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Biosensors of Inorganic Lead Exposure and Effect in an Adult Amphibian

Marcos A. Arrieta,[1] Liliana Bruzzone,[2] Carina Apartín,[2] Carolina E. Rosenberg,[3] Nilda E. Fink,[1] Alfredo Salibián[3,4]

[1] Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Calle 47 y 115, 1900 La Plata, Argentina
[2] Departamento de Química, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, 1900 La Plata, Argentina
[3] Comisión de Investigaciones Científicas de la Provincia de Buenos Aires, 1900 La Plata, Argentina
[4] Programa de Ecofisiología Aplicada, Universidad Nacional de Luján, 6700 Luján, Argentina

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Abstract. Lead (Pb) is a ubiquitous environmental pollutant, widely distributed, representing a high toxicological and ecotoxicological risk. Several morphological, functional, and biochemical parameters have been proposed as biomarkers of effect and exposure to Pb. The information related to adverse effects of Pb is not abundant for adult amphibians. These animals are of interest, because during their development they move from aquatic to terrestrial habitats, which may be polluted by the metal since they are receptors of products generated by anthropogenic activities. Previous studies carried out on the adult South American toad *Bufo arenarum* (Amphibia, Anura) showed that it has a high tolerance to lead and studied the effect of sublethal doses of the metal on the erythrocyte osmotic fragility and δ-ALAD activity. It was also shown that after a single injection of Pb, a significant increase in the number of reticulocytes was produced, suggesting the suitability of those cell counts as a biomarker of exposure to the metal; its impact on the immune system of the toads was also studied. In this work we extend our early studies on the same species evaluating the chronic effect of sublethal Pb (equivalent to 5.6% of the 120-h LD-50) on free erythrocyte protoporphyrin (FEP) and blood Pb and δ-ALAD activity; blood lead was positively associated with a significant decrease in the enzyme activity and to an increase in the FEP level. Pb concentration in target organs (liver, spleen, femur, and kidney) and the total cumulated amount as well as its impact over the mass of those organs were also determined. In addition, the magnitude of the possible depuration through urine and intestine was evaluated. Our results showed that FEP, δ-ALAD, and blood Pb are reliable biosensors of chronic metal intoxication, the former being the marker with the highest sensitivity.

Lead (Pb) is the most abundant of the heavy metals in the Earth's crust; it is an important environmental pollutant due to its industrial and domestic use, representing a high toxicological and environmental risk (Tong *et al.* 2000; WHO 1977, 1989). It is one of the most hazardous agents for human health; it can exert severe and chronic adverse effects interfering with biochemical, physiological, morphological, and behavioral parameters (Juberg *et al.* 1997; US EPA 1985; WHO 1995); lead and inorganic lead compounds are carcinogens in experimental animals, being classified as possible carcinogens in humans (Group 2B) (IARC 1987). It has no known essential role in animals, and there is no metabolic role for lead.

In contrast with the known effects on vertebrates, Pb is shown to have only a few toxic effects on the invertebrates (see Beeby and Richmond 2001; Beeby *et al.* 2002).

Problems derived from Pb pollution in developing countries are associated with the permanent presence and circulation of high amounts of the metal in many habitats like soil, surface and ground water, sediments, and air, which, in turn, are produced by the remotion of the metal from natural deposits due to human activities and the massive and sustained use of fossil biomass, particularly in urban environments (Albert and Badillo 1991; Romieu *et al.* 1997). Several authors have reported that high amounts of Pb are still present in soils of urban conglomerates in the region (Camilión *et al.* 1996; Catoggio 1991; Garcia Fernández *et al.* 1990; Mañay *et al.* 1999).

Exposure to lead can result in significant adverse effects to multiple organ systems. It is not accumulated homogeneously within the body. Instead, it circulates throughout the body and is distributed across several physiologically different compartments. In mammals, the main targets for Pb are red blood cells and their precursors, as well as soft tissues like kidneys and central and peripheral nervous tissue. Other sites like bones, spleen, and liver are also organs, where important amounts of the metal can be stored, producing a number of disorders.

It is well known that in both vertebrates and invertebrates Pb induces the synthesis of macromolecules known as stress proteins, although Pb is not a selective stimulus for that process. It has been interpreted that the induction of those proteins could be attributed to the disruption of Ca metabolism or to its binding to–SH groups in target organs (Clarkson 1993; Goering and Fisher 1995; Knigge and Köhler 2000).

