Lead-inhibited adenylate cyclase: a model for the evaluation of chelating agents in the treatment of cns lead toxicity

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Lead exposure has been recently reported to affect the concentrations and turnover rate of the putative neurotransmitters, noradrenaline and dopamine (Michaelson, Greenland & Roth, 1974; Silbergeld & Goldberg, 1975; Silbergeld & Chisolm, 1976). It is as yet unclear whether such alterations in catecholamine metabolism result from a direct effect on neutrotransmitter synthesis (or degradation) or only indirectly from an alteration in catecholamine receptor activity. Current evidence suggests that the synaptic receptors for noradrenaline and dopamine may be intimately associated with the membrane-bound enzyme adenylate cyclase, and that activation of this enzyme, resulting in the intracellular formation of adenosine 3′,5′-monophosphate (cAMP), may mediate the synaptic effects of the catecholamines (Klainer, Chi & others, 1962; Greengard, 1976; Nathanson, 1977). Lead has been reported to be a potent inhibitor of brain adenylate cyclase, and it has been suggested that interference with cAMP metabolism and its associated synaptic mechanisms may be a factor in some of the behavioural manifestations of lead toxicity (Nathanson & Bloom, 1975, 1976). In support of this hypothesis are the results of recent electrophysiological studies in the cerebellum which show that low concentrations of lead block the cAMP-mediated inhibitory effect of noradrenaline on Purkinje cell discharge but fail to affect the non-cAMP-mediated effects of acetylcholine and α-aminobutyric acid on neuronal discharge (Taylor, Nathanson & others, 1976).

The efficacy of chelating agents currently employed in the treatment of lead toxicity has been determined primarily on the basis of their mobilization of lead from peripheral tissues or of their action on lead-induced abnormalities of heme biosynthesis (Selander, Cramer & Hallberg, 1966; Hammond, Aronson & Olson, 1967; Chisolm, 1968; Friedheim, Corvi & Wakker, 1976). There is little information presently available about the effects of these agents on lead-induced alterations of central nervous system (cns) metabolism. Because of the potent inhibition of brain adenylate cyclase by lead and the possible involvement of this enzyme in the physiology of synaptic transmission, it seemed of potential value to use the reactivation of lead-inhibited adenylate cyclase as a measure of the possible efficacy of various lead chelating agents in the treatment of the cns manifestations of lead toxicity.

The procedures used to assay brain adenylate cyclase activity have been described previously (Nathanson & Bloom, 1976). Briefly, 1 mm thick slices of cerebral or cerebellar cortex from 6-wk old male Sprague-Dawley (Zivic-Miller) rats were homogenized (15 mg ml^{-1}) in 6 mM tris maleate buffer (pH 7·4). Aliquots (1 mg wet weight) of this homogenate were preincubated for 2 min at 30° in a medium containing 80 mM tris maleate (pH 7·4); 10 mM theophylline; 6 mM $MgSO_4$; and 10 μM $PbCl_2$, in a final volume of 0·3 ml. After cooling to 0°, various chelating agents were added and the tubes preincubated for an additional 2 min at 30°, following which the enzyme reaction was started by the addition of 1·5 mM ATP (final concentration). Incubation was for 3 min in a shaking water bath, and the reaction was terminated by boiling for 2 min. After low speed centrifugation to remove insoluble material, cAMP in the supernatant was measured by the method of Brown, Elkins & Albano (1972) with appropriate blanks. Under the experimental conditions, adenylate cyclase activity was linear with respect to time and enzyme concentration; and, in the presence of theophylline, interference from phosphodiesterase activity was insignificant. The concentration of lead used (10 μM) was that which caused somewhat greater than a 50% inhibition (I50) of basal enzyme activity in the Zivic-Miller strain of Sprague-Dawley rat (Table 1). (The I50 of adenylate cyclase for lead differed somewhat among various stocks of Sprague-Dawley rats). This concentration had no effect on the protein binding assay for cAMP nor on non-enzymatic conversion of ATP to cAMP, potential sources of artifact which may occur at higher lead concentrations (Nathanson & Bloom, 1976).

