Delta-aminolevulinic acid dehydratase (ALAD) activity in blood of *Bufo arenarum* (Anura)

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The aim of the present investigation was to standardize a method for measuring δ-aminolevulinic acid dehydratase (ALAD) activity in circulating red blood cells of adult ***Bufo arenarum*** *kept in controlled environmental conditions, and to obtain reference basal values suitable for environmental monitoring of lead exposure. The normal ALAD activity for* ***B. arenarum*** *was 131.86 ± 14.47 U per liter of red blood cells (n = 38, mean ± SEM; interval 72.98 - 236.33). In animals exposed to lead, ALAD activity decreased as lead dose increased.*

Key terms: *δ-aminolevulinic acid dehydratase, Anura,* ***Bufo arenarum****,* ***delta–ALAD****, erythrocyte, lead, toad*

Biomarkers are essential to assess the environmental and health risks of exposure to potentially toxic chemicals. The development and use of biomarkers has recently become of major interest (20). Particularly, changes of blood enzyme activities can be useful as biochemical markers of toxicity for heavy metals, among them the bioaccumulation of lead, which has become one of the main human health and environmental problems in Latin America (16).

The inhibition of δ-aminolevulinic acid dehydratase (ALAD) in red blood cells has become accepted as a standard bioassay to detect acute and chronic lead exposure (13, 21, 23). It is one of the enzymes involved in the heme biosynthesis pathway which is recognized as essential to maintain hemoglobin content in erythrocytes. It catalyzes the condensation of *delta*-aminolevulinic acid to form porphobilinogen (PBG) and requires Zn^{++} and intact sulphydryls. Because it is the most abundant enzyme in that system, it is unlikely to play a regulatory role (15).

The above mentioned metabolic pathway was directly or indirectly shown to be present both in endothermic and ectothermic vertebrates (6, 8, 10, 12, 17).

The aims of the present investigation were to standardize a method for measuring ALAD activity in circulating red blood cells of adult *Bufo arenarum*, kept in controlled environmental conditions, and to obtain reference basal values suitable for environmental monitoring of lead exposure.

Since the life cycle of amphibians occurs both in freshwater and on land, they may be threatened by environmental

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pollutans simultaneously during the early developmental stages as well as in adult conditions. The presence of elevated amounts of lead may severely affect their survival, because of its toxicity to the red blood cells and their precursors, the central and peripheral nervous system and the kidneys. Thus, amphibians constitute one of the most vulnerable points of the natural trophic webs. For this reason, they are useful as indicators of the environmental conditions, by means of the measurement of specific markers when subjected to sublethal exposure to lead.

Adult male specimens of *Bufo arenarum* were collected in the neighborhood of La Plata (Argentina) and were divided in two groups. Toads from the first group (n = 82; mean body weight ± SD: 128.0 ± 35.0 g) were used as a source of samples for the standardization of the technique; the second group (n = 38; 130.6 ± 21.5 g) was used to determine normal values of *delta*-ALAD activity under our experimental conditions.

Toads were kept in the laboratory for 3-7 days at 20-24°C, in individual cages containing tap water which was renewed once daily. They were maintained unfed during the adaptation period.

Blood was obtained by heart puncture with heparinized plastic syringes and placed in disposable polyethylene tubes. All samples were kept overnight at 4°C until used. The hematocrit was determined by centrifugation at 12000 g for 5 min in capillary tubes.

The method followed for the determination of the enzyme activity was based on the European Standardized Method (5). The main changes introduced in the assay were the volume of blood used, the pH of the reaction medium and the incubation temperature.

Assays were performed with different volumes of blood (10, 25, 50, 75, 100 and 200 µL), previously mixed with distilled water and buffered aminolevulinic acid (0.01 M) at each one of the selected pH (final volume: 3.5 ml). The selected sample size was 75 µL (Fig 1), a volume allowing appropriate readings of absorbance. Burch and Siegel (6) recommended for humans a volume of 200 µL of blood, suggesting that it may be reduced to 50 or 100 µL.

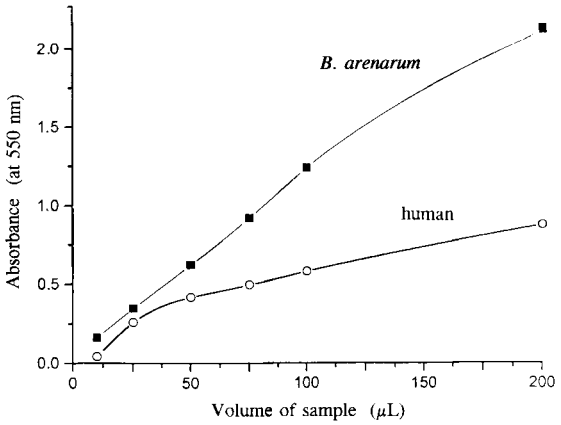

Fig 1. Blood ALAD activity -as absorbance- in *Bufo arenarum*, compared with the activity of normal human blood.

The effect of temperature on enzyme activity was examined by incubating blood of *B. arenarum* at 22 and 38°C for 60 min: while the activity was nil at 22°C, it was the highest at 38°C. Several authors (5, 6) incubated hemolysates at 38°C, while others -as Batlle and Magnin (2)- did it at 37°C.

The effect of pH on the activity was checked by adjusting the reaction media (150 mM phosphate) to the following values: 4.4 (n=5), 4.6 (n=5), 5 (n=5), 5.2 (n=5), 5.4 (n=6), 5.8 (n=9), 6 (n=7), 6.4 (n=5), and 6.8 (n=6). The number of samples tested at each pH is indicated in parenthesis.