Several functional, morphological, and biochemical parameters have been proposed as biomarkers of exposure and effect to Pb (Sakai 2000; WHO 1993). Among them, the more important are δ-aminolevulinic acid dehydratase (δ-ALAD) ac-

Correspondence to: Dr. Nilda E. Fink; *email:* fink@biol.unlp.edu.ar

tivity, the hematocrit, alterations of the hematological profile, and immune function. The blood Pb and the free erythrocyte protoporphyrin concentrations, as well as the contents of this metal in tissues and fluids, must also be mentioned.

The effect of both inorganic and organic lead compounds on heme synthesis has been recognized as the earliest response to lead toxicity. The quantification of free erythrocyte protoporphyrin (FEP) and the measure of δ-ALAD activity are sensitive and widely used indicators of Pb exposure. The biosynthesis of heme, the prosthetic group of hemoglobin, is selectively impaired by the binding of the metal, thus affecting particular steps, inhibiting the activity of this enzyme, and affecting the synthesis of protoporphyrin IX and the hemoglobin (Daniell *et al.* 1997; Woods 1995). It is important to indicate that Pb inhibition of δ-ALAD has been described in human beings and in many other vertebrates.

It is interesting to mention that the inactivation of δ-ALAD produces the accumulation of pro-oxidant products that can trigger oxidative stress (e.g., oxidation of the membrane components). Some of the adverse effects caused by Pb like hemolytic anemia may be, at least in part, attributed to oxidative stress and lipid peroxidation. Several authors have postulated that differences in the resistance to oxidative stress and lipid oxidation could explain differences in susceptibility to lead between species (Mateo and Hoffman 2001).

A survey of the available literature reveals that the information related to these toxicological and ecotoxicological aspects of Pb is limited in the case of adult amphibians (Devilliers and Exbayat 1992; Linder and Grillitsch 2000; Schuytema and Nebeker 1996). These vertebrates are of interest because during their early development they breed and feed in water, in a continuous transition from aquatic to terrestrial habitats, which may be polluted by the metal from different sources. Because of this integration of their lifestyles between terrestrial and aquatic habitats, some amphibian populations, especially those of periurban areas, confront the risk derived from their sustained contact with relative hot spots of Pb concentration over all their life stages, first taking it up directly from water (Rice *et al.* 2001, 2002). Later, in their subsequent stages and, finally, as adults, other substrates like soils and sediments or dietary sources may contribute significantly to their exposure (Carey *et al.* 1999). These characteristics may be one of the reasons for the decline in amphibian populations worldwide reported to be happening in different parts of the world for several decades (Boyer and Grue 1995; Houlahan *et al.* 2000; Mann and Bidwell 1999). Hence, the amphibians can be key bioindicators to assess the status of the environment affected by chemical stressors mobilized from their insoluble deposits by anthropic activities and may be valuable tools in ecotoxicological monitoring programs.

In previous studies carried out on the South American toad *Bufo arenarum* (Amphibia, Anura), we determined LD-50 of Pb (Arrieta *et al.* 2000a) and studied the sublethal effect of the metal on the erythrocyte osmotic fragility (Rosenberg *et al.* 1998a). We also described a technique to determine the δ-ALAD activity in the blood of this anuran (Perí *et al.* 1998a). In addition, a relevant increase in the number of reticulocytes after Pb injection in sublethal amounts was shown, suggesting the suitability of changes in those cells count as a biomarker for exposure to Pb (Perí *et al.* 1998b). More recently we studied the effects of the metal on some indicator parameters of the immune system function from the same species (Chiesa *et al.* 1999; Rosenberg *et al.* 1998b, 2000; Rosenberg 2001).

This paper reports our observations on the chronic effect of Pb injected in sublethal amounts to adult specimens of *Bufo arenarum,* evaluating simultaneously FEP, blood Pb, blood δ-ALAD activity, and Pb concentration in target organs, urine, and feces; we also determined the total cumulated amount of the metal in several organs and its impact, in particular, on the mass of target organs.

