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Storage Method for Blood that Provides Stability of Erythrocyte 5-Aminolevulinic Acid Dehydratase of Carp *Cyprinus carpio* when Used as an Indicator of Water Lead Pollution

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5-Aminolevulinic acid dehydratase (ALA-D) in the blood of carp *Cyprinus carpio* has been certified for use as an indicator of water lead pollution. The present study was undertaken in order to find storage conditions for blood samples which provide stability of, and allow a standarized expression unit for ALA-D activity, since the provision of these is essential for accurately assessing water lead pollution in a field survey, when using carp ALA-D extensively as the indicator enzyme of water lead pollution. Carp blood ALA-D levels were stable for at least 4 days when the blood was stored in a refrigerator (4°C), an ice bath, or a dry-ice bath. The three expression units of ALA-D activity, that is, per ml of RBC, ml of blood, and g of Hb, were found to be related as follows: nmol PBG/ml RBC/h = 3.6 Xnmol PBG/ml blood/h = 0.256 × nmol PBG/g Hb/h

INTRODUCTION

The enzyme 5-aminolevulinic acid dehydratase (ALA-D, EC 4. 2. 1. 24) in fish blood is useful as an indicator of water lead pollution, since its activity is inhibited by lead contamination (Larsson et *al.*, 1985; Hodson et al., 1977). ALA-D in the blood of carp *Cyprinus carpio* has been previously certified for use as an indicator of water lead pollution (Nakagawa et al., 1995a,c). In order to use carp ALA-D extensively for assessing water lead pollution in fresh-water areas, the blood samples are collected from fish caught in the survey field, taken to the laboratory, stored under certain conditions, and then ALA-D activity in the stored blood samples is measured, according to the circumstances. Therefore, a storage method for these blood samples which provides stability of ALA-D activity is absolutely essential.

Blood ALA-D is an enzyme which is present in erythrocytes, and it has been verified that carp blood ALA-D is also present in erythrocytes (Nakagawa et al., 1995d). Its activity has been expressed as "nmol of porphobilinogen (PBG)" which is formed from aminolevulinic acid by 1 ml of erythrocyte (Hodson et al., 1977). Since it is impossible to measure the hematocrit value (Hct) of the stored blood samples, a new expression unit of this activity is necessary to replace that of "per volume of erythrocyte".

The present study was undertaken in order to establish a storage method for blood samples which provides stability of, and a new expression unit for ALA-D activity, since the provision of these is essential for accurately assessing water lead pollution in a field

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survey, when using carp ALA-D extensively as the indicator enzyme of water lead pollution.

MATERIALS AND METHODS

Test fish and exposure test

The carp used as test fish were of 8.2 −12.4 cm in body length and 31.2−40.6 g in weight. Acclimatization of the test fish to the experimental conditions, and the exposure tests for water lead were performed according to methods described in our previous paper (Nakagawa *et al.,* 1995a). Since it is commonly stated that the average level of water hardness of river water in Japan is 30 ppm $CaCO_3$, dilution water of 30 ppm $CaCO_3$ was prepared by the addition of deionized water to tap water (about 80 ppm $CaCO_3$) obtained from the Tatara river in Fukuoka city. A stock solution of lead nitrate (10 mg/ml as Pb) was diluted with this dilution water to produce nominal water lead concentrations of 10, 50, and 100 ppb, respectively. The dilution water was also used as the control water. The exposure tests were performed for two weeks at room temperature of between 20−23 ℃.

Measurement of blood ALA-D activity

ALA-D activity was measured by a modified method which does not use $HgCl_2$, as in our previous report (Nakagawa et al., 199513). In addition, blood collection from fish and measurement of Hct also followed methods previously reported (Nakagawa *et al.,* 1995a).

Experimental procedures for observing stability of blood ALA-D under various storage conditions

Blood samples were collected from 5-7 fish. These were then mixed immediately, divided into quantities of 50 μl in 2-ml Eppendorf test tubes, and stored in a refrigerator (4 ℃), an ice bath, or a dry-ice bath. After storage times of 0, 1, 2, 3, or 4 days, ALA-D activity in the blood samples was measured. In addition, some blood samples were stored for 3 weeks in either an ice bath, or a dry-ice bath, and ALA-D activity in these blood samples was measured at set interval times.

The stability of blood ALA-D from lead-contaminated fish was studied only under the storage conditions of an ice bath. In the case of lead-contaminated fish, the blood sample collected from each fish was divided between five of the test tubes as described above, and these were then stored in an ice bath only.

