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Effect of Mercury on Rabbit Myelin CNP-ase *In Vitro*

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ABSTRACT: *2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) catalyzes hydrolysis of 2',3'-cyclic nucleotides to form the corresponding 2'-monophosphates. Rabbit myelin fraction with CNPase specific activity between 30-40 µmoles/min/mg protein was incubated in the presence of various inorganic and organic heavy metal compounds: $HgCl_2$; $(CH_3Hg)OH$; $Pb(NO_3)_2$; $Pb(C_2H_3O_2)_2.3H_2O$; $(C_2H_5)_2Pb$; $(C_2H_5)_3SnCl$. The enzyme has been shown to be almost exclusively sensitive to mercurials in µM concentration range. This would arise from the high solubility of mercurials in organic solvents, which allows them to penetrate into hydrophobic regions of the enzyme to react with active sulfhydryl groups. CNP-ase inhibition by methylmercury was biphasic: (1) A reversible, non-competitive inhibition with an apparent K_i = 1 µM occurred after a 5 min preincubation time of the enzyme with the inhibitor. (2) In the case of longer preincubation time, as well as in the presence of $HgCl_2$, the graph of enzyme activity versus protein concentration intercepted the abscissa to the right of the origin, indicating that mercurials are irreversible inhibitors of the enzyme. After 45 min of preincubation of the inhibitors with the enzyme 1 nmol of $HgCl_2$ completely blocks CNP-ase activity equivalent to 15.6 µg of myelin protein, whereas 1 nmole of Met-Hg blocks activity in 9.9 µg proteins. This apparently irreversible inhibition of CNP-ase activity by HgCl2 could be fully restored by the use of an excess of hydrophobic low molecular weight thiols, lipoic acid being the most efficient. Dithiothreitol, a hydrophilic complexing agent, was potent to reverse the inhibition caused by Met-Hg only during the short time experiments. Both low molecular weight thiols, and also EDTA in the case of inorganic mercury could prevent the inhibition of CNP-ase by mercurials, if preincubated for 15 min with the inhibitors, prior to the addition of the enzyme. The irreversible type of inhibition of CNP-ase by Met-Hg was only partially reversed in the presence of low molecular weight thiols. This suggests that the formation of a metal-mercaptide complex is not the only mechanism of inhibition by methylmercury. The possibility of lipid peroxidation triggered by methylmercury with subsequent inhibition of the enzyme activity was not supported by the experimental results. In fact, myelin associated CNP-ase activity appears to be very resistant to the structural membrane alterations caused by lipid peroxidation.*

Key Words: CNP-ase, Myelin, Organic Mercury, Inorganic Mercury, Heavy Metals, Lipoic Acid

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INTRODUCTION

The neurotoxic effects of mercury poisoning are well documented and include degenerative changes in myelin and nerve fibers. Morphologically, an axoplasmic degeneration and a demyelination can be visualized (Chang, 1977 - a review). When comparing inorganic and organic mercury poisonings, different kinds of morphological changes were observed, but organic mercury was considered to be the most neurotoxic agent (Berlin and Ullberg, 1963). Mercury compounds can cross the blood-brain barrier and cell membranes. They are capable of reacting with sulfur leading to inactivation of many enzyme systems, particularly those requiring reduced thiol groups for their activity.

