Comparison of the in vivo and in vitro effects of lead on the pH-activity relationship of human erythrocytic δ -aminolaevulinic acid dehydratase

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ABSTRACT The effect of lead in vitro on the pH-activity relationship of human erythrocytic δ -aminolaevulinic acid dehydratase (δ -ALAD) and on the assessment of lead exposure with ratios of δ -ALAD activity measured at specific pH values was investigated. The addition of lead nitrate to whole blood at concentrations ranging from 0.40 to 8.1 μ mole Pb^{2+} /l, for periods of contact ranging from 16 h to 20 days at 4°C, resulted in a time and dose dependent shift of the enzyme's pH optimum to a more acid value. The pH optimum shift obtained at raised lead concentrations or after long periods of contact at 4°C, in both, closely approximated that observed in vivo. The loss of enzyme activity, however, was significantly less in vitro than that in vivo for similar whole blood lead concentrations. These findings indicate that the presence of trace amounts of lead in blood collection devices can seriously affect results obtained with the pH activity ratio method of assessing lead exposure.

A small increase in an individual's endogenous blood lead concentration may result in a measurable reduction in the activity of the erythrocytic enzyme δ -aminolaevulinic acid dehydratase (δ -ALAD, prophobilinogen synthase, EC 42124) assayed at pH 6.4 in phosphate buffer.¹ The degree of inhibition observed correlates closely with the concomitant concentration of lead in blood and, as such, has been proposed by many as a basis for biologically monitoring exposure to low lead concentrations.¹⁻⁵

Nikkanen *et al* have shown that lead has another less apparent effect on the enzyme in vivo.¹ That is, the enzyme's pH optimum shifts to more acid values as lead concentrations exceed normal values. This effect has been confirmed by Tomokuni⁶ and ourselves.⁷

According to the few studies conducted to date,¹⁻⁸ the action of lead in vitro on the enzyme in human blood does not cause a shift in the pH optimum. Instead, enzyme activity decreases uniformly across the pH range. Nikkanen explained this occurrence by proposing that lead produces its effects in vivo via

an allosteric type inhibition and suggested that the metal interacts with the enzyme in a different manner in vitro.¹

Recently we have proposed a biological test for lead intoxication which entails the use of a ratio of δ -ALAD activities measured at two carefully selected pH values.⁹ The results of previous studies notwithstanding, it is clear that any changes in the pH activity relationship caused by adventitious lead, such as may be present in blood collecting and storage devices,¹⁰ would seriously affect the test results. Furthermore, the lengthy period of blood storage (> 2 weeks at 4°C) allowed by the proposed pH activity ratio method protocol increases the time of contact with exogenous lead and could favour such an occurrence. For this reason, we have re-examined the effects of lead in vitro on δ -ALAD to ascertain whether or not it could affect the δ -ALAD pH-activity relationship under the proposed pH-activity ratio test conditions.

Materials and methods

BLOOD SAMPLE COLLECTION

Thirty millilitre blood samples were collected by venepuncture with a disposable 30 ml plastic syringe (Becton Dickinson and Co, Rutherford, NJ) from six volunteer non-smokers with no known exposure

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to lead. The blood samples were immediately pooled in a plastic container containing enough sodium heparinate (Abbott Laboratories Ltd, Montreal, Quebec, Canada) to yield a final solution of 500 USP units per ml of blood.

IN VITRO EXPERIMENT

Ten millilitre aliquots of the pooled blood sample were transferred to eight polystyrene culture tubes (No 2570, Corning Glass Works, Corning, NY 14835) containing 25, 50, 75, 150, 200, 250, 300, and 500 μ l of a solution of 33.56 μ g lead (5N9, Ventron Alfa Division, Danvers, MA 01923) as the nitrate salt per ml of saline solution prepared from ARISTAR grade sodium chloride (British Drug House). Ten millilitre aliquots of the remaining blood sample were added to polystyrene culture tubes containing 25, 50, 75, 100, 150, 200, 250, 300, and 500 μ l saline solution. All samples were gently mixed for one hour at 4°C on a Multipurpose rotator (Scientific Industries, Inc). Each blood sample, control and contaminated, was further subdivided into 10 equal portions and stored in 1.5 ml plastic micro tubes (Brinkmann Instruments, Inc) at 4°C until analysed.