Materials and Methods

Male adult toads with a mean body weight of 130 g, captured from the surroundings of La Plata City, Argentina, were individually placed in plastic containers with approximately 100 ml freshwater/kg body weight. Then the toads were acclimated for 1 week at a constant temperature (20 ± 2°C) under a photoperiod of 12D:12L. After acclimatization, heparinized blood was obtained by cardiac puncture from anesthetized frogs to determine basal levels of Pb in blood. All the animals continued under the same acclimatization environmental conditions during the lead dosing period. Toads were fed by force once a week with bovine meat, and water was renewed daily.

Then the animals were randomly divided into two groups. The group under treatment received weekly injections of aqueous Pb solutions (as acetate), at a rate of 50 mg $Pb \cdot kg^{-1}$ body weight, for 5 weeks. Each weekly dose of lead was equivalent to 5.6% of the 120-h LD-50 determined previously in our laboratory (Arrieta *et al.* 2000a). A control group of toads, injected with an equivalent dose (as acetate) of aqueous sodium acetate solutions and at the same frequency, was run simultaneously. The injected volume of lead and sodium acetate solutions was 0.1–0.2 ml/100 g body weight.

At the end of the sixth week, i.e., 1 week after dosing stopped, urine samples were taken by abdominal pressure and then animals were double pithed. Additional blood samples were collected by ventricular puncture; whole organs (liver, kidney, and spleen) were removed, and samples of intestinal contents were obtained. Whole femur from the right leg was taken out.

Organs were dried at 100°C until a constant weight to obtain dry masses. Hematocrit, blood Pb, and δ-ALAD activity were determined in samples collected on heparin. Levels of FEP were determined in blood aliquots collected on EDTA · Na_2. Hematocrit was recorded using a capillary tube reader following centrifugation on a clinical hematocrit centrifuge; results are indicated as percentages.

Pb concentration in fluids and content of organs was determined on aliquots of 150–500 μl for blood, 1 ml for urine, and 5–100 μg (dry weight) for whole organs and intestinal contents. Samples were digested with approximately 1 ml of HNO_3 for 12 h at room temperature and were later heated for 2 h at 60–70°C until they became limpid. Once digestion was completed, samples were filtered through Whatman No. 1 and MSI 0.45 μ nitrocellulose disks. The distilled water used was nanopure (MilliQ). Pb content was determined by atomic absorption spectrometry, using a Varian SpectrAA, Model AA300 (Varian, Lexington, MA). The equipment was calibrated with a curve prepared from a commercial Pb standard (1000 mg/L AA standard; J.T. Baker); dilutions were carried out in nanopure water. The detection limit for Pb in samples was 0.1 μg Pb/g dry mass. The standard calibration curves to check linearity were carried out with filtered samples, with adequate amounts of Pb (as nitrate) added. Regression calibration curve was $y = -0.006 \pm 0.0575x$ ($r = 0.993$, $p = 0.007$). Pb concentration in distilled and nanopure water was <0.010 mg/L, while in fresh water it was <0.150 mg/L; the injected sodium acetate solutions had 0.100 mg Pb/L.

Simultaneously to the sample tests, duplicated tubes containing only nitric acid, as well as other tubes containing blood with known

amounts of lead added, were run. Both served as negative and positive controls, respectively, for the treatment received by the samples.

δ-ALAD activity (EC 4.2.1.24; δ-aminolevulinic acid dehydratase) was determined in duplicate, in accordance to the technique developed in our laboratory (Peri *et al.* 1998a). Assays were performed on aliquots of 75 μl of blood and the medium pH was adjusted to 5.4. Ten minutes after the addition of Ehrlich reagent, the absorbances were read at 555 nm in a Shimadzu UV–visible spectrophotometer, Model 1603 (Shimadzu, Kyoto, Japan). Activity, expressed as U/L erythrocytes (RBC)/h, was calculated using the following equation:

$$\text{δ-ALAD activity} = \text{Abs} \times 100 \times \text{DF/Htc\%} \times 60 \times 0.062$$

where Abs = absorbance of sample, 2 = conversion factor of δ-ALA to PBG, DF = dilution factor, 60 = incubation time (min), and 0.062 = extinction coefficient (L/μmol × cm).