Five chelating agents were evaluated in these studies: 2,3-dimercapto-1-propanol (BAL), β,β-dimethylcysteine (D-penicillamine); ethylenediaminetetraacetic acid (EDTA); meso-2,3-dimercaptosuccinic acid (DMS) and β-mercaptoethanol. Fig. 1 shows the effects of the compounds on lead-inhibited adenylate cyclase activity

Table 1. *Inhibition by lead chloride of basal adenylate cyclase activity in an homogenate of rat cerebellar cortex.*

Lead (μM)	Activity (pmol cAMP mg^{-1} min^{-1})
0	280 ± 22*
1	211 ± 3
2	180 ± 6
5	158 ± 2
10	115 ± 7
20	95 ± 5
50	79 ± 2
100	27 ± 4

* The values shown are the means (± mean deviation) for 2 to 4 replicate samples, each assayed in duplicate.

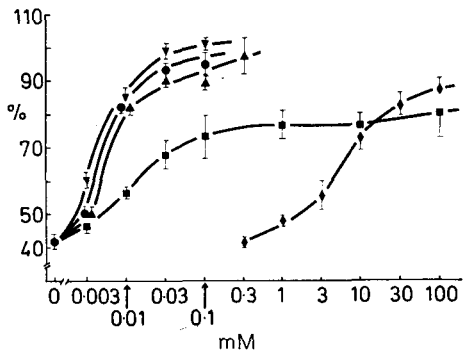

FIG. 1. Effect of various chelating agents (mM) on lead-inhibited adenylate cyclase activity (% of control) in a rat cerebellar homogenate. 10 μM $PbCl_2$ alone reduced enzyme activity to 42 $\pm$ 3% of control (control activity = 220 pmol cAMP mg^{-1} protein min^{-1}). The addition of increasing concentrations of ▼ ethylenediaminetetraacetic acid; ● meso-2,3-dimercaptosuccinic acid; ▲2,3-dimercapto-1-propranol; ■ β,β-dimethylcysteine (penicillamine); or ◆ β-mercaptoethanol restored enzyme activity to varying degrees. The control activity for which each data point was calculated was that in the presence of chelating agent alone (Fig. 2). The values shown are the means ($\pm$ mean deviation) for 2 to 4 replicate samples, each assayed in duplicate.

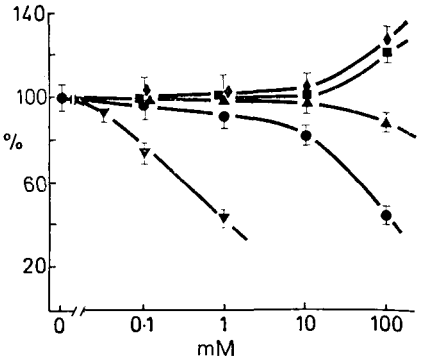

FIG. 2. Effect of various chelating agents (mM) on basal (lead-free) adenylate cyclase activity (% of control) in rat cerebellum. In most cases, the low concentrations of chelating agents necessary for reactivation of lead-inhibited activity (Fig. 1) had little effect on basal enzyme activity. However, at higher concentrations (above) these same agents did alter activity. Control activity was 220 pmol cAMP mg^{-1} protein min^{-1}. The values shown are the means ($\pm$ mean deviation) for 2 replicate samples, each assayed in duplicate. Symbols as in Fig. 1.

in rat cerebellum. In the presence of 10 μM $PbCl_2$ alone, basal enzyme activity was decreased to 42% that of control. EDTA was the most potent of the chelating agents in restoring enzyme activity—a concentration of less than 10 μM caused a 50% restoration of lead-inhibited adenylate cyclase activity, and 30 μM completely reactivated the enzyme. DMS and BAL were also quite effective in reversing the effects of lead: at 10 μM both agents were as potent as EDTA, but somewhat higher concentrations of DMS (100 μM) and BAL (300 μM) were required for complete reactivation of the lead-inhibited enzyme. For all three of these compounds, full reactivation of enzyme activity occurred at concentrations of the chelating agent which had no effect on control (i.e., lead-free) enzyme activity (Fig. 2).