δ -ALAD pH-ACTIVITY CURVES

Two hundred microlitres of each well mixed blood sample were placed into three 5 ml Falcon polypropylene tubes and 1.4 ml of 0.1% Triton X-100 (Sigma Chemical Co, St Louis, MO) solution added. The haemolysate was vortexed for 30 sec and allowed to stand in an ice bath for 3 min to complete lysis.

One hundred microlitres of lysate were added to each one of a series of 400 μ l disposable polypropylene tubes (Brinkmann Instruments, Inc) containing 100 μ l each of the substrate, δ -aminolaevulinic acid (Sigma Chemical Co, St Louis, MO) (0.125 mM), and phosphate buffers (0.3M) pH 5.8 to 7.4, preincubated at 37°C for 30 min. The mixtures were capped, vortexed, and the micro tubes placed in a water bath at 37°C for 60 min. The remainder of the assay was performed as described elsewhere.¹¹

A pH-activity curve was obtained for δ -ALAD at each blood lead concentration and for each corresponding control blood sample after 16 h, 2, 4, 7, 10, 14, and 20 days of storage at 4°C.

It should be noted that one set of samples was fractionated into plasma and erythrocytes after 1 h of contact with added lead at 4°C. The erythrocytes were washed repeatedly with ice cold saline and stored at 4°C for nine days before the derivation of the δ -ALAD pH activity curve for each sample to determine if plasma borne lead had a role in the

effects observed.

δ -ALAD ACTIVITY RATIO TESTS

Recently, we have proposed a novel biological test for lead intoxication which involves the measurement of erythrocytic δ -ALAD activity at two carefully selected pH values and the use of the ratio of the activity values obtained as an index of lead exposure.⁹ In our laboratory this test has proved to be a plausible alternative to the usual tests for lead intoxication—namely, blood lead concentration and free erythrocytic protoporphyrin level. The effect of exogenous lead present in trace amounts in blood collecting devices on one such δ -ALAD activity ratio—namely,

$$\frac{\delta\text{-ALAD activity measured at pH } 6.4}{\delta\text{-ALAD activity measured at pH } 7.2}$$

was studied. The enzyme activity values at pH 6.4 and 7.2 were obtained for each test sample during the derivation of δ -ALAD pH activity curves described above.

BLOOD LEAD ANALYSIS

The analysis of the lead in each blood, plasma, or erythrocytic cytosol sample was performed as described elsewhere.¹¹ The erythrocytic membrane was separated from the cytosol by centrifugation of the haemolysates at 11 500 rpm for 30 min before analysis.

The blood lead values were verified by their comparison with results obtained by another clinical laboratory for the same samples. In all instances the results agreed within $\pm 7\%$.

Results

Attempts were made to simulate gross lead contamination of blood samples, as described earlier. The results of one of the several tests performed are shown in fig 1a-1b. After 16 h of contact at 4°C, an apparent progressive shift of the pH maximum occurs from its initial value at pH 6.5 to 6.1 accompanied by a significant decrease in enzyme activity concomitant with increasing exogenous lead concentrations (fig 1a). As storage time increases, a pH optimum shift to pH 6.0 and a change in the overall shape of the profile analogous to that observed for in vivo lead (fig 2) takes place at ever lower concentrations of adventitious metal (fig 1b). The optimum shift and overall profile shape usually observed in the blood of lead intoxicated individuals (fig 2) are approximated at all concentrations of exogenous lead after 20 days of contact at 4°C (fig 1b). It is noteworthy that an apparent increase in activity occurs at pH 6.0 at all concentrations after 16 h of contact.