FEP levels were determined following Piomelli's (1973) fluorometric method. Briefly, 20 μl of the sample was added to 0.1 ml of 5% Celite suspension in saline (0.6% NaCl), then 2 ml of ethyl acetate: acetic acid (4:1) was added, with shaking in vortex for 10 s. HCl (2 ml) was added to the supernatant obtained after 30 s of centrifugation, followed by mixing with vortex for 10 s. Finally the FEP concentration in the acid phase was determined, measuring the fluorescence intensity in an Aminco–Bowman SPF spectrofluorometer (Aminco, Silver Spring, MD) equipped with an off-axis ellipsoidal mirror condensing system. The excitation source was a 150-W xenon arc lamp and the detector was a Hamamatsu R928 photomultiplier tube. Excitation and emission wavelengths employed were 394 and 606 nm, respectively.

A standard solution of 0.015 μg/ml coproporphyrin I was used as a reference. This solution was obtained by diluting a coproporphyrin I solution prepared from 5 μg coproporphyrin I (Sigma, St. Louis, MO) to which 10 ml of 1.5 *N* HCl was added and heated for 5 min at 100°C. As a blank, a tube treated in the same way as the samples was used, but replacing the 20 μl of blood with the same volume of saline solution. The results are reported as μg/dl.

All chemicals were of analytical grade. All measurements were carried out in duplicate.

Comparison of the means from values pertaining to both control and lead-injected groups of toads was performed using Student's *t* test. For all statistical analysis, the Statgraphics Plus software (Manugistics, Rockville, MD) was employed. Statistical significances were determined at $p < 0.05$. All data are given as mean ± standard error ($\bar{X}$ ± SEM).

Linear regression using log-transformed data was used to investigate correlations among blood Pb, FEP, and δ-ALAD activity. The significance of the correlations was tested by means of one-way analysis of variance.

Results and Discussion

Bufo arenarum is an anuran species highly tolerant to lead (as acetate) (Arrieta *et al.* 2000a,b), which can be related to its particular accumulation and excretion dynamics. Regarding this aspect it is important to indicate that in this set of experiments the mortality of injected toads during the experimental period was null, confirming that the assayed dose was sublethal and tolerable under chronic exposure laboratory conditions. Several reasons may explain this fact (Mulvey and Diamond 1991). In the case of amphibians, acquisition of tolerance may be due to different factors, among them their relatively short generation times and effective selection processes at the most sensitive life stage; both factors may contribute to reducing the time of effective contact with pollutants and/or leaving in the populations those individuals that were able to overcome the intense ecological selective effect of metals' toxicity. Thus the overall process will cause an adaptation of a particular population to the environmental chemical stressors. In addition, it must be pointed out that amphibians typically have a small home range with nonmigratory habits, which is an ecological characteristic that makes them useful biomarkers of environmental lead within a localized area.

Blood lead level is the biological index most often used as an indicator of exposure. At the end of the experimental period (sixth week), blood Pb in injected toads was significantly augmented (an almost-fourfold increase) with respect to control animals (Table 1). In addition, it is interesting to note that this parameter in control toads measured at the beginning of the experiments was 2.7 ± 0.2 μg/dl ($n = 10$), which was not significantly different ($p = 0.30$) from the value found for the same group of toads 6 weeks later (2.3 ± 0.2 μg/dl). In other words there was no evidence of a depuration process while in contact with clean media.

The fact that on their arrival to the laboratory the blood of toads contained some residues of Pb supports the idea that the animals used in this set of experiments were possibly captured in a polluted area; we have previously shown that the Pb found in the blood of experimental animals cannot be attributed to possible secondary active uptake from the surrounding freshwater used in the laboratory (Arrieta *et al.* 2001). With regard to this point it is likely that the environmental exposure may be prominent for metals like lead that are known to have a relatively long biological half-life, in the range of years to decades, and are not under homeostatic control. Interestingly, in most articles devoted to the study of different aspects of lead exposure and its effects in invertebrates and in vertebrates, including humans, the presence of the metal in important measurable amounts in fluids and tissues of the nonexposed control groups was reported, indicating the occurrence of an unavoidable exposure due to a high environmental background level (see Berzins and Bundy 2002; Calderon-Salinas *et al.* 1996; Duydu *et al.* 2001; Maldonado-Vega *et al.* 1996).

