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Inhibition of bovine plasma amine oxidase by lead

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A variety of symptoms are noted in patients suffering from acute lead poisoning. These include anemia [1], impaired motor function [2] and encephalopathy characterized by seizures [3, 4]. The biochemical defects giving rise to these symptoms are beginning to be defined. As a result of lead intoxication, blood lead levels rise. Lead may interact with a variety of serum and cellular enzymes to either enhance or inhibit their activity [5]. Furthermore, after lead crosses the blood–brain barrier, it has the potential to alter the metabolism of various compounds of the central nervous system. Studies using animals indicate that, after exposure to lead, the concentrations of several biogenic amines are altered in the brain [6, 7].

Recently, work in this laboratory has demonstrated that inorganic lead can inhibit two enzymes involved in norepinephrine metabolism, namely phenylethanolamine-*N*-methyltransferase [8] and dopamine-β-hydroxylase* *in vitro*. We wished to extend our studies regarding the effects of lead of amine metabolism to include a degradative enzyme. Because blood lead levels rise dramatically as a result of lead intoxication, we chose serum amine oxidase (benzylamine oxidase or amine:oxygen oxidoreductase, deaminating, E.C. 1.4.3.6). This enzyme catalyzes the deamination of biogenic amines according to the reaction:

$$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + NH_3 + H_2O_2$$

The serum enzyme, unlike mitochondrial monoamine oxidases, is soluble and contains copper [9]. The exact nature of the active site group(s) is uncertain. Sulfhydryl [10] or imidazolium groups [9] have been implicated. Either of these functional groups could be affected by heavy metals. Dooley and Coolbaugh [11] report that micromolar concentrations of Cu^{2+} can inhibit this enzyme *in vitro*.

To date, no reports have appeared regarding the effects of inorganic lead on serum amine oxidase. In the present study we describe the kinetics of lead inhibition of the bovine plasma amine oxidase *in vitro*.†

Materials and methods

Materials. DL-Penicillamine, 1,3-dithiothreitol, benzylamine, tranylcypromine,‡ serotonin, kyuramine, pargyline and amine oxidase (partially purified from bovine plasma) were obtained from the Sigma Chemical Co., St. Louis, MO. 5-Hydroxy[side chain-2-^{14}C]tryptamine creatine sulfate (50–60 mCi/mmole) was purchased from the Amersham Corp., Arlington Heights, IL.

Assay methods. The assay for plasma amine oxidase activity was based on the method of McEwen and Cohen [12] with several modifications. Unless otherwise noted, the enzyme was exposed to $Pb(NO_3)_2$ for 10 min at 37° prior to addition of the reaction mixture. In experiments in which a chelator was also added, the enzyme was incubated with $Pb(NO_3)_2$ after which chelator was added and the samples were incubated at 37° for 30 min prior to addition of reaction mixture. The assay mixture contained 5 μmoles benzylamine and 40 μmoles Tris–HCl (pH 7.2) in a final volume of 1 ml unless otherwise noted. Controls were run in the absence of enzyme or benzylamine. The mixture was incubated for 90 min at 37°. The reaction was stopped by addition of 100 μl of 60% perchloric acid. Cyclohexane (1 ml) was added, and the tubes were vortexed and allowed to stand at room temperature for 15 min. After a second mixing they were centrifuged in an IEC Clinical Centrifuge for 10 min. The absorbance of the cyclohexane layer was determined at 242 nm using a Gilford model 2220 spectrophotometer. The data represent the means of duplicate experiments that differed by less than 10%.

One unit of enzyme activity is defined as the amount of enzyme catalyzing an absorbance increase of 0.001/min at 242 nm and 37°. This corresponds to 75 pmoles benzylaldehyde formed per min per ml.

In assays using kynuramine as substrate, the method of Harada *et al.* [13] was followed except that 75 mM Tris–HCl (pH 7.4) was used and the final volume was 1 ml. With serotonin, the only modification of the method of Honecker *et al.* [14] was the use of 0.1 M Tris–HCl (pH 7.4) and a final volume of 250 μl.

Results and discussion

Prior to the $Pb(NO_3)_2$ inhibition studies, the conditions of enzyme assay were verified. The oxidation of benzylamine was linear over the 90 min assay, and the rate of oxidation was proportional to the concentration of enzyme. Addition of 0.16 mM tranylcypromine to 7 μg protein abolished 84% of amine oxidase activity. Pargyline at a final concentration of 0.3 mM did not inhibit enzyme activity. This is consistent with data on the pig plasma enzyme [15].

The inhibition of plasma amine oxidase by $Pb(NO_3)_2$ was independent of the exposure time. When 28 μg protein was exposed to 0.3 μmole (0.3 mM) $Pb(NO_3)_2$ for intervals between 0 and 30 min prior to addition of reaction mixture, the enzyme activity was inhibited 86% in all cases. Samples incubated under identical conditions, but without $Pb(NO_3)_2$, showed no loss of enzyme activity. Therefore, we routinely incubated the enzyme with $Pb(NO_3)_2$ for 10 min prior to addition of reaction mixture. Under these conditions, amine oxidase activity was inhibited completely with 0.4 μmole (0.4 mM) $Pb(NO_3)_2$ (Fig. 1).