The 2'3'-cyclic nucleotide 3'-phosphodiesterase (EC 3.1.4.37-CNPase), an enzyme which is located preferentially in myelin, was demonstrated by Sprinkle and co-workers (Hells and Sprinkle, 1981; Sheedle *et al.*, 1984; Sprinkle and Kneer, 1981) to be sensitive to mercury inhibition *in vitro*. This observation suggests that injury of myelin-connected CNPase may be a primary event in the development of toxic demyelination. The enzyme catalyzes the hydrolysis of 2'3'-cyclic nucleotides to form the corresponding 2'-monophosphates (Drummond and Keith, 1962). The enzyme has been shown to be an integral protein of myelin and a major component of Wolfram protein doublet (Drummond and Dean, 1980; Sprinkle *et al.*, 1980). CNPase has been exploited as a myelin marker, however, nothing is known about the enzyme's function in the myelin membrane. Some data are consistent with the role of CNPase in myelin protein synthesis (Starich and Dreiling, 1980), and in myelin formation during development (Snyder *et al.*, 1983; Sprinkle *et al.*, 1978; Danks and Matthieu, 1979). The enzyme may also be involved in demyelinating neuropathies in animals (Bourre *et al.*, 1982; Kurihara *et al.*, 1971) and humans (Foster and Caret, 1983; Reikkinen *et al.*, 1972; Sprinkle and McKhann, 1978). Heretofore, no report has appeared regarding the mechanism of action of different mercurials on that enzyme. Our work, concerning the reaction mechanism of CNPase inhibition *in vitro*, leads to the discovery of some interesting differences between the effects of inorganic and organic compounds on isolated rabbit myelin fraction and shows specific alteration by organic mercury and not by other organometals (lead and tin).

MATERIAL AND METHODS

Heavy metals were examined for their ability to affect the CNPase activity in rabbit brain myelin fraction isolated as previously published (Norton and Poduslo, 1973). Fractions of myelin were stored at -20°C and thawed just before measurement of the enzyme activity.

CNPase activated with 0.5% Triton X-100 was exposed to mercurials for the time and concentration indicated, and then assayed with 2'3'-cyclic AMP, based on the method of Kurihara and Tsukada (1967) with some modifications. Briefly, solution of 0.03 M adenosine 2'3'-cyclic phosphate (0.05 ml) was added to a mixture containing 0.05 ml of 0.2 M Na_2HPO_4 - 0.1 M citric acid buffer (pH 6.2) and 0.05 ml of an enzyme solution of appropriate dilution in 0.5% Triton X-100 and incubated at 37°C. A sample containing the substrate but no enzyme served as a control. The reaction was stopped by the addition of 0.01 ml of 1mM $HgCl_2$ and chilled in ice. The samples were applied on TLC plates of DC-Alurolle Silico Gel F_{254} (Merck) in a volume varying from 0.02 to 0.1 ml, depending on the substrate concentration that was used and then developed in saturated ethyl-acetate, propanol, NH_3 and water (90:60:40:30). The spots of adenosine 2'phosphate and adenosine 2'3'-cyclic phosphate were located by using a UV lamp, cut out and eluted with 4 ml of 0.01 N HCl. The optical density of the supernatant was read at 260 mu after centrifugation.

In kinetic studies, the substrate concentration varied from 1.25 to 20 mM and the time of incubation varied from 5 to 10 min. In experiments with inhibitors, incubation time was prolonged to 30-45 min, after confirming the linearity of the reaction during this period. Standard assay conditions were chosen to be 10 mM substrate concentration and a 5 min incubation time at 37°C as recommended by Tsukada and Suda (1980). As the enzyme is competitively inhibited by its reaction product, the rate of reaction was proportional to the enzyme concentration up to 50% hydrolysis of

the substrate.

The specific activity of the enzyme was defined as μmol of product formed per min, per mg protein at 37°C. Protein was determined according to the method of Lowry (1951) using bovine serum albumin as a standard.

RESULTS

Inhibition of CNPase by methyl mercury (Met-Hg) and mercuric chloride increased both with the time of exposure and the inhibitor concentration (Figure 1). However, $HgCl_2$ was the most potent inhibitor. For the same protein concentration, 1 μM final concentration of $HgCl_2$ caused 40% enzyme inhibition after 5 min exposure and the activity was completely inhibited after 45 min. After 45 min 40% inhibition was observed at 0.5 μM concentration of $HgCl_2$. Organic mercury caused 50% loss of enzyme activity after 5 min preincubation in the presence of 2.5 μM inhibitor. When the time of exposure was increased to 45 min, the inhibition was 100% at 2.5 μM and 50% at 1 μM.

