prepared immediately before use by dissolving 4 g of $FeSO_4 \cdot 7H_2O$ in 100 ml of a solution containing 1% ammonium molybdate in 0.6 M H_2SO_4. The mixture was kept in the ice bath for 1 h before absorbance was read at 750 nm. A blank, into which the sample was introduced only after addition of TCA, was used to correct for the spontaneous hydrolysis of ATP and also the phosphate content of the sample.

All enzymatic determinations were performed immediately after preincubation of the samples under peroxidation conditions and were run simultaneously with MDA measurement. The enzymatic reaction velocities were linear over the incubation period indicated above, and proportional to the protein concentrations in the ranges used in the experiments (see Refs. 3, 9).

Proteins were determined using the Bio-Rad Protein Assay according to the standard procedure. Myelin samples were dissolved in 1% SDS (sodium dodecyl sulphate) then diluted to 0.1% with SDS before estimation.

Gel electrophoresis of myelin proteins

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by addition of 'sample' buffer (0.1 M Tris-HCl pH 6.8, 2% SDS, 0.1 M dithiothreitol, 10% glycerol, 0.001% Bromophenol blue) and heating at 100 °C for 2 min. Discontinuous 1 mm thick slab gels were composed of stacking gel (4.5% acrylamide, 0.12% bisacrylamide, 0.125 M Tris-HCl pH 6.8, 0.1% SDS) and a separating gel (12% acrylamide, 0.32% bisacrylamide, 0.375 M Tris-HCl pH 8.8, 0.1% SDS). The gels were calibrated using molecular weight marker kits (Combithek-Boehringer Mannheim) and stained with Coomassie blue (0.25% w/v in 50% methanol, 7% acetic acid).

Statistical methods

A Student's *t*-test for small samples was used to evaluate significant differences [2].

Results

The TBAR content in the myelin preparations slightly exceeded the level found in freshly prepared brain homogenates (Table I). Neither the storage conditions, sonication procedure, nor incubation of myelin suspension without iron, influences the TBAR level (results not shown). The addition of iron to the incubation mixture resulted in an increased formation of TBAR (Table II): 200 μM Fe^{3+} with 25 μM vitamin C produced the greatest increase in TBAR levels, but also produced visible precipitation. The addition of 200 μM Fe^{2+} to an incubation mixture containing 100 μM H_2O_2 was less effective, but in this case no precipitation of the sample was observed up to 1 h of incubation. Interestingly, the mixture of Fe^{2+} and Fe^{3+} (100 μM and 50 μM, respectively) also increased the TBAR levels by up to 300% of the control (from 4.6 to 14.4 nmol TBAR/mg protein). The observed increase in TBAR was due to the effect of iron on lipid peroxidation, since addition of either Fe^{2+}, Fe^{3+}, ascorbic acid or H_2O_2 at the end of the incubation was without effect on TBAR levels.

Myelin-associated Na^+,K^+-ATPase was significantly inactivated during 15 min preincubation at 37 °C, while myelin was suspended in H_2O adjusted to pH 7.0. Buffering of the samples with 10 mM Tris-HCl prevented this inhibition, but also diminished TBAR formation during peroxidation by about 50%. However, as shown in Fig. 1, the increase of TBAR was still time dependent and significant. Moreover, under these conditions the protein electrophoretic pattern of peroxidized myelin did not change (Fig. 2). Thus, incubation of myelin suspended in 10 mM Tris buffer with 200 μM Fe^{2+} and 100 μM H_2O_2 was chosen as the routine peroxidizing condition and used in further experiments.

The specific activity of the enzymes in the control brain homogenate and myelin of normal mice and in the brain homogenate of the shiverer mutant are shown in Table III. Enzyme activities in the shiverer homogenate were all significantly lower than in the controls.