It was of interest to determine whether the Pb^{2+} inhibition of amine oxidase was reversible. When the concentration of $Pb(NO_3)_2$ was held constant at 0.3 mM and the amount of enzyme was varied, the resulting graph intercepted the origin (Fig. 2). This suggests that Pb^{2+} was acting as a reversible inhibitor [16]. To confirm this, we exposed the lead-treated enzyme to various compounds known to bind Pb^{2+} very tightly. When 11 units of enzyme activity were exposed to 0.3 μmole Pb^{2+} for 30 min, the

* S. Barsoum and M. L. Caspers, manuscript in preparation.

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‡ Abbreviations: tranylcypromine, *trans*-2-phenylcyclopropylamine; serotonin, 5-hydroxytryptamine; and pargyline, *N*-methyl-*N*-2-propynylbenzylamine.

activity was inhibited 79% (2.3 units remained). Addition of 2 μmoles phosphate (pH 7.2) completely restored enzyme activity. Addition of phosphate to control samples not exposed to Pb^{2+} did not affect enzyme activity. In other experiments, 8 μmoles DL-penicillamine when added to control samples reduced enzyme activity 51% to 5.6 units.

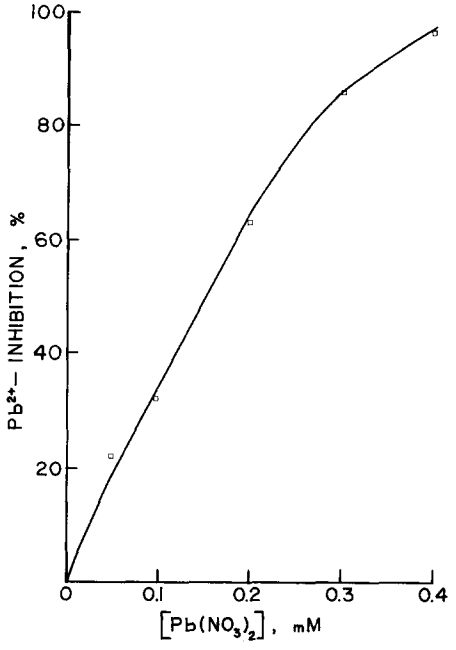

Fig. 1. Inhibition of plasma amine oxidase as a function of total $Pb(NO_3)_2$ concentration. The protein content was 28 μg. $Pb(NO_3)_2$ and enzyme were incubated for 10 min prior to enzyme assay using 5 mM benzylamine as substrate. Each point represents percent inhibition relative to samples containing 15 units of enzyme activity treated similarly but without $Pb(NO_3)_2$.

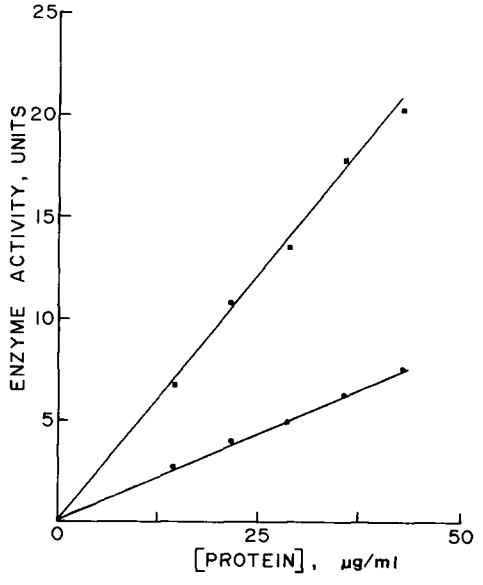

Fig. 2. Amine oxidase activity as a function of protein concentration. The enzyme was exposed to H_2O (■—■) or 0.3 μmole $Pb(NO_3)_2$ (●—●) for 10 min at 37° prior to enzyme assay.

When the enzyme was exposed to Pb^{2+} first, followed by DL-penicillamine, enzyme activity was also 5.6 units. Thus, the DL-penicillamine restored Pb^{2+}-induced inhibition of amine oxidase. Although EDTA and 1,3-dithiothreitol chelate Pb^{2+}, the presence of 8 μmoles of either of these compounds caused substantial inhibition of the enzyme in control samples (84% and 97%, respectively), presumably by removing copper which is essential for enzyme activity [17]. Tabor *et al.* [18] note that 4 mM EDTA did not inhibit the enzyme activity in 1.5 to 3 mg protein. In our experiments 8 mM EDTA was incubated with 0.028 mg protein. This greater EDTA:protein ratio may account for the 84% inhibition of amine oxidase activity.

A Lineweaver–Burk plot of amine oxidase activity as a function of benzylamine concentration in the presence of various concentrations of $Pb(NO_3)_2$ demonstrates that Pb^{2+} acted as a noncompetitive inhibitor (Fig. 3). The apparent Michaelis constant, K_m, for benzylamine in the presence of ambient O_2 was 2.2 mM. This value is slightly higher than the value of 1.4 mM reported by Oi *et al.* [19]. Variations in assay conditions may account for this discrepancy. The inhibition constant, K_i, for Pb^{2+} was determined to be 46 μM (Fig. 3 inset).