Experimental procedures for observing stability of blood Hb under storage conditions

The Hb concentration of carp blood samples was measured using a method which converts Hb to cyanmethemoglobin (HiCN) by the addition of a coloring reagent (0.78 mM potassium cyanide plus 0.61 mM potassium ferricyanide) from the Hb-test kit (Wako Pure Chemicals Ind). Blood samples were collected from one fish, and these were then divided into quantities of 20 μl and placed in 10-ml test tubes. Five ml of the coloring reagent were added to each blood sample and the blood samples containing the reagent

were stored either at room temperature (20 “C) or in a refrigerator. After storage times of 0, 1, 2, 3, or 4 days, the Hb concentration in the blood samples was measured.

Analysis of blood lead

Blood lead was measured using ICP-MS at the Center of Advanced Instrumental Analysis, Kyushu University, after blood samples had been wet-digested, as described in our previous report (Nakagawa et al., 1995a).

Statistical analysis

Data were analyzed for statistical significance by the Student’s t-test. Significance differences were established at the 5% level.

RESULTS AND DISCUSSION

The stability of carp blood ALA-D under various storage conditions

Carp blood ALA-D was stable for at least 4 days when the blood was stored in a refrigerator, an ice bath, or a dry-ice bath (Fig. 1). Moreover, blood ALA-D activity was not depressed significantly for a period of 3 weeks when the blood was stored in an ice bath (Fig. 2). When the blood was stored in a dry-ice bath, blood ALA-D activity began decreasing significantly after 5 days, but became stable 12 days later (Fig. 2), although the cause of this decrease in activity was unknown. Granick *et al.* (1973) reported that ALA-D activity in human blood samples was constant for 24 hours, decreased by 15% after 5 days and by 30% after 12 days when the blood sample was stored at 4 “C. No activity was lost when the blood was stored in liquid N_2, however, 50% of the activity was

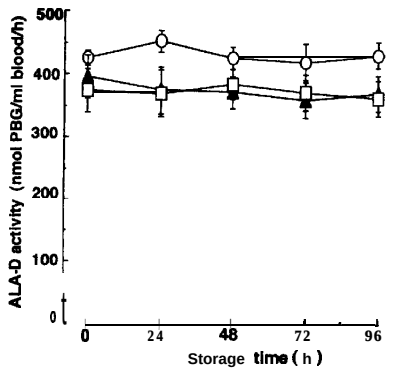

Fig. 1. The stability of carp blood ALA-D under various storage conditions. Values indicate the mean obtained from five measurements, while the vertical bars show the SE of the mean. 0, refrigerator; A, ice bath; □, dry-ice bath.

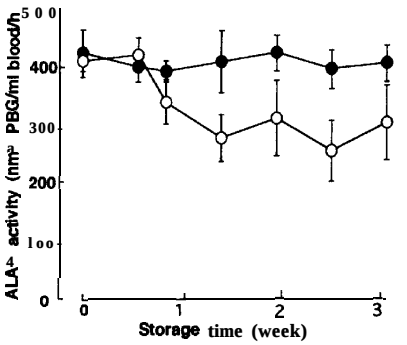

Fig. **2.** The stability of carp blood ALA-D under various storage conditions. Values indicate the mean obtained from five measurements, while the vertical bars show the SE of the mean. ●, ice bath; 0, dry-ice bath.

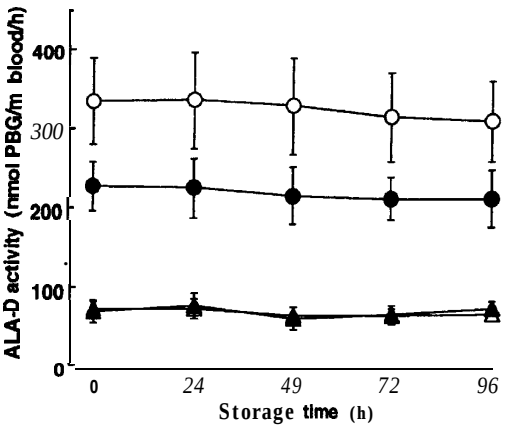

Fig. 3. The effect of storage on the blood ALA-D activity of lead-contaminated carp. Values indicate the mean obtained from the measurements of five fish, while the vertical bars show the SE of the mean. Water lead (ppb): A, 100; △, 50; ● ,IO; **0**, 0 (control water).

lost by day 2 if stored at -20 ℃ under air. It is thought that the characteristics of blood ALA-D in humans and in carp are not quite the same. However, judging from information regarding storage conditions for blood with a view to preventing any loss of activity such as that described above, it is apparent that the levels of ALA-D in carp blood samples stored in an ice bath are much more stable than those of blood samples stored in a dry-ice bath, or at 4 "C.