In our case the δ-ALAD activity was significantly reduced in blood of injected toads, the remaining activity being 27% that of the controls. This result was similar to that reported in our previous works in the same species (Peri *et al.* 1998a; Rosenberg *et al.* 1998a). Other authors have also shown the inhibition of the enzyme under both laboratory and field conditions (Stanley and Roscoe 1996; Vogiatzis and Lombourdis 1999).

To our knowledge, this is the first time that the FEP concentration in *Bufo arenarum* has been reported. In the injected animals it was increased almost ninefold compared to controls.

Comparison of the changes in δ-ALAD activity and the concentration of FPE shows that in adult *Bufo arenarum* under our experimental conditions, these parameters appear to be appropriate biomarkers for the diagnosis of experimental Pb exposure. Moreover, as deduced from the magnitude of the relative changes registered in treated and control FEP, δ-ALAD, and blood Pb (Table 1), it may be concluded that in this species FEP is a more sensitive marker than δ-ALAD and blood Pb.

Our results reflect a well-defined and precise log-linear relationship between blood Pb and both FEP level and δ-ALAD activity. The equation pertaining to the log of FEP vs. log of blood Pb concentration regression was $\log y = 0.6987 + 1.3376 \log x$ ($r = 0.70$); the log of δ-ALAD vs. log of blood Pb

Table 1. Hematological and biochemical parameters and Pb concentrations in tissues and fluids of *Bufo arenarum* treated with sublethal doses of Pb (data expressed as $\bar{X} \pm$ SE)

Parameter	Controls (C)	Treated (T)	T/C	*p*
Blood Pb (mg/dl)	2.3 ± 0.2 (10)[a]	8.8 ± 0.9 (19)	3.80	<0.001
δ-ALAD (U/L RBC/h)	138.4 ± 23.1 (13)	37.5 ± 5.8 (15)	0.27	<0.001
Free erythrocyte protoporphyrin (μg/dl)	11.7 ± 1.9 (11)	103.6 ± 22.5 (14)	8.85	<0.002
Hematocrit (%)	35.7 ± 2.8 (13)	20.3 ± 2.5 (15)	0.57	<0.001
Liver Pb (μg/g dry weight)	16.0 ± 3.8 (17)	6116.2 ± 876.9 (26)	382.3	<0.001
Kidney Pb (μg/g dry weight)	138.9 ± 24.6 (17)	1158.6 ± 112.1 (26)	8.34	<0.001
Spleen Pb (μg/g dry weight)	393.5 ± 66.3 (17)	3469.3 ± 386.7 (26)	8.81	<0.001
Femur Pb (μg/g dry weight)	9.0 ± 2.6 (17)	27.4 ± 6.3 (25)	3.04	<0.02
Urine Pb (μg/dl)	1429.4 ± 301.4 (14)	1632.6 ± 273.9 (22)	1.14	NS
Intestinal content Pb (mg/g dry, weight)	0.84 ± 0.21 (10)	2.19 ± 0.52 (14)	2.61	<0.05

[a] Number of samples in parentheses.

Table 2. Total Pb content (μg) in organs of *Bufo arenarum* treated with sublethal doses of Pb (data expressed as $\bar{X} \pm$ SE)

Organ	Controls (C)	Treated (T)	T/C	*p*
Liver	15.9 ± 4.0 (17)[a]	6995.1 ± 806.7 (26)	439.94	<0.001
Kidney	6.5 ± 0.9 (17)	156.4 ± 27.0 (26)	24.06	<0.001
Spleen	8.3 ± 1.5 (17)	74.8 ± 7.1 (26)	9.01	<0.001
Femur	7.7 ± 2.3 (17)	25.5 ± 6.1 (26)	3.31	<0.05

[a] Number of samples in parentheses.

Table 3. Mass (g dry weight) of organs of *Bufo arenarum* treated with sublethal doses of Pb (data expressed $\bar{X} \pm$ SE)

Organ	Controls (C)	Treated (T)	T/C	*p*
Liver	1.024 ± 0.069 (17)[a]	1.354 ± 0.118 (26)	1.32	<0.05
Kidney	0.057 ± 0.005 (17)	0.075 ± 0.006 (26)	1.32	<0.03
Spleen	0.029 ± 0.007 (17)	0.025 ± 0.002 (26)	0.86	NS[b]
Femur	0.768 ± 0.043 (17)	0.833 ± 0.005 (26)	1.08	NS

[a] Number of samples in parentheses.
[b] Not statistically significant.

concentration equation was $\log y = 2.3747 - 0.6721 \log x$ ($r = 0.64$). Both correlation coefficients were highly significant ($p < 0.05$).