Peroxidation of the samples for 15 min (Table III) resulted in inhibition of 5′-nucleotidase and ATPase activity in the homogenate and myelin fractions, but not of CNPase. 5′-Nucleotidase was inhibited to 70–80% of control values in the homogenate and myelin fraction, while Na^+,K^+-ATPase was strongly inhibited (less than 20% of control) only in myelin. The enzyme in brain homogenate was less sensitive to peroxidation and de-

TABLE I

TBAR formation in brain homogenate and myelin under peroxidizing conditions

Myelin was suspended in 10 mM Tris-HCl buffer (pH 7.4) at a concentration of 1 mg/0.9% NaCl, 10 mM Tris-HCl buffer (pH 7.4). Samples were incubated for 15 min at 37 °C with or without 0.2 mM Fe^{2+} and 0.1 mM H_2O_2 designated as 'peroxidizing conditions'. Production of TBAR was determined as described under Material and Methods. Values are means ± S.D. of the number of experiments shown in brackets. Significance levels compared with controls: * $P < 0.01$, ** $P < 0.001$.

	nmol TBAR/mg protein	
	homogenate	myelin
Control without incubation	3.37 ± 0.67 (6)	5.39 ± 1.09 (10)
Control after incubation	4.16 ± 1.46 (3)	4.93 ± 0.57 (3)
Incubation under peroxidizing conditions	10.77 ± 1.88 * (3)	10.98 ± 1.57 ** (6)

TABLE II

TBAR formation in the myelin fraction in different iron-containing systems

Production of TBAR was determined as described under Material and Methods in myelin suspended in H_2O adjusted to pH 7.0 immediately prior to use. Samples (200 μl) containing 0.5 mg protein/ml were incubated at 37 °C for 30 min. Mean results of three replicas ± S.D. are shown. Significance levels as compared with control ($P < 0.01$) are marked by asterisk.

Incubation with addition of	nmol TBAR (mg protein)	% of control
None	4.6 ± 0.45	100%
0.2 mM Fe^{2+}	6.6 ± 0.4	144%
0.2 mM Fe^{2+} + 0.1 mM H_2O_2	19.5 ± 5.1 *	413%
0.2 mM Fe^{3+} [a] + 25 μM vitamin C	30.0 ± 2.0 *	652%

[a] During incubation aggregation of the material was observed.

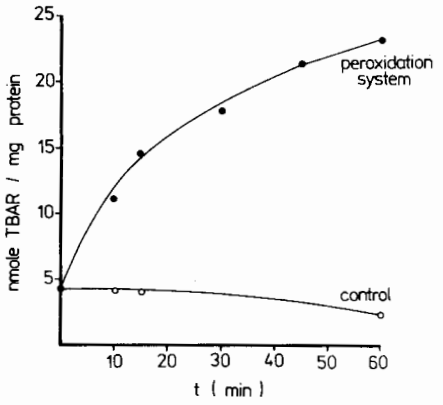

Fig. 1. Time course of TBAR formation during incubation of myelin (0.5 mg protein/ml) in control medium containing 10 mM Tris-HCl buffer (pH 7.4) and after addition of 0.2 mM Fe^{2+} and 0.1 mM H_2O_2 (peroxidation system).