When kynuramine or serotonin was used as substrate, Pb^{2+} also acted as a noncompetitive, reversible inhibitor of amine oxidase (data not shown).

The site(s) on the enzyme that interacts with Pb^{2+} remains to be elucidated. However, histidine or cysteine residues may be involved in the catalytic cycle of the enzyme [9, 10], and Pb^{2+} could bind to either of these functional groups. The binding of Pb^{2+} to a sulfhydryl would be much tighter than to an imidazolium side chain [20, 21]. The association constant for Pb^{2+}-cysteine is 10^6 times greater than for the Pb^{2+}-histidine complex [22, 23]. With this tight binding, one might expect irreversible inhibition of the enzyme if Pb^{2+} were binding to sulfhydryls. In the case of phenylethanolamine-*N*-methyltransferase, an enzyme that has a sulfhydryl at the active site [24], Pb^{2+} does inhibit the enzyme irreversibly [8]. Further studies are needed to examine these possibilities.

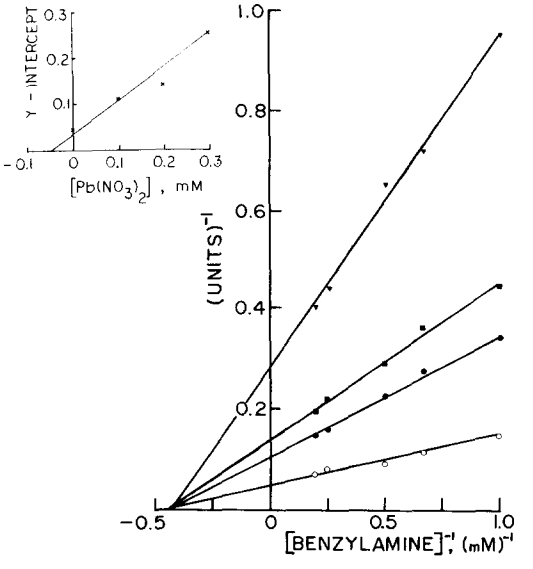

Fig. 3. Lineweaver–Burk plot of enzyme activity as a function of benzylamine concentration. The protein content was 28 μg. The final concentrations of $Pb(NO_3)_2$ were 0 (○—○), 0.1 mM (●—●), 0.2 mM (■—■) and 0.3 mM (▼—▼). Inset: Replot of the y-intercept ($1/V_{max}$) values versus the concentration of $Pb(NO_3)_2$. The x-intercept in this figure is equal to $-K_i$ [16]. The line was obtained through linear regression analysis.

It would be of interest to know if the plasma amine oxidase is inhibited in actual cases of lead poisoning. While it is difficult to draw conclusions regarding the effects of lead *in vivo* based on these *in vitro* results, a rough calculation can be made. In patients with clear evidence of lead toxicity, blood levels of lead range from 100 to 1000 μg Pb^{2+}/100 ml whole blood [25]. More than 90% of the lead is believed to sequester in erythrocytes [26]. Thus, 0.5 to 5 μM lead would remain in serum. Not all of this lead would be available to inhibit the amine oxidase because other serum proteins such as albumin can bind Pb^{2+} [20]. Our data indicate that the amine oxidase activity inhibited 10% in the presence of 25 μM Pb^{2+}. While it seems unlikely that this enzyme would be inhibited by lead levels sufficient to cause the clinical disorder, the definitive answer can only come from studies correlating enzyme activity with blood lead levels.

In summary, Pb^{2+} acted as a noncompetitive, reversible inhibitor of bovine plasma amine oxidase *in vitro*. The inhibition constant (K_i) for $Pb(NO_3)_2$ was 46 μM.

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Effect of valinomycin on human peripheral blood lymphocytes

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Valinomycin, a cyclic dodecadepsipeptide that enhances ion permeability of artificial membranes selectively for K^+, has been reported to inhibit phytohemagglutinin (PHA)-induced lymphocyte transformation in human peripheral blood lymphocytes [1, 2] as judged by depressed [^{3}H]thymidine ([^{3}H])TdR) incorporation into DNA. The inhibitory effect of valinomycin on mitogenic transformation was overcome by increasing the external K^+, suggesting that interaction of PHA with specific receptors on the lymphocyte cell membrane may involve mechanisms affecting cation fluxes and membrane potential [2]. The mechanism of inhibition of mitogenesis by valinomycin remains unsettled.

The present investigation was designed to ascertain whether non-lectin mitogens, which do not require the presence of specific binding sites for their action, would also be unable to induce blastogenesis in the presence of valinomycin. The inhibitory action of valinomycin may occur at a step(s) subsequent to initial stimulation and be totally non-specific as to the agent or technique used to induce transformation. The effect of increasing the external K^+ concentration on inhibition of mitogenesis by valinomycin was also examined, along with the effect of valinomycin on [^{3}H]TdR and [^{3}H]uridine incorporation by maximally transformed cells, in order to distinguish between the degree of actual inhibition of lymphocyte