Although various levels of ALA-D activity in blood samples collected from lead-contaminated fish were observed, it was also certified that no activity was lost during 4 days when those blood samples were stored in an ice bath (Fig. 3). Therefore, when using carp blood ALA-D for assessing water lead pollution in field surveys, blood samples collected from fish caught in the field should be divided immediately into quantities of 50 μl in the Eppendorf test tubes as described above, and these are stored in an ice bath. Consequently, carp blood ALA-D activity will remain stable.

Relationship between ALA-D activity and blood lead concentration, and water lead concentration

The relationship between ALA-D activity and blood lead concentration, and water lead concentration is shown in Fig. 4. Blood lead concentrations increased with increasing water lead concentrations. On the other hand, blood ALA-D activity in fish exposed to water lead concentrations of 10, 50, and 100 ppb decreased to about 70, 23, and 25% of that of the control fish, respectively. It has been proposed that fish blood ALA-D could be used as an effective short-term indicator to estimate the long-term effects of water lead on fish (Hodson et *al.*, 1977). It has been pointed out that a water lead concentration of 10 ppb (Environmental Water Quality Standards relating to Human Health in Japan) is a

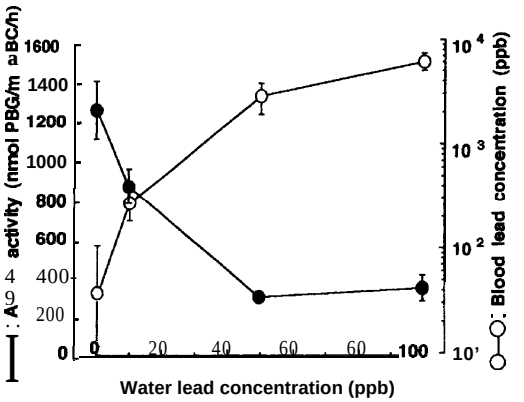

Fig. 4. Relationship between ALA-D activities and lead concentrations of carp blood, and water lead concentrations. Values indicate the mean obtained from the measurements of five fish, while the vertical bars show the SE of the mean.

concentration that may cause chronically sublethal effects to carp as described in our previous report (Nakagawa ***et al., 1995c).*** From the present results, it was also verified that the above standard value of water lead may lead to chronic toxicity in carp. Since blood ALA-D activities decreased and blood lead concentrations increased with increasing water lead concentrations, the ALA-D activities were negatively correlated to blood lead concentrations.

Stability of blood Hb under storage conditions

No change in the Hb concentrations in carp blood samples was observed during 4 days when the blood samples containing the reagent were stored at room temperature or in a refrigerator (Fig. 5).

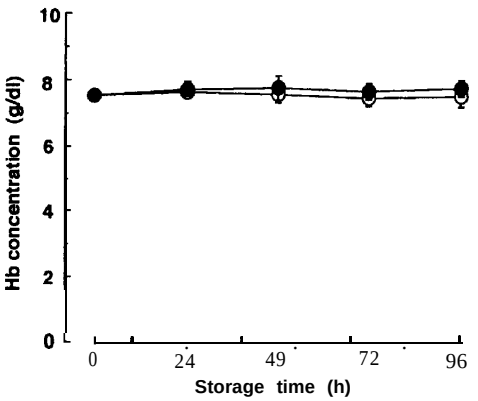

Fig. 5. The stability of Hb concentration of carp blood under various conditions of storage. Values indicate the mean obtained from five measurements, while the vertical bars show the SE of the mean. ●, room temperature; ***0,*** refrigerator.

A standarized expression unit for ALA-D activity

ALA-D activity in fish blood has been expressed as follows: nmol, μg, or nM of PBG which is formed from aminolevulinic acid during 1 hour, as 1) per ml of RBC, that is, nmol PBG/ml RBC/h (Hodson et al, 1977); 2) per ml of total blood, that is, μg PBG/ml blood/h (Johansson-Sjobeck and Larsson,1978; Haux et al., 1986); and 3) per mg of Hb, that is, nM PBG/mg Hb/h (Dwyer et al., 1986). Although it is impossible to measure Hct in blood samples which have been stored in an ice-bath, it is possible to measure Hb concentrations. