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INHIBITION BY LEAD OF PHENYLETHANOLAMINE-*N*-METHYLTRANSFERASE

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Abstract—Phenylethanolamine-*N*-methyltransferase (PNMT) catalyzes the conversion of norepinephrine to epinephrine. The enzyme, obtained from bovine adrenal gland, was incubated with $PbCl_2$ at 23° for various times prior to assay at 37°. Inhibition developed slowly and reached a maximum after 45 min. In the presence of 4.5 nmoles $PbCl_2$ (15 μM), 5.8 μg protein was inhibited 50%, and inhibition was complete at 18 nmoles $PbCl_2$ (60 μM). At maximum inhibition the $PbCl_2$: protein ratio was 3.1 nmoles $PbCl_2$/μg protein. In the presence of $PbCl_2$, the graph of enzyme activity versus protein concentration intercepted the abscissa to the right of the origin, indicating that lead is an irreversible or very slowly reversible inhibitor. The activity of PNMT which had been exposed to $PbCl_2$ (2.6 or 5.2 nmoles $PbCl_2$/μg protein) was not restored by the addition of EDTA, DL-penicillamine or 1,3-dithiothreitol even when the concentration of the chelator was in 10 to 200-fold mole excess over $PbCl_2$. DL-Penicillamine and 1,3-dithiothreitol were unable to prevent $PbCl_2$ inhibition of the enzyme when combined with $PbCl_2$ 1 hr before addition of enzyme. EDTA could prevent 40% of $PbCl_2$ inhibition but a decrease in total enzyme activity was noted in the presence of this chelator. Dialysis of the $PbCl_2$-inhibited enzyme against buffer alone or buffer plus DL-penicillamine did not result in restoration of PNMT activity.

The neurotoxic effects of lead poisoning are well documented and include encephalopathy characterized by seizures, coma and behavioural disorders [1, 2], as well as peripheral neuropathy with impaired motor function [3, 4]. The biochemical basis of these disorders is uncertain but *in vivo* studies indicate that the concentration of several neurotransmitters is altered in the brains of animals exposed to lead [5, 6]. One such neurotransmitter, norepinephrine, is increased in amount after lead exposure [7, 8].

One of the steps in norepinephrine metabolism involves methylation to epinephrine using the enzyme phenylethanolamine-*N*-methyltransferase (*S*-adenosyl-L-methionine: phenylethanolamine-methyltransferase, PNMT,* EC 2.1.1.28). This enzyme has been identified in many species [9, 10] including humans [11]. In mammals it is present in highest amount in the adrenal medulla [9, 12] although lesser amounts have been found in several other tissues including brain [13, 14]. Many regions of the brain exhibit PNMT activity including the hypothalamus, cerebral cortex and cerebellum [15–17]. PNMT is believed to possess an essential sulfhydryl group because inhibition of enzyme activity occurs *in vitro* in the presence of *p*-chloromercuribenzoate, [9], *N*-ethylmaleimide and mercury [11].

To date, no reports have appeared regarding the effects of lead on PNMT, and it is the subject of the present study. Kinetic data indicate that lead irreversibly inhibits this enzyme.†

* Abbreviations: PNMT, phenylethanolamine-*N*-methyltransferase; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-[2-(5-phenyloxazolyl)]benzene; and Cellosolve, ethyleneglycol monoethyl ether.

† Part of this work was presented to the American Society of Biological Chemists, June 2–5, 1980, New Orleans, LA, U.S.A.; *Fedn Proc.* **39**, 2215 (1980).

MATERIALS AND METHODS

Materials. Disodium EDTA was obtained from the Fisher Scientific Co., Detroit, MI. The Amersham Corp., Arlington Heights, IL, was the source of $^{210}Pb(NO_3)_2$ and *S*-adenosyl-L-[methyl-^{3}H]methionine (15 Ci/mmole). DL-Normetanephrine HCl, PNMT (obtained partially purified from bovine adrenal medulla), and *S*-adenosyl-L-methionine were obtained from the Sigma Chemical Co., St. Louis, MO. Prior to use, *S*-adenosyl-L-methionine was purified one a Biorad AG1-X8 (200–400 mesh, HCO_3^- form) column as described by Shapiro and Ehninger [18] to remove *S*-adenosylhomocysteine, a potent inhibitor of PNMT [19]. All other reagents were of analytical grade when available.