The graph of pH of the reaction medium *vs* enzyme activity showed a maximum activity at pH 5.4 (Fig 2). For ectothermic vertebrates as fishes, Hodson *et al* (10) have reported an optimum pH of 6.2 for several species, while Burch and Siegel (6)

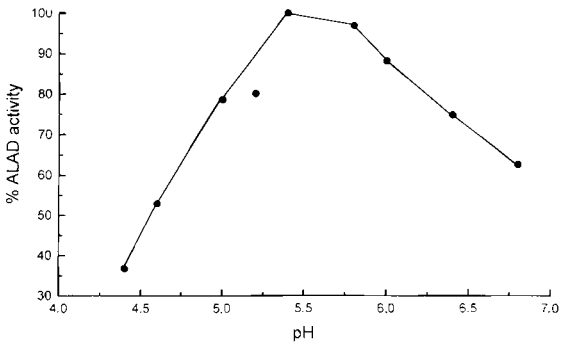

Fig 2. Relative activity of blood ALAD (%) in *Bufo arenarum* at different pH.

have found highest activity in humans at pH 6.7. Granick *et al* (9) recommended to adjust the pH at 6.0-6.4 to decrease the activity of uroporphyrinogen-1-synthetase, an enzyme that utilizes PBG and reduces the apparent ALAD activity (6).

In all cases, the reaction was stopped by the addition of 10% TCA; after centrifugation (10 min at 500 g), 1.5 ml of Ehrlich's reagent was added to 1.5 ml of supernatant and absorbances at 555 nm were read after 10 min in a Shimadzu UV-1603 spectrophotometer. All measurements were carried out in duplicate. Activity, expressed as units per liter of red blood cells (RBC), was calculated using the following equation:

ALAD activity (U/L RBC) =
Abs x 100 x 2 x DF / Htc% x 60 x 0.062

where: Abs = absorbance of sample; 2 = conversion factor of delta-ALA to PBG; DF = dilution factor; Htc = hematocrit; 60 = incubation time (min); and 0.062 = extinction coefficient (L/µmol x cm).

Data on blood ALAD activity in anurans are not frequent (7, 18). Ireland (12) reported for *Xenopus laevis* an activity of 13.53 ± 1.09 U (mean ± SEM; n = 6), using 50 µL of blood and following the method of Weissberg *et al* (22). More recently, the same parameter was measured in three specimens of the green frog (*Rana calamitans*) (19). The normal ALAD activity for *Bufo arenarum* was 131.86 ± 14.47 U/L RBC (mean ± SEM, n = 38), interval of ALAD activity 72.98-236.33 (Fig 3).

Under our experimental condition, the ALAD activity in toads' blood was always higher than the activity measured in a sample of a healthy human adult (Fig 1). This fact was interpreted as a consequence of the higher metabolic rate of nucleated erythrocytes (11).

In a recent series of preliminary experiments made to validate the test as a biomarker, the effect of sublethal concentrations of lead on the enzyme activity was measured (14). An activity decrease of 32% and 62%, relative to controls, was obtained for doses of 10 and 50 mg $Pb \cdot kg^{-1}$, respectively (Table I).

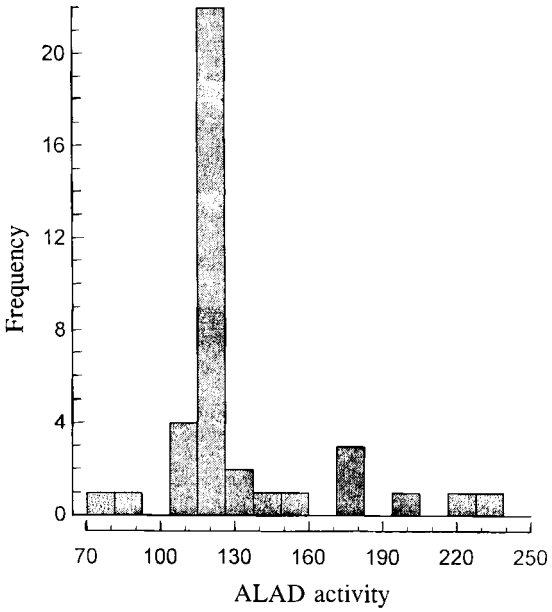

Fig 3. Frequency distribution of blood ALAD activity (U/L RBC) in *Bufo arenarum*.

Table I

Erythrocyte δ-aminolevulinic acid dehydratase (ALAD) activity in lead-injected *Bufo arenarum*. Data as means ± SEM's.

Animals	n	ALAD activity (U/L RBC)
Control	12	148.43 ± 15.81
Lead injected*		
10 mg $Pb \cdot kg^{-1}$	12	100.94 ± 7.81
50 mg $Pb \cdot kg^{-1}$	8	57.46 ± 6.70

* Animals injected with single dose of lead acetate, and controls with the equivalent amount of sodium acetate. Samples tested in duplicate at 7 days post-injection.

The value of ALAD activity that we determined as basal in *B. arenarum* is comparable to that reported for bald eagle *Haliaeetus leucocephalus*, which was 188.3 ± 12.5 U/L (mean ± SEM; n = 5), under similar assay conditions (11). It is worthwhile to mention that ALAD activity in some other bird species resulted almost 40% below that found for *Bufo arenarum*. ALAD activities are 64.8 in male rock doves (*Columba livia*) (8); 51.34 and 57.06

in hens (*Hybro* sp.) (3, 4); and 58.55 in *Gallus domesticus* (1). Since authors performing these determinations used the European Standardized Method, their results are comparable to ours.

It can be hypothesized that these important differences might be the expression of a particular biochemical adaptation mechanism, indicative of dissimilar sensitivity to the adverse effects of lead exposure.

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