Hematocrit in Pb-injected animals was 43% lower than in controls. This statistically significant result can be interpreted as a consequence of the anemia secondary to the inhibitory effect of the metal on the erythropoiesis. It is worth mentioning that in another group of experiments carried out in our laboratory, the administration of Pb for a shorter time did not provoke significant changes in the hematocrit with respect to controls (Arrieta *et al.* 2000b; Perí *et al.* 1998b; Rosenberg *et al.* 1998a). These findings are consistent evidence in favor of exposure time-dependent responses. As Rice *et al.* (1999) suggested for lead-exposed *Rana catesbeiana* tadpoles, it may be postulated that lead-injected toads can be considered to be in an hypoxic condition because of the decrease in their hemoglobin levels and the erythrocyte damage.

It is known that lead accumulates mainly in soft tissues. In our case, when comparing Pb contents in the studied organs (Table 1), on a dry matter basis, the highest concentration pertains to the liver; its content was 382 times higher than the controls. Kidney, spleen, and femur also appeared to be repository sites of the metal, although in much lower proportions. It is interesting to consider that in amphibians, the three mentioned organs display hematopoietic function. Consequently, the impact of the accumulated lead may be responsible for the impaired hematological and immunological parameters (Varela and Sellares 1937).

In considering lead incorporated into bones two aspects must be distinguished, the tissue (a) as a repository and (b) as a source of body metal burden due to its mobilization into the circulation. Based on our figures of the Pb content in femur, even after a relatively short-term exposure, the bone acted as a modest storage site of the metal. However, because of our experimental design, characterized by the regular administration of the toxin for 6 weeks, it is possible that the steady state between circulating and deposited Pb was not achieved.

A comparable order was obtained when absolute Pb depositions in the organs were analyzed (Table 2): the higher values were in liver and kidney, with treated (T)/control (C) toad ratios of 439.9 and 24.1, respectively, followed by the contents in spleen and femur. Except for urine Pb content, all differences between evaluated parameters in control and injected toads were statistically significant.

Increased tissue concentrations of lead may not necessarily be associated with augmented proportional toxicity outcomes. It is accepted that adverse effects of most metals will arise only when the sequestration capacity of particular protective metal-binding macromolecules, known as metallothioneins, are overcharged (Amiard and Cosson 1997). In this respect, the ability of lead to induce selective high-affinity Pb-binding proteins was shown in several vertebrates (see Conner 1994; Quintanilla-Vega *et al.* 1995; Schumacher *et al.* 1997). However, similar findings for amphibians are lacking.

It is interesting that the tissue concentration of lead in most cases did not appear to be strictly proportional either to its original endogenous basal amount or to the blood concentration reached at the end of the experimental period. This fact must be interpreted as evidence of the existence of net accumulation processes which appear to be relevant in some organs.

In experiments designed similarly to those described here, carried out on the same species, the effect of Pb, both hematological (cell blood counts, serum protein electrophoretic fractions) and immunological (quantification of natural and immune anti-sheep red blood cell antibodies)