Assay methods. PNMT was assayed according to the method of Axelrod [9] with minor modifications. In addition to enzyme, the final assay mixture contained 0.3 μmole DL-normetanephrine HCl, 10 μmoles phosphate (pH 7.9), and 12.5 nmoles *S*-adenosyl-L-methionine in a total volume of 300 μl. Similar results were obtained if 6–10 μmoles imidazole HCl (pH 7.9) replaced phosphate. Controls were run in the absence of normetanephrine or enzyme. Incubation was carried out for 1 hr at 37°. The reaction was stopped by addition of 0.5 ml of 0.5 M borate buffer (pH 10) and 5 ml of toluene–isoamyl alcohol (3:2, v/v). The tubes were vortexed for 30 sec, and 4 ml aliquots of the organic layer were removed and dried prior to counting in a Packard model 3375 liquid scintillation spectrometer. The scintillation fluid consisted of 8 g PPO, 0.2 g POPOP, 1 liter toluene (scintanalyzed) and 1 liter Cellosolve (purified). PNMT was exposed to $PbCl_2$ for periods of 0–120 min at 23° prior to addition of reaction mixture. Likewise, in experiments using EDTA,

DL-penicillamine or 1,3-dithiothreitol, these chelators were added prior to assay. The activity of the enzyme not exposed to $PbCl_2$ was unaffected over these time periods. The data represent the mean of duplicate samples that differ less than 8%.

Dialysis experiments. Plexiglass microcells of the type frequently utilized for equilibrium dialysis were used. Each half-cell contained four chambers of 100 μl volume. The chambers were coated with epoxy adhesive-sealant (Easy Epoxy Repair Kit, K-256, CONAP, Inc., Olean, NY) and allowed to dry for 24 hr prior to use. The two half-cells were separated by dialysis tubing and clamped tightly together to prevent leakage.

Enzyme (46 μg protein) was exposed to 0.3 mM $PbCl_2$ or H_2O for 1 hr at 23°. The final volume was 400 μl. Next, 90 μl of the enzyme or enzyme—$PbCl_2$ solution was placed in one side of the microcells. The other side contained 90 μl of 0.033 M imidazole HCl/0.15 M KCl (pH 7.9) or 90 μl of the imidazole/KCl buffer containing 0.02 M DL-penicillamine. Dialysis was performed at 23° with mechanical shaking for the duration of the experiment. The buffer or buffer–DL-penicillamine was replaced at 1, 3, 5.5 and 22 hr. After 24 hr, 40 μl aliquots of enzyme solutions were removed and assayed for PNMT activity as described above. In all cases, duplicate samples were assayed. Aliquots of the undialyzed, starting preparations also were assayed for PNMT activity.

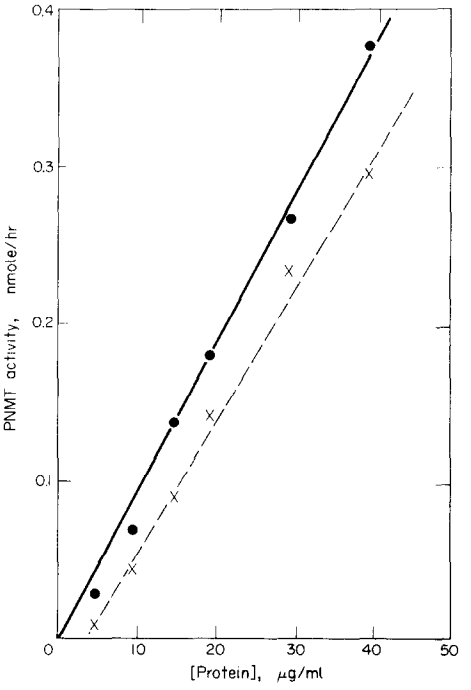

Fig. 2. PNMT activity as a function of protein concentration. PNMT was exposed to H_2O (●——●) or 3 nmoles $PbCl_2$ (×---×) at 23° for 1 hr prior to enzyme assay. PNMT activity is expressed as nmoles metanephrine produced/hr.

RESULTS

Inhibition of PNMT activity by $PbCl_2$ increased as a function of exposure time. When 5.8 μg protein was treated with 3 nmoles $PbCl_2$, inhibition developed slowly and reached a maximum of 25% after 45 min (data not shown). In all cases, enzyme samples incubated under identical conditions but without $PbCl_2$ showed no loss of enzyme activity. At the same protein concentration, addition of 4.5 nmoles (15 μM final concentration) $PbCl_2$ resulted in 50% inhibition of PNMT activity. Activity was completely inhibited in the presence of 18 nmoles (60 μM) $PbCl_2$ (Fig. 1).

