

Patuxent Wildlife Research Center
Laurel, MD 20708

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TECHNICAL OPERATING PROCEDURE

PROCEDURE TITLE: ALAD (delta-aminolevulinic acid dehydratase) determination in red blood cells.

AREA OF APPLICABILITY: This procedure may be used for determining ALAD activity in the blood of vertebrates as an initial bioindicator of recent lead exposure.

SCOPE: This procedure has been successfully used for determining red blood cell ALAD activity using the whole blood of different species of birds (adults, nestlings, and embryos), small mammals, and fish. Other candidate species include amphibians and reptiles.

PRINCIPLE: ALAD in a completely hemolysed sample of whole blood is incubated with excess substrate (ALA; delta-aminolevulinic acid). ALAD catalyses the condensation of two ALA molecules to form porphobilinogen. The reaction is allowed to proceed for a set time and then stopped by the addition of trichloroacetic acid. Porphobilinogen, when mixed with Ehrlich's reagent, forms a purple color which can be measured spectrophotometrically at 555 nm. The quantity of porphobilinogen formed is directly proportional to the ALAD activity which in many species is inversely proportional to the log of the blood lead concentration over the lowest range of lead exposure (Burch and Siegel, 1971; Pain 1987);

PROCEDURE:

1. Reagents

- a. Solution A (0.1 M Na_2HPO_4) 1.78 g of disodium hydrogenphosphate dissolved in 100 ml distilled water.
- b. Solution B (0.1 M NaH_2PO_4) 1.38 g of sodium dihydrogenphosphate dissolved in 100 ml of distilled water.
- c. Sodium phosphate buffer (0.1 M)- prepare 100 ml by mixing solution A with solution B to attain the proper pH for a given species.
- d. Substrate Solution - This is prepared fresh prior to each assay by mixing 167.6 mg of aminolevulinic acid hydrochloride (ALA) (Sigma Chemical Co., St. Louis, MO) into 100 ml of the sodium phosphate buffer.
- e. Ehrlich's reagent-prepare under a hood. Dissolve 2.5 g of p-dimethylaminobenzaldehyde (Sigma Chemical Co.) into 50 ml of glacial acetic acid. Then 24.5 ml of perchloric acid (70%, SGI 0.7) is added. Allow to cool and make volume to 100 ml with glacial acetic acid. Store in a dark bottle and refrigerate. Can be stored for 30 days, but discard if any brown color appears.
- f. Trichloroacetic acid solution (10% W/V in distilled water).

2. Sample Collection and Storage

Venous blood is collected in heparinized tubes, hematocrits are determined and an aliquot of whole blood is snap frozen in liquid nitrogen (-176°C) (dry ice may also be used). Blood samples are then stored in an ultracold freezer (-80°C). Assays should be performed within two months. It is important that an adequate number of concurrent blood samples are collected from reference control animals of the same species, sex, and age.

3. Assay Incubation Procedure

- a. Two aliquots of blood (0.1 ml) are each diluted with 1.4 ml of distilled water in clean plastic tubes (suitable for centrifugation). The tubes are vortexed for 15 seconds to insure complete hemolysis and homogeneity of suspension.
- b. The tubes as well as a water blank are then placed into a water bath at 38°C for 10 min. After 10 min., 1 ml of the freshly prepared substrate solution is added to each tube and mixed. The tubes are left to incubate in the water bath in darkness for one hour (porphobilinogen is light sensitive).
- c. After the one hour incubation, 1 ml of trichloroacetic acid solution is added to each tube and mixed to stop the reaction. The tubes are then centrifuged at 2000 rpm for 10 minutes.
- d. One ml of the clear supernatant is then pipetted off into a clear tube or cuvette. Add 1 ml of Ehrlich's solution down the side of each tube under a hood and stir each with a rod. Five to seven minutes later, sample absorbance is read against a water blank at 555 nm in a 1 cm cell using a spectrophotometer.

4. Calculations of Enzyme Activity

$\frac{\text{Absorbance at 555 nm} \times 1881 \times 2}{\text{hematocrit}} = \text{ALAD activity (nmol ALA used/min/ml RBC)}$

Activities of subjects should be compared with the reference or control activities that were run concurrently.

5. Data Interpretation

Delta-aminolevulinic acid dehydratase (ALAD) is a cytosolic enzyme found in many tissues and active in the biosynthetic pathway of heme necessary for maintenance of hemoglobin content in erythrocytes and for cytochromes in various other tissues. ALAD is widely recognized as the enzyme in heme synthesis that is the most sensitive to lead exposure. Inhibition of ALAD activity by lead results in a block in the usage of delta-aminolevulinic acid (ALA) with decreased porphobilinogen formation, and subsequent decline in heme synthesis. The ALAD activity

of peripheral red blood cells may be the most sensitive biological indicator of lead effects that is readily quantifiable. Historically, numerous human health studies examining venous blood samples from both adults and children revealed a highly significant negative correlation between the log of ALAD activity and blood lead concentration from 10 to about 90 ug/100 ml (Goyer and Mushak, 1977).