Figure 2 shows the effect of methylmercury on the enzyme activity as a function of time of exposure at a fixed concentration (1 μM) of inhibitor. The inhibition increased from about 30% to 90% between 5 min and 40 min.

In all cases, enzyme samples incubated under identical conditions, but without mercurials, showed negligible changes in CNPase activity. Inhibition of CNPase after a 5 min preincubation with methylmercury was found to be reversible by plotting the Vmax versus the amount of enzyme protein at a fixed inhibitor concentration (Figure 3). From Lineweaver-Burk double reciprocal plot that is shown in Figure 4 we found that the inhibition was of the non-competitive type. The Km was not influenced by inhibitor and remained 6.5 mM. Plotting the reciprocal of reaction velocity versus inhibitor concentration (Dixon plot), we found the Ki for methylmercury to be about 1 μM.

Contrary to the above effect of Met Hg observed only after short (5 min) time of exposure, the inhibition of CNPase after longer preincubation with the organic as well as with inorganic mercurials proved to be irreversible (Figure 5). From the data shown on the diagram we can calculate that 1 nmol of $HgCl_2$ blocks completely a CNPase activity equivalent to 15.6 μg of myelin protein, whereas 1 nmole of Met Hg blocks the activity of 9.9 μg protein.

As was pointed out previously, this effect was time-dependent and all the calculations were made after 45 min of exposure of the enzyme to the inhibitor.

Hydrophilic chelators (EDTA, DTT) were unable to reverse the effect of $HgCl_2$ when

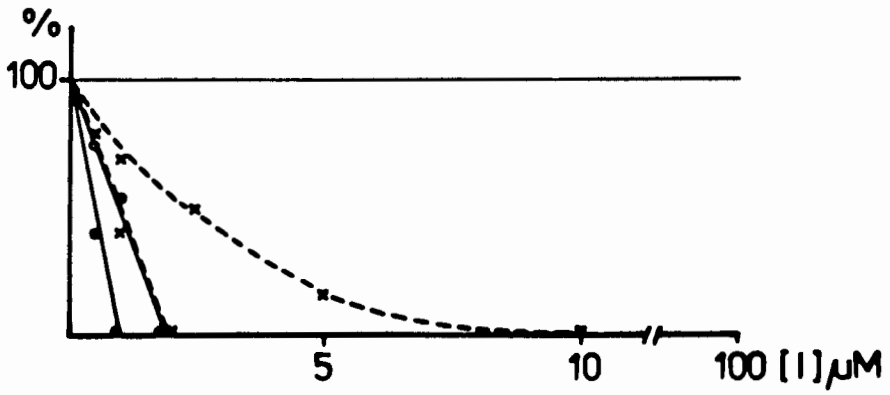

FIG. 1. $HgCl_2$ (•) and Met Hg (x) inhibition of CNPase activity in rabbit myelin fraction measured in different inhibitor concentration. The 5 min (- - - -) or 45 min (——) exposure to inhibitors at 37°C preceded the enzyme assay. The activity of CNPase is expressed as a percentage of control measured in the absence of inhibitor. Each point represents a mean of triplicate determination.

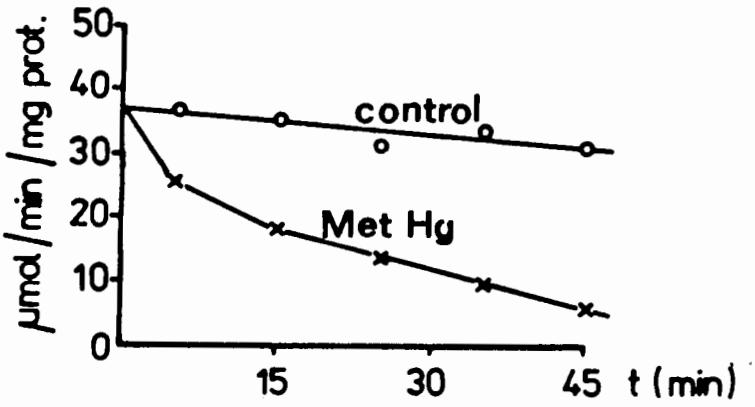

FIG. 2. Met Hg Inhibition of rabbit myelin CNPase as a function of preincubation time with the enzyme at 37°C and 1 μM inhibitor concentration.

added after the exposure of enzyme to inhibitor (Table 1). In the case where chelators were added in a 100- or 200-fold molar excess for 15 min prior to the enzyme assay, only a small improvement of the enzyme activity was noticed in the case of 1,3 dithiothreitol. A non-thiol chelator such as EDTA had no effect on the inhibition of inhibited CNPase activity by $HgCl_2$. The hydrophobic low-molecular weight thiol, lipoic acid, in concentration of 0.1 mM fully reversed the effect of $HgCl_2$ on CNPase and even increased the enzyme activity above the control (triton activated) value. DTT, EDTA and lipoic acid were able to prevent the enzyme inhibition by $HgCl_2$ when added in 100- or 200-fold molar excess over $HgCl_2$ 15 min prior to the addition of the enzyme. The CNPase activity in control samples was not affected by the EDTA or DTT in the above (0.1 mM) concentrations. However, lipoic acid itself increased the myelin CNPase activity up to 110%.

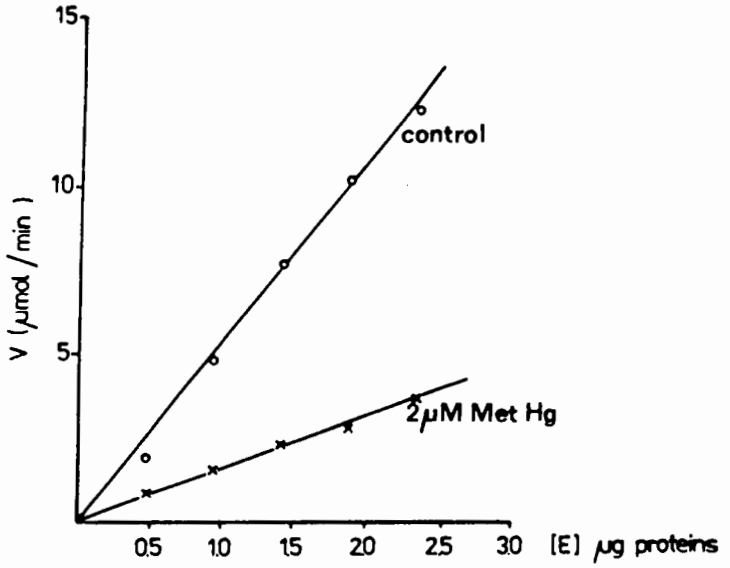

FIG. 3. A plot of CNPase reaction velocity as a function of protein concentration. Rabbit brain myelin fraction was exposed to H_2O or 2 μM Met Hg at 37°C for 5 min prior to enzyme assay.

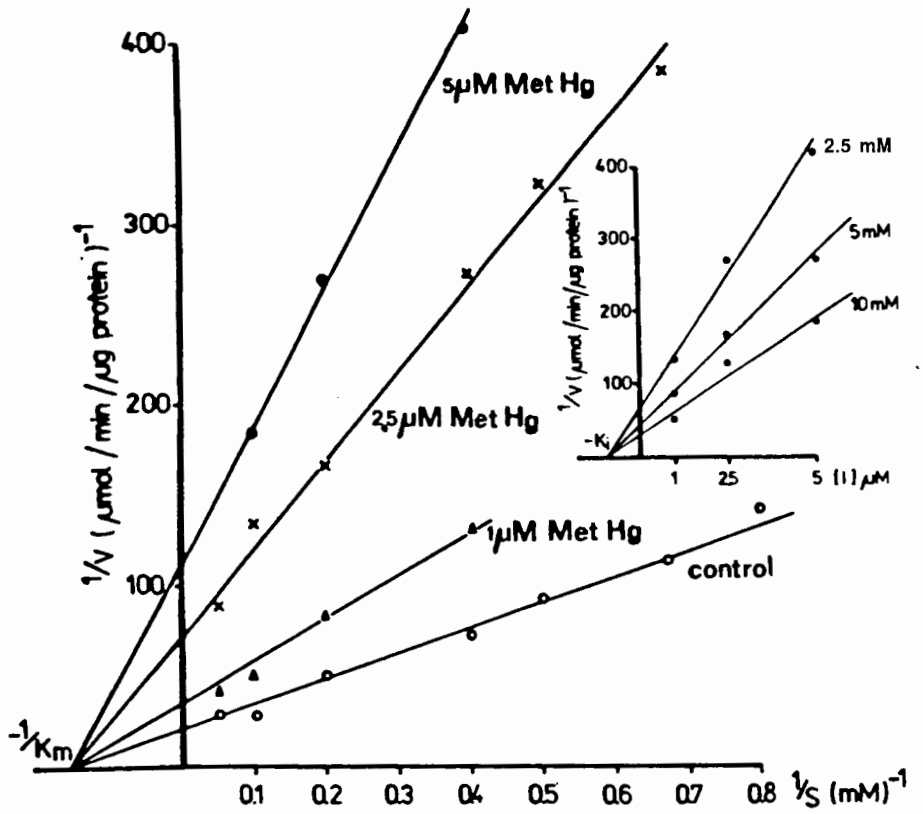

FIG. 4. The effect of different Met Hg concentrations on the Lineweaver-Burk double reciprocal plot of CNPase velocity and substrate concentration. On the right side is shown a Dixon plot supporting a non-competitive type of inhibition.

TABLE 1. Effect of Chelators on CNPase Inhibition by Inorganic and Organic Mercury.

	Concentration (μM)		EDTA (no thiol-chelator)			DTT (Hydrophilic thiol-complexing agent)			Lipoic Acid (Hydrophilic thiol-complexing agent)		
		Exposure time min	Inhibition	Reversibility	Prevention	Inhibition	Reversibility	Prevention	Inhibition	Reversibility*	Prevention**
			% of control activity								
$HgCl_2$	0.5	5	51	55	98	76	83	100	--	--	--
	1.0	5	--	--	--	44	57	100	--	--	--
	0.5	45	38	35	100	40	48	100	34	110	109
	1.0	45	0	0	94	0	16	100	0	113	111
Met Hg	1.0	5	87	87	86	77	102	100	--	--	--
	2.0	5	30	35	30-40	43	100	100	50	100	113
	1.0	45	55	0	0	55	83	100	--	--	--
	2.0	45	0	0	0	0	23	100	0	70	115

* Reversibility of inhibition was estimated by addition of chelators into the incubation medium for 15 min, after exposure of the enzyme to inhibitor for the time that is indicated in the table.

** Prevention of inhibition was measured by preincubation of inhibitor with chelator in the absence of the enzyme for 15 min and further incubation with the enzyme for the time indicated in the table. Chelators were added at a concentration of 0.1 mM.

In the case of methylmercury, EDTA in 50 or 100 fold molar excess over inhibitor concentrations was completely ineffective and could not reverse or prevent inhibition of CNPase. On the other hand, both lipoic acid and DTT could prevent the enzyme inhibition by Met Hg when added 15 min prior to the enzyme addition. These agents were also able to reverse the inhibition when added after a short time (5 min) exposure to the enzyme to methylmercury.