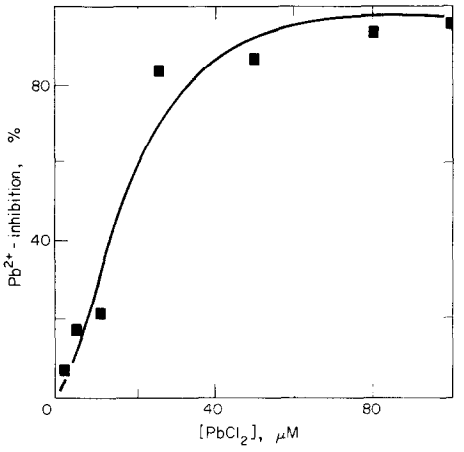

Fig. 1. $PbCl_2$ inhibition of PNMT activity at different $PbCl_2$ concentrations. Protein content was 5.8 μg. A 1-hr exposure to $PbCl_2$ at 23° preceded PNMT assay. The abscissa shows total $PbCl_2$ concentration. Values are percent inhibition relative to a control sample exposed under the same conditions but with H_2O instead of $PbCl_2$. The activity of the control was 0.26 nmole metanephrine produced/hr.

Alternatively, when the concentration of $PbCl_2$ was held constant at 3 nmoles (10 μM) and the amount of protein was varied, 100% inhibition occurred at a protein concentration of 3.5 μg/ml (Fig. 2). The $PbCl_2$:protein ratio needed for 100% inhibition of PNMT activity as determined from this experiment was 2.9 nmoles $PbCl_2$/μg protein. This value was in close agreement with the value of 3.1 nmoles $PbCl_2$/μg protein as determined from Fig. 1.

Chelators were unable to reverse the $PbCl_2$-induced inhibition of PNMT activity when added after exposure of the enzyme to $PbCl_2$ (Table 1, column A). $PbCl_2$:protein ratios of 2.6 and 5.2 nmoles $PbCl_2$/μg protein were chosen to give 84 and 100% inhibition respectively. The chelators were added in 10- to 200-fold mole excess over $PbCl_2$ for 1 hr prior to PNMT assay. In no instance was any significant reversal of $PbCl_2$ inhibition noted. When $PbCl_2$ and either DL-penicillamine or 1,3-dithiotheritol in 200-fold mole excess over $PbCl_2$ were incubated for 1 hr prior to addition of enzyme, these chelators were unable to prevent $PbCl_2$ inhibition of PNMT activity (Table 1, column B). EDTA in 67- or 100-fold mole excess over $PbCl_2$ was able to partially prevent inhibition of the enzyme. However, control samples of the enzyme not exposed to $PbCl_2$ showed up to a 50% loss of activity in the presence

Table 1. Inability of chelators to restore or prevent loss of PNMT activity

$PbCl_2$ (μmoles)	Chelator (μmoles)	% Inhibition A*	% Inhibition B†
	EDTA‡		
0.030	0.3	92	
0.015	0.3	95	
0.015	1.0	92	60
0.015	1.0	87	43
	DL-Penicillamine§		
0.030	0.3	100	
0.015	0.3	100	
0.015	3.0	89	81
	1,3-Dithiothreitol§		
0.015	3.0	93	93
0.030	None	95	95
0.015	None	84	84

* PNMT (5.8 μg protein) was exposed to $PbCl_2$ for 1 hr at 23°. Chelator was added and another 1-hr incubation preceded enzyme assay. In both A and B, values are percent inhibition relative to control samples treated with chelator but not $PbCl_2$. Values for percent inhibition in the absence of chelators are included for comparison.

† $PbCl_2$ and chelator were preincubated at 23° for 1 hr prior to addition of PNMT (5.8 μg protein). Another 1-hr incubation preceded PNMT assay.

‡ The total activity of lead-free PNMT samples treated with 0.3, 1.0 or 1.5 μmoles EDTA decreased 20, 45 and 48%, respectively, when compared to identical samples but without EDTA.

§ The activity of lead-free PNMT samples remained virtually the same (0.015 nmole metanephrine produced/hr) whether or not DL-penicillamine or 1,3-dithiothreitol was present.

of EDTA. The PNMT activity in control samples was not inhibited by the addition of DL-penicillamine or 1,3-dithiothreitol.

Dialysis experiments were performed in an attempt to restore enzyme activity by removal of $PbCl_2$. Because of the small amount of enzyme, microcells were used. Preliminary experiments using $^{210}Pb(NO_3)_2$ indicated that a large percentage of Pb^{2+} adhered to the walls of the plexiglass chambers.