There are several reasons why erythrocyte ALAD activity is considered to be the most suitable index of exposure to lead. ALAD activity reflects immediate exposure to blood lead whereas samples of bone and other tissues are usually attainable only at postmortem and reflect chronic rather than immediate exposure. The mobility of lead in blood permits a greater degree of biological activity than stored lead in tissue such as bone, leading to more immediate physiological damage. Advantages of the ALAD assay over blood lead analysis as a first line screen for lead exposure include small sample volume, relatively inexpensive cost, ease of performance using basic and highly portable lab equipment, high reproducibility, and high sensitivity in the lowest range of blood lead concentrations. The assay has been adapted to detect lead contamination in a wide range of species including numerous bird species, small rodents, rabbits, fish, and even invertebrates.

Utilization of ALAD for Experimental Wildlife Studies

Waterfowl

Significant negative correlations have been reported between ALAD activity and lead concentration in mallards where 0.2 ppm lead in the blood resulted in greater than 50% inhibition of erythrocyte ALAD activity following dosing with lead shot; retention of lead shot for at least 24 hours resulted in reduced activity for 4 weeks (Dieter and Finley, 1979). Inhibition of over 80% was reported after one week in ducklings with blood lead of 0.4 ppm wet weight due to experimental consumption of lead in automotive waste oil (Eastin et al 1983). Mallard embryos were also found to exhibit ALAD depression following topical egg exposure to only 5 ul of waste oil. Other studies have revealed black ducks to be equally or more sensitive than mallards to lead shot ingestion as reflected by subsequent erythrocyte ALAD depression (Rattner et al., 1989).

Raptors

Dosing of bald eagles, unfit for captive propagation, with lead shot resulted in inhibition of red blood cell ALAD by nearly 80% within 24 hours with a mean blood lead concentration of 0.8 ppm (Hoffman et al., 1981). When day-old American kestrel nestlings were orally dosed for 10 days with metallic lead, 2.5 mg/kg ingestion resulted in 50% inhibition of the enzyme (Hoffman et al., 1985). Eastern screech owls receiving lead acetate at a concentration causing 50% mortality exhibited over 90% ALAD depression (Beyer et al., 1981).

Other Avian Species

When ring doves were exposed either orally or via i.p. injection over two days, a significant negative correlation between blood lead concentration and ALAD activity occurred independent of mode of administration (Scheuhammer, 1987). Lead exposure has also been reported to cause ALAD inhibition in pheasants, red-winged black birds, cowbirds, grackles, and in northern bobwhite and their embryos (Beyer et al. 1988).

Mammals

The degree of ALAD activity in mammals has been correlated with the percentage of reticulocytes in the circulation which is species dependant, resulting in a wider activity range than seen in birds where erythrocytes are nucleated. ALAD has been used as an indicator of lead exposure in experimental studies using small rodents and rabbits (Mouw et al., 1975).

Fish

Limited studies with fish have shown a dose dependent decrease in erythrocyte ALAD activity.

Field Study Validation and Utility of ALAD Assay

Waterfowl

Dieter et al., (1976) were the first to apply the diagnostic use of the ALAD assay to wild waterfowl, demonstrating a highly significant inverse correlation between ALAD activity and blood lead concentration in canvasback ducks trapped on the Chesapeake Bay. Similarly Pain (1987) demonstrated effects in black ducks on the Chesapeake Bay; hematological screening techniques were compared for three species of wild waterfowl and it was concluded that an adapted ALAD assay proved the most accurate and useful predictor of blood lead level and acute exposure.

Other Avian Species

Red blood cell ALAD activity was lower in highway nesting barn swallows than in their rural counterparts and related to highway lead concentrations (Grue et al., 1984). Nestling european starlings along highways were more sensitive to lead exposure than adults as reflected by depression of red blood cell ALAD and depressed hemoglobin concentration (Grue et al., 1986). Others have shown a significant field relationship in feral pigeons between lead exposure and ALAD in urban locations. Near the site of a zinc smelter where soil was contaminated with lead, a variety of passerine species exhibited lower ALAD activities than did corresponding species from a control area (Beyer et al.,

1985). Similar observations were reported in Tundra swans and in osprey near a lead mining site in Idaho.

Mammals

Shrews and mice in the vicinity of a zinc smelter with lead contaminated soil showed depression of ALAD but correspondingly higher body burdens of lead than did controls from a more pristine location (Beyer et al., 1985). A similar relationship was demonstrated for lead exposed urban rats in contrast to rural rats (Mouw et al., 1975).

Fish

Field studies have demonstrated ALAD inhibition in fish from lead polluted lakes.

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