However, after a longer time of exposure (45 min), only a partial restoration of CNPase activity was observed after treatment by either lipoic acid or 1,3-dithiothreitol.

The biphasic effect of low molecular weight thiols after methylmercury inhibition, as well as the full recovery obtained by immediate treatment with DTT or lipoic acid, suggests the primary formation of a mercaptide-metal complex, followed by inhibition of different character. Therefore, the possible involvement of lipid peroxidation in the inhibition of CNPase was tested; however, pyrogallol, an active oxygen scavenger, had no effect on methylmercury-induced CNPase inhibition (Table 2). Also *in vitro* peroxidation of isolated myelin fraction, triggered by a Fenton reaction mixture (Table 3) had no effect on myelin CNPase activity.

Taking into consideration that a high dilution of myelin samples could account for its low tendency to peroxidize, we added exogenous arachidonic acid to the reaction medium. The extent of lipid peroxidation was followed by continuous monitoring of conjugated dienes formation at a wavelength of 233 nm. The free radicals were generated in 25 mM Tris pH 7.4; 0.1% lubrol; 1 mM reduced glutathione, 0.1 mM arachidonate and 50 µg of proteins in a final volume of 1 ml. Lipid peroxidation was stopped at different times by the addition of 2 mM phytate. We only observed a substantial activation CNPase by 0.1 mM arachidonate.

The observed differences in enzyme activity between the control and the free radical generating system were stable during the time of active lipid peroxidation as monitored by the changes in OD_{233}. Thus, that difference does not seem to be related to lipid peroxidation. In additional experiments, we found that phytate (a free-radical scavenger) could inhibit CNPase by about 30%.

TABLE 2. Effect of Pyrogallol on the Inhibition of CNPase Activity by Methylmercury.

		CNPase activity µmol/min/mg protein
Control	a	48.0
	b	45.5
+ 0.5% Pyrogallol	a	49.0
	b	45.0
+ 2 µM Met Hg	a	17.7
	b	3.3
+ 2 µM Met Hg 0.5% Pyrogallol	a	18.5
	b	3.5

The incubation system for estimation of CNPase activity is described in "Methods". Preincubation time of the whole system but without substrate : 5 min (a) and 45 min (b).

TABLE 3. Effect of a Free Radical-Generating System on CNPase Activity in Myelin.

	CNPase µmol/min/mg/protein
Control 1	39.0
Control 2	43.5
Control 3	40.5
Experiment	43.0

Preincubation of myelin samples was carried out for 45 min in 25 mM Tris/HCl pH 7.4, 0.1% lubrol, 0.5 mM ascorbic acid, 0.1 mM FeCl2 and at a protein concentration of 0.2 mg/ml.
Control 1 was run with the whole system but in the presence of 2mM phytate for stopping peroxidation.
In control 2 the myelin sample was incubated in buffer and detergent alone.
Control 3 was identical to control 2, except for the addition of 2 mM phytate.

DISCUSSION

Rabbit myelin fraction, activated with 0.5% Triton X-100, exhibited a relatively high specific CNPase activity (30 to 40 µmoles per mg protein per min). This activity could be compared with fully activated enzyme from rat brain myelin as reported by Tsukada and Suda (1980). The optimum pH for the enzyme was similar to that reported for the enzyme from rat

experiments were carried out using 0.1% lubrol, and detergents are known to suppress the inhibitory activity of fatty acids. The differences in the inhibitory potency between bivalent mercuric ions and monovalant organomercurials can be explained by the fact that mercuric ions can react with 1 or 2 sulphydryl equivalents, whereas, methylmercury would react with only one thiol group of the enzyme.

More interesting was the finding of the biphasic character of methylmercury inhibition with a full reversal observed only after a "short-term" exposure of the enzyme.