Table 2. Inability to restore PNMT activity by dialysis*

Dialysate	% Activity†
Imidazole	2
Imidazole + DL-penicillamine	8

* PNMT was exposed to $PbCl_2$ (2.6 nmoles $PbCl_2$/μg protein) for 1 hr prior to 24 hr of dialysis at 23°. The procedure is described in Materials and Methods. Values are percent activity relative to enzyme samples dialyzed in similar fashion but without exposure to $PbCl_2$. The activities of the controls were 0.26 nmole metanephrine produced/hr. The activity of the enzyme was not changed significantly during the course of this experiment.

† Activity of the lead-treated enzyme from the original incubation was 5% that of the lead-free control when not dialyzed.

Thus, the chambers were coated with an epoxy sealant. The activity of PNMT was not affected by this sealant. Dialysis of an enzyme–$^{210}Pb(NO_3)_2$ solution (5.2 nmoles Pb/μg protein) for 5 hr against either imidazole or imidazole–penicillamine resulted in removal of 80% of the ^{210}Pb. After 18 hr, 95% of the ^{210}Pb had been removed.

The activity of PNMT exposed to $PbCl_2$ (2.6 nmoles $PbCl_2$/μg protein) for 1 hr prior to dialysis was not restored when dialyzed against either imidazole or imidazole-penicillamine for 24 hr (Table 2). The activities of control samples not exposed to $PbCl_2$ were not altered significantly during the dialysis period.

DISCUSSION

The inhibition of PNMT activity by $PbCl_2$ appeared to be irreversible. The observation that $PbCl_2$-induced inhibition of the enzyme developed slowly suggests that the total amount of active enzyme was slowly decreasing. Additional support for this hypothesis comes from a plot of PNMT activity in the presence and absence of $PbCl_2$ (Fig. 2). The line obtained in the presence of $PbCl_2$ has the same slope as the uninhibited enzyme but crosses the abscissa to the right of the origin suggesting irreversible inhibition [20]. Chelators such as EDTA, DL-penicillamine and 1,3-dithiothreitol in large molar excess over $PbCl_2$ were unable to restore enzyme activity when added after exposure of the enzyme to $PbCl_2$. Furthermore, DL-penicillamine and 1,3-dithiothreitol were unable to prevent $PbCl_2$ inhibition of the enzyme, and EDTA only partially prevented inhibition. Care must be taken when interpreting the EDTA results because EDTA reduced the total activity of control samples not exposed to $PbCl_2$. Finally, the activity of PNMT inhibited by $PbCl_2$ was not restored by dialysis against buffer or buffer plus chelator. These data demonstrate that the $PbCl_2$-induced inhibition of PNMT activity was irreversible.

Kitabchi and Williams [11] reported that Hg^{2+} reversibly inhibits PNMT and suggested that the enzyme has a sulfhydryl group at its active site which is necessary for enzymatic activity. The binding of Pb^{2+} to this sulfhydryl may account for the inhibition. Equilibrium binding data would be needed to determine the number of Pb^{2+} binding sites as well as the association constant(s). These studies must wait until a highly purified enzyme preparation can be obtained so that non-specific lead binding can be eliminated.

The finding that Pb^{2+} plus DL-penicillamine or 1,3-dithiothreitol still inhibited the enzyme (Table 1, column B) raises the possibility that the chelators can actually enhance Pb^{2+} inhibition or that the Pb^{2+}–inhibitor complex is also inhibitory. The first point has been ruled out. In experiments in which PNMT activity was inhibited 50% by $PbCl_2$ alone, no increase in inhibition was seen if $PbCl_2$ plus chelator was used instead (data not shown). The latter possibility still may exist. Measurement of Pb^{2+} association constants in the presence and absence of chelators will be useful in answering this question.

It is difficult to draw conclusions regarding effects of Pb^{2+} on PNMT activity *in vivo* based on these *in*

vitro studies. $PbCl_2$, however, did irreversibly inhibit PNMT activity *in vitro* in the nmoles $PbCl_2/\mu g$ protein range. If sufficient concentrations of Pb^{2+} could reach the cellular location of PNMT in the adrenal medulla and brain, then presumably irreversible inhibition could result. This could contribute to alterations in the concentrations of norepinephrine and epinephrine in these tissues. Also, in the present study exposure to $PbCl_2$ was of short duration, whereas *in vivo* chronic exposure frequently occurs. What effect this would have on the enzyme or on the amount of Pb^{2+} needed for inhibition is unknown. Studies are underway to assess the *in vivo* effects of lead.

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