Fig 1 Effects of varying concentrations of lead added to adult human blood on erythrocytic δ -ALAD pH-activity relationship after 16 hours (a) and 20 days (b) at 4°C. (●) Control blood, 0.53 μM lead/l; (Δ) 0.40, ($\blacktriangle$) 0.81, ($\circ$) 1.22, ($\blacksquare$) 2.43, ($\square$) 3.24, ($\diamond$) 4.05 and (+) 8.10 $\mu\text{M Pb}^{2+}$ /l blood added.

Fig 2 Erythrocytic δ -ALAD pH-activity relationship for the blood of an individual with no known exposure to lead (●) (0.43 μM lead/l) and firing range instructor occupationally exposed to lead fumes (2.17 μM lead/l) (▲).

Fig 3 Relationship between residual δ -ALAD activity determined at selected pH values for blood of adults occupationally exposed to lead and blood lead concentrations. Control population consisted of 21 individuals with no known exposure to lead (blood lead concentration $0.58 \pm 0.10 \mu\text{M/l}$); 13 firing range instructors constituted the exposed group (blood lead concentration $0.87 - 3.02 \mu\text{M/l}$).

The relation between blood lead concentrations and enzyme activity measured at pH 6.0, 6.4, and 6.8 (arbitrarily selected) is best depicted in figs 3 and 4 for *in vivo* and *in vitro* lead respectively. For *in vivo* lead, a rather rapid decrease in enzyme activity at blood lead concentrations $> 0.8 \mu\text{M Pb/l}$ followed by a tendency to plateau at $> 3.0 \mu\text{M Pb/l}$ takes place at pH 6.4 and 6.8. A more gradual and less pronounced decrease in enzyme activity with increasing blood lead concentrations occurs at pH 6.0. *In vitro* lead approximates the relationship between residual enzyme activity and blood lead "*in vivo*" only after relatively long periods of contact at 4°C (fig 4). Data obtained by other workers,¹²⁻¹⁴ who performed similar lead *in vitro* tests at single pH values, approximate closely to the results presented here.

Not surprisingly, the action of *in vitro* lead on δ -ALAD yields a comparable progression in the value of the ratios of enzyme activity measured at

Fig 4 Effect of varying concentrations of Pb^{2+} added to adult human blood (lead concentration $0.52 \pm 0.10 \mu M/l$) on activity of δ -ALAD measured at selected pH values after varying periods of storage at $4^\circ C$. Each point represents mean of results obtained from three separate trials.

pH 6.4/pH 7.2 from normal values to that expected for the blood of lead poisoned individuals. The values obtained for this ratio, after 20 days of contact, approach those observed at similar blood lead concentrations in vivo. Results obtained for the 6.4/7.2 ratio are shown in fig 5.

Discussion

The results presented here resolve a peculiarity in current reports on δ -ALAD—namely, that there is a significant difference in the lead poisoned enzyme behaviour depending on whether the lead was acquired in vivo or in vitro.¹ Our results show clearly that provided sufficient time elapses in the in vitro experiment there is no such difference. Previous work resulting in the differential conclusion was apparently reached because insufficient time was allowed to elapse for the full in vitro effect to be felt. Figure 1a and b shows this conclusion particularly clearly. Explanations to account for the in vivo-in vitro difference are therefore apparently no longer required, and subsequent work can now focus on the uniform way in which lead affects enzymic behaviour.

Fig 5 Effects of varying concentrations of Pb^{2+} added to adult human blood (lead concentration $0.54 \pm 0.1 \mu M/l$) on ratio of δ -ALAD activity measured at pH 6.4/pH 7.2 after different periods of storage at $4^\circ C$. ($\square$) 0, ($\blacktriangle$) 3, ($\triangle$) 6, ($\bullet$) 10 and ($\circ$) 20 days. Results obtained from lead exposed individuals are shown for comparison ($\blacksquare$).