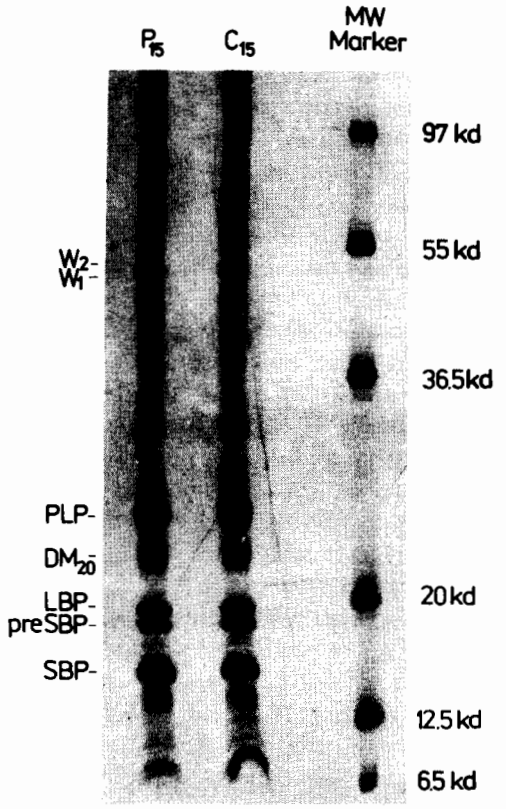

Fig. 2. Gel electrophoresis. Left: pattern of myelin proteins after 15 min incubation in the control (C_{15}) and in the peroxidation (P_{15}) system. Right: molecular weight (MW) standard. Coomasie blue stain. W_1W_2, Wolgram protein doublet; LBP, large basic protein; SBP, small basic protein; PLP, proteolipid protein.

creased to 73% of control during 15 min of preincubation.

The effect of several iron chelators and free radical scavengers on TBAR formation and ATPase activity in the mouse myelin fraction is presented in Fig. 3. An effective chelator, desferal, inhibited lipid peroxidation completely; however, the enzyme activity was still reduced to about 50% of control. In contrast, phytic acid only partially inhibited TBAR formation, but enzyme activity was well preserved. Hydroxyl radical scavengers such as mannitol, and an increase of the Tris concentration to 50 mM, did not significantly affect lipid peroxidation in our system. Superoxide dismutase (SOD) also failed to inhibit TBAR formation and significantly prevent enzyme inactivation. On the other hand, the increase of the Tris concentration protected against inhibition of ATPase, whereas mannitol and EDTA had no effect.

All the compounds used in this part of the study were found to be without influence on the thiobarbituric acid assay.

Discussion

The Fenton reaction (Fe^{2+} in the presence of H_2O_2) was used as a source of reactive oxygen species. Iron is believed to be an initiator of peroxidation in certain pathological states such as ischemia [15], idiopathic Parkinson's disease and Reye's syndrome [29]. In the present study TBAR formation was used as an indicator of lipid peroxides formation and breakdown in vitro. TBAR, especially that estimated in the presence of BHT, has recently been accepted as an indicator of lipid peroxidation. The endogenous level of TBAR in brain homogenate was within the range observed by others in cerebral slices [5] and homogenate [19] when homogenization was performed routinely with no special precautions taken. The endogenous level of TBAR in myelin was slightly higher than that in the homogenate, possibly indicating that some peroxidation of the material occurred during fractionation.

TABLE III

Influence of peroxidizing conditions on enzymatic activity in brain homogenate and myelin fraction

Peroxidation in samples containing 0.1 mg protein in 0.2 ml was initiated with 0.2 mM Fe^{2+} and 0.1 mM H_2O_2. After 15 min at 37°C, further peroxidation was prevented by addition of 1 mM phytic acid. Enzymatic activity was immediately estimated as described in the Material and Methods. Values are means of the number of experiments given ± S.D. Significance levels compared with controls: * $P < 0.05$; ** $P < 0.001$.

		No. of experiments	5′-Nucleotidase (μmol P_i/h per mg prot.)	Na^+, K^+-ATPase (μmol P_i/h per mg prot.)	CNPase (μmol P_i/mg prot. per min)
Homogenate (normal mice)	control activity	6	1.40 ± 0.03	9.04 ± 0.36	2.97 ± 0.09
	activity after peroxidation	3	1.02 ± 0.08 **	7.07 ± 0.54 *	3.02 ± 0.21
	(% of control)		(73%)	(78%)	(102%)
Homogenate (shiverer mice)	control activity	3	1.20 ± 0.10	5.60 ± 0.44	1.86 ± 0.26
	activity after peroxidation	3	0.98 ± 0.20 *	3.64 ± 0.92 *	1.95 ± 0.17
	(% of control)		(82%)	(65%)	(105%)
Myelin (normal mice)	control activity	10	2.75 ± 0.12	8.50 ± 0.44	20.1 ± 0.66
	activity after peroxidation	3	2.06 ± 0.21 **	1.30 ± 0.65 **	21.1 ± 0.52
	(% of control)		(75%)	(15%)	(105%)