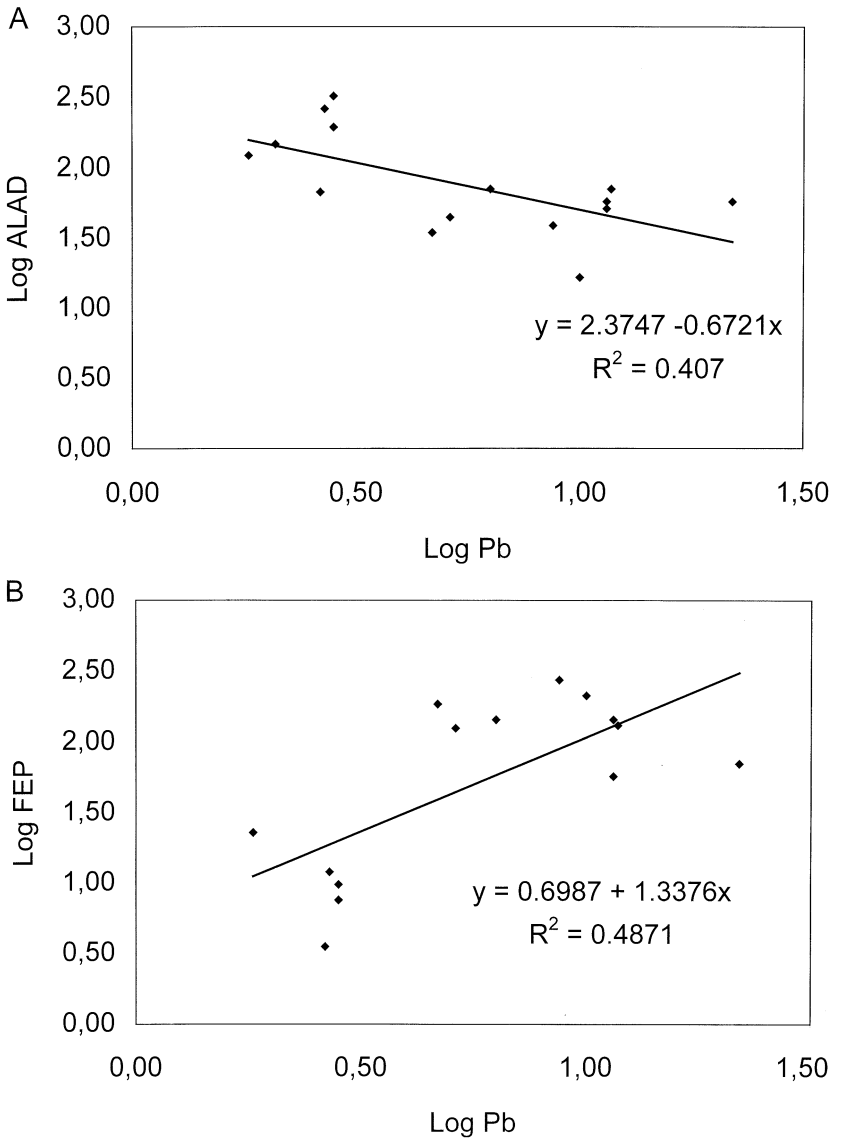

Fig. 1. Free erythrocyte protoporphyrin (FEP) and blood δ-ALAD activity of *Bufo arenarum* treated with sublethal doses of Pb: (A) linear regression of log δ-ALAD activity vs. log Pb blood concentration; (B) linear regression of log FEP vs. log Pb blood concentration

parameters were tested. In the lead-injected toads, an increase in the number of white blood cells and a decrease in red blood cells and in the hematocrit, as well as in the production of natural antibodies, were found (Rosenberg 2001; Rosenberg *et al.* 1998b, 2000). These findings show that the adverse effects of sublethal amounts of Pb also affect other hematological parameters besides the immune function of the toads.

The concentrations of lead found in control toads suggest that urine may play a role in the depuration of Pb previously accumulated in the field. However, in injected animals the concentration of lead excreted in this way presented only modest, nonsignificant increases, suggesting that the lead administered on the regular 6-week schedule was deposited in target tissues rather than excreted through urine. The urine/blood concentration ratios were 0.62 for control toads and 0.19 for injected animals; these figures show that, on balance, the urinary excretion is negligible.

The results obtained for the metal concentrations in the intestine showed that part of the Pb depuration may occur through fecal deposition, probably via biliary secretion.

Regarding the changes in the dry mass of analyzed organs (Table 3), it was observed that Pb induced a marked and significant increase in liver and in kidney. On the contrary, the alterations in spleen and femur were not significant. One possible explanation of our findings is that the intoxication was accompanied by degenerative processes (Williams and Iatropoulos 2002). Comparable results have been reported in rats (Cory-Slechta 1990), even after a very short exposure to lead.

Finally, it must be remembered that age and gender influence the biological levels of environmental chemicals; in our case all used animals were homogeneous in both age and sex. Consequently the impact of those factors can be disregarded.

Overall, we conclude that in adult *Bufo arenarum* experimentally intoxicated with sublethal doses of Pb, a marked decrease in δ-ALAD activity and an increase in the free erythrocyte protoporphyrin (FEP) concentration appeared to

be very sensitive biochemical markers to assess Pb exposure. The main target organs appear to be liver and kidney.

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