The fact that such a long time is required for maximum in vitro effect is itself of interest. A possible explanation for this delay could be that the plasma bound lead, bound to albumin or globulin, or as a peptised lead phosphate sol¹⁵ could have difficulty penetrating the erythrocytic membrane to gain access to the enzyme in the cytosol. For this reason, we have carried out the lead analysis shown in the table. These data, and those of others,^{14,16} do not provide any support for this explanation, showing that equilibrium between the two fractions occurs early^{17,18} and does not change appreciably over a seven day period.

To determine if plasma borne lead plays a part in the effects observed, plasma was removed from blood samples previously treated with lead salt after one hour of contact. The red cells were washed repeatedly with cold saline and the pH activity relationship for the δ -ALAD in the washed erythrocytes determined in the usual fashion. As the results in fig 6 attest, the optimum shift observed after nine days

Effect of storage at 4°C upon the distribution of varying concentrations of Pb²⁺ added to human blood

Hct %	Amount added (μM/l)	Whole blood concentration (μM/l)	Plasma concentration (μM/l)	Plasma/whole blood (%)	Erythrocyte* concentration (μM/l)	Erythrocyte/whole blood (%)
Day 1						
42	8.10	8.58 ± 0.5	7.74 ± 1.2	64.3	5.99 ± 0.5	29.3
	6.48	6.80 ± 0.6	6.94 ± 0.8	59.2	3.94 ± 0.2	24.3
	4.05	4.05 ± 0.6	3.75 ± 0.2	53.7	2.96 ± 0.2	30.7
	1.62	1.37 ± 0.2	1.48 ± 0.1	52.7	1.63 ± 0.1	49.9
	0.41	0.43 ± 0.2	0.45 ± 0.2	43.9	0.72 ± 0.1	70.3
	—	0.24 ± 0.1	N.D.	—	0.58 ± 0.1	—
Day 7						
42	8.10	9.01 ± 0.9	8.09 ± 0.1	54.7	5.37 ± 0.3	26.3
	6.48	6.53 ± 0.7	6.06 ± 0.2	51.7	4.05 ± 0.2	25.0
	4.05	3.95 ± 0.7	3.75 ± 0.2	53.7	3.18 ± 0.2	33.0
	1.62	1.32 ± 0.2	1.39 ± 0.2	58.8	1.45 ± 0.1	44.5
	0.41	0.51 ± 0.1	0.42 ± 0.1	47.8	0.72 ± 0.1	59.3

*Refers to lead concentration in the erythrocytic cytosol only. The erythrocytic membrane was removed before analysis.

of contact is caused in large part by erythrocyte borne lead.

Ninety per cent of erythrocyte borne lead is reportedly bound to the haemoglobin molecule¹⁶ and a portion of the remaining lead probably interacts with the less abundant δ-ALAD. On the basis of findings by Barnard *et al.*,¹⁹ it may be postulated that this lead interacts most readily with the enzyme's active site geminal thiol groups and produces the rapid loss of activity observed both in vivo and in vitro. The results presented here notwithstanding, it is not yet apparent whether this interaction or other similar metal-protein interaction is also responsible for the pH optimum shift observed early in vivo and

approximated much later in vitro. Evidently, further work is required to find an explanation for both the delayed in vitro effect and the occurrence of the pH optimum shift.

The results presented here would indicate that utmost care must be taken to ensure that blood collection devices are lead free since the adventitious metal will eventually lead to results which approximate those obtained in vivo with the pH activity ratio method of assessing lead exposure. The impact on results obtained with the usual δ-ALAD assay¹¹ will be correspondingly less since this determination is usually performed within 24 h of blood sample collection.

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Fig 6 Effects of varying concentrations of erythrocyte borne lead on δ-ALAD pH-activity relationship nine days after addition of Pb²⁺ to adult human blood; plasma was removed one hour after addition of lead salt. (●) control blood, 0.58 μM Pb/l; test blood (Δ) 1.5, (▲) 1.9, (○) 2.96, (■) 5.99 μM Pb²⁺ added/l blood.

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