Mercurials combine very strongly with sulfhydryl groups. The dissociation constants of thiol-mercurial complexes under the conditions that were used in our experiments are 10-20 or less. For this reason, the activity of sulhydryl enzymes is irreversibly inhibited, as was demonstrated with $HgCl_2$. This altered activity can often be restored to a certain extent by treatment with competing ligands, particularly with low-molecular weight thiols.

That dithiothreitol (DTT) did not restore CNPase activity after inhibition by inorganic mercury in our experiment is probably due to its hydrophilic character. Previous works on CNPase have demonstrated that the enzyme is tightly bound to myelin (Tsukada and Suda, 1980), since it cannot be extracted by detergent or organic solvents. Accordingly, CNPase would be associated with highly hydrophobic regions of the membrane that would not be accessible to hydrophylic low molecular weight thiols such as DTT. Only hydrophobic molecules such as lipoic acid are able to penetrate and displace the mercaptide complexes formed in the enzyme. Lipoic acid at 0.1 mM concentration also activated myelin CNPase above the control values. This suggests that enzyme sulfhydryl groups are partially oxidized in the native myelin or during the isolation procedure.

In contrast, CNPase inhibited by methylmercury during the short-time enzyme exposure was fully restored by both hydrophilic and hydrophobic low molecular weight thiols. This observation is in agreement with the finding of Sprinkle and Knerr (1981) on purified CNPase. These authors favored a reversible type of organomercurials inhibition through metal complexation by sulfur-containing amino-acids, *i.e.*, cysteine at the enzyme active site. They mentioned, however, that CNPase inhibition that resulted from the use of other organomercurials (thimerosal) during enzyme isolation, could not be restored by a treatment with thiol-reducing agents. In the light of our study, it seems that there are two different types of inhibition. The first and only one to be fully reversed by hydrophilic as well as hydrophobic thiols, appears only after a short-time treatment of the enzyme with organomercurials. The observed differences with inorganic mercury (Table 1 and Figures 2, 3 and 4) suggest that the primary binding of monovalent organic mercury to non-sulfhydryl protein groups should be considered. The formation of such transient complexes, weaker than in the case of inorganic mercury, occurs probably at a certain distance from the hydrophobic enzyme active site where the most of low-molecular weight thiols could penetrate. However, this transient, reversible complex would be eventually masked by an irreversible process of inhibition. Membrane lipids peroxidation initiated by methyl radical could be excluded as a cause of such an irreversible inhibition. There still exists, however, the possibility that methyl radical itself (CH_3), generated in close vicinity of enzyme active center would play a role in the irreversible enzyme inhibition. Methylation of the enzyme active groups, once achieved, would account for the irreversible blocking of the active site. Homolytic cleavage of the covalent bound of alkyl-mercury compounds was reported to occur in brain (Inverson and Hierlihy, 1974).

Other heavy metals (lead and tin) studied at concentrations 100-fold higher than mercurials have negligible effect on myelin CNPase activity. The neurotoxic effect of triethyltin (TET), *in vivo*, was established for a tissue concentration of approximately 10^{-5}M (Kutsman *et al.*, 1963). In our experiment, this compound lead only to about 20% inhibition, at a concentration of 10^{-4}M after a 30 min preincubation of the enzyme with TET. In previous studies, it was reported that brain CNPase was not inhibited by TET (Hassengar and Kroon, 1973). This is in agreement with the results that we obtained without preincubation of the enzyme with inhibitor.

Lead compounds, both organic and inorganic, did not inhibit CNPase up to 10^{-4}M

concentration. Higher concentrations did not seem to have any physiological significance, as the lead concentration in brain does not exceed 30-40 μM (Lefauconnier *et al.*, 1980) after *in vivo* administration of high doses of lead acetate.

The relative high reactivity of mercurials with CNPase as compared with other heavy metals, may be related to their high solubility in organic solvents (Robinson *et al.*, 1967), which allows them to penetrate the hydrophobic region of that intrinsic protein of myelin.

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