Penicillamine was moderately effective in restoring enzyme activity (50% reactivation occurred at less than 100 μM) but failed to reactivate the enzyme completely, even at a concentration of 100 mM. β-Mercaptoethanol was an effective reactivator of lead-inhibited adenylate cyclase, but was much less potent than the other agents tested (10 mM β-mercaptoethanol was necessary to restore 50% of enzyme activity). At these higher concentrations (10 mM), both penicillamine and β-mercaptoethanol caused a small (20–30%) stimulation of control adenylate cyclase activity (Fig. 2). (These effects on enzyme activity in the absence of lead were used, in all cases, as the control values in calculating the reactivation of lead-inhibited activity.)

Although low concentrations of EDTA and DMS had no effect on control (i.e., lead-free) enzyme activity, higher concentrations of these two agents caused significant inhibition of basal enzyme activity (Fig. 2). These effects (which were insignificant at the concentrations necessary for reactivation of the lead-inhibitied enzyme) were possibly due to interaction with either enzyme cofactor (Mg^{2+}) or substrate (ATP). Results similar to those described in Figs 1 and 2 for cerebellar homogenates were obtained also with broken cell preparations of rat cerebral cortex.

BAL, EDTA and penicillamine have been used, with varying degrees of success, in the clinical treatment of lead toxicity (Boulding & Baker, 1957; Chisolm, 1968; Beattie, 1974). Calcium-EDTA has been reported to mobilize more lead from the body than penicillamine (Selander, 1967), and the combination of calcium-EDTA and BAL appears particularly effective in the treatment of acute lead encephalopathy (Chisolm, 1968). Penicillamine, on the other hand, seems to carry a lower degree of toxicity than the other agents, especially when used chronically (Selander & others, 1966; Chisolm, 1968). Recently, Friedheim & others (1976) have investigated the use of the water-soluble BAL analogue, DMS, and have found it superior to either EDTA or penicillamine in reducing brain lead concentrations in lead-treated rats. With the exception of penicillamine, none of these chelating agents has been used in the treatment of chronic lead toxicity, and there have been as yet no published reports of the effect of chronic chelation therapy on the behavioural abnormalities associated with low-dose lead toxicity.

The present results demonstrate clear differences in the effectiveness of various chelating agents in reactivating lead-inhibited adenylate cyclase from the brain. The ultimate clinical usefulness of such agents in the

treatment of the cns manifestations of lead toxicity will, of course, depend on additional factors. For example, because of the differential absorption, binding and intracellular uptake of these compounds (see Chisolm, 1968), it is possible that the *in vivo* reactivation of brain adenylate cyclase may differ somewhat from that seen *in vitro*. Nonetheless, the procedure outlined here offers a rapid, convenient and physiologically meaningful assay for the initial screening of potentially effective chelating agents for use in the treatment of cns lead toxicity.

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The segregation of granules during tableting

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In the manufacture of tablets segregation of dissimilar granules can occur during the flow of granules from the hopper and also within the hopper as a consequence of vibration. As the drug content of granules of differing size can vary significantly (Lachman & Sylwestrowicz, 1964; Selkirk, 1976), segregation of granules could therefore contribute to variations in the drug content of tablets. To investigate this variation a series of batches of granules have been made from blends of two size fractions of lactose granules. One part of each blend contained the dye eosin so that variation in the ratio of granules from each size fraction, in the final tablet, could be studied.

Preparation of granules. A number of 2 kg batches of granules were prepared using a conventional massing and screening technique. The binder was 5% w/w solution of polyvinylpyrrolidone (220 cm^3) with or without eosin (1·0 g). The damp mass was forced through a 1·0 mm sieve dried at 60° in trays in an oven fitted with a fan and rescreened through a 1·0 mm sieve using a Jackson-Crockatt granulator. The dried granules were sieved into 5 size fraction >1·0 mm; 1·0 mm–710 μm (fraction A); 710–500 μm (fraction B); 500–250 μm (fraction C) and <250 μm. The sieve fractions from each batch were combined to give sufficient granules in each size fraction for the subsequent tableting experiments.

Assay of granules. Fractions A, B and C were assayed for eosin content, 5·0 g of granules were dissolved and made up to 500 cm^3 with water. The resulting solutions were assayed colorimetrically, the eosin contents were 0·490, 0·508 and 0·501% for batches A, B and C respectively.

Preparation of tablets. The tablets were prepared from 500 g batches of granules lubricated with 0·5% sodium lauryl sulphate (and 0·5% magnesium stearate if necessary) using a Manesty E3 machine. Each batch except one consisted of a 50:50 blend of two size