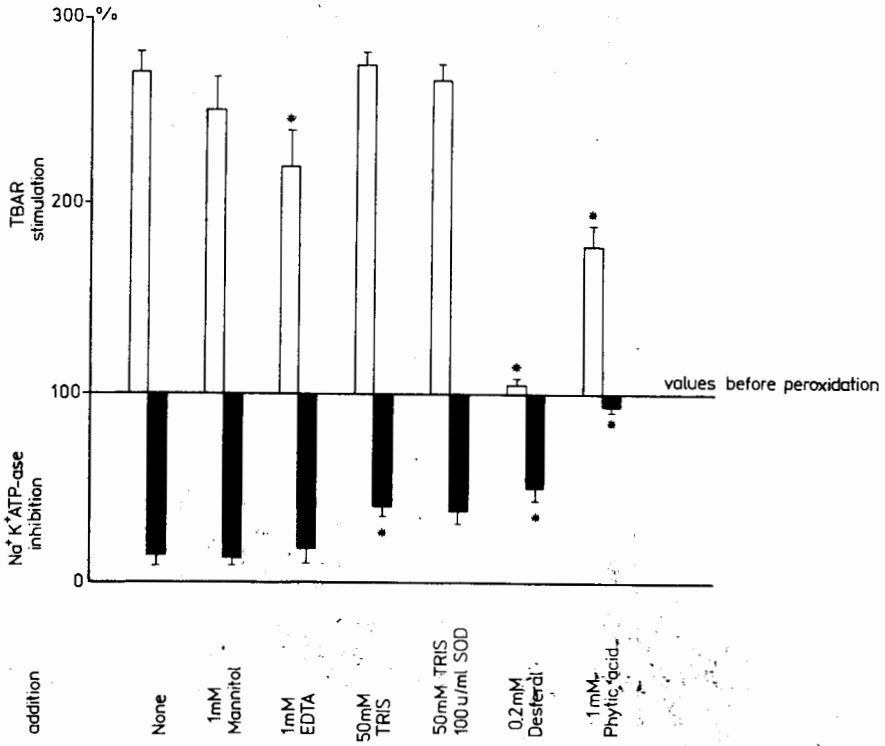

Fig. 3. Effect of iron chelators and oxygen radical scavengers on TBAR formation and inhibition of Na^+,K^+-ATPase in myelin fractions under peroxidizing conditions. Peroxidation was performed in 0.2 mM Fe^{2+}, 0.1 mM H_2O_2 and 10 mM Tris-HCl (pH 7.4) for 15 min at 37 °C. Samples contained 0.5 mg protein/ml. Initial values for Na^+,K^+-ATPase were 9.2 μmol P_i/h per mg P. Initial values for TBAR level were 4.52 nmol/mg P. The results are the mean of three parallel samples with standard deviations indicated. Significant differences ($P < 0.05$) from values obtained under routine peroxidizing conditions are marked by asterisk.

Under the conditions used in this work, the damage of myelin proteins due to iron-induced peroxidation seems to be less general than that described by others [6,16]. Each of the enzymes studied reacted differently, Na^+,K^+-ATPase being most sensitive and CNPase totally resistant. Moreover, the myelin-specific structural proteins did not aggregate in our iron-generated peroxidation system, as they did in Cu^{2+}-induced myelin peroxidation [16], suggesting inhibition of ATPase and 5′-nucleotidase as the initial events in the peroxidative damage of myelin.

From our experiments, it is still unclear whether Na^+,K^+-ATPase inhibition in myelin was due to the lipids surrounding the enzyme or to a direct reactive oxygen attack on the enzyme active center(s). When using iron chelators and free radical scavengers, we did not observe a strict correlation between TBAR formation and enzymic inhibition suggesting, rather, that the protein molecule are a possible target of oxidative attack. Desferal completely inhibited lipid peroxidation, but was only partially effective in preventing ATPase inhibition, whereas phytic acid was very effective in preventing inhibition with only partially reduced TBAR formation.

Unfortunately, we still do not have a good indicator of the peroxidative changes that occur in proteins. It has been suggested that many enzymatic effects of various exogenous compounds on enzymatic or protein-binding properties work through the oxidative mechanism [1,17,22,27]. The disruption of the myelin structure with protein aggregation observed in the Cu^{2+}-induced peroxidative system has also been attributed to direct peroxidative attack on the protein moiety [16]. It has been reported that sarcoplasmic reticulum Ca^{2+}-ATPase was strongly inhibited by iron/vitamin C in vitro and by vitamin E deficiency in vivo [24]. These authors suggest a direct alteration in the protein enzymatic component.

The particular sensitivity of myelin ATPase to peroxidation could be explained by its specific molecular properties and its difference from the enzyme predominant in the brain homogenate. Two different isoenzymatic forms of Na^+,K^+-ATPase have been reported to occur in the brain [33]. These forms exhibit different sensitivities to ouabain inhibition with half-maximal inhibition in the range 10^{-7} to 10^{-5} M ouabain, respectively [34]. According to our results, the form prevailing in brain homogenate would be less sensitive to peroxidative damage than that associated with myelin.

As for the other myelin-related enzyme, 5′-nucleo-

tidase, moderate sensitivity to peroxidation was observed. The knowledge of the subcellular localization of 5′-nucleotidase and Na^+,K^+-ATPase is still incomplete. However, recent studies using electron microscopic cytochemistry and subcellular fractionation procedures suggest their association with myelin [3,14,25]. The other possible source of Na^+,K^+-ATPase in the myelin fraction could be contamination by the axolemma, which has been reported to contain measurable concentrations of this enzyme [8]. Values for Na^+,K^+-ATPase, 5′-nucleotidase and CNPase activity measured in the homogenate and myelin fractions were close to those reported previously for mouse and rabbit brain [3,9].

The 2′3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) has been shown to be an integral protein of myelin and a major component of the Wolfram protein doublet [12,31]. However, its specific enzymic function is still unknown. The substantial increase of CNPase activity in the myelin fraction (seven times over that in homogenate) is an argument for the relative purity of the myelin fraction used. CNPase was totally resistant to peroxidation in our system. This is in contrast to the observation of Konat et al. [16] on the inhibition of CNPase by peroxidation in brain slices. The authors suggest that CNPase associated with premyelin membranes is more sensitive to oxidative attack than that associated with myelin. To test this hypothesis we investigated enzyme activity in the dysmyelinated shiverer mutant mouse, known to possess CNPase activity associated preferentially with premyelin membranes [4]. However, was CNPase was not affected by iron-induced peroxidation in this mutant homogenate either. Thus, the possibility that premature, myelin-like membranes are more susceptible to oxygen species than mature myelin could be ruled out.

In conclusion, and regardless of the radical generating system used in our study and the still hypothetical source of iron in the brain, numerous observations suggest that $Fe^{2+} \leftrightarrows Fe^{3+}$ interconversion is necessarily required (Table I). We have used the combination of Fe^{2+} and H_2O_2 to initiate peroxidation because we have noted its moderate effect on myelin to be most suitable for our goal, i.e., recognition of the susceptibility of myelin enzymes to the peroxidation reaction. Ferric iron (Fe^{3+}) initiated an even stronger peroxidation of myelin lipids in the presence of a reducing agent (Table I), but in this case aggregation of myelin proteins occurred. On the basis of our data, we could not resolve which type of iron-catalyzed reaction (if any) is most probable for the brain in situ, we believe, however that when peroxidation has been initiated (regardless of the nature of the initiating factor) in the glucose vicinity of myelin membranes, the enzymes under investigation would react in a manner similar to that observed in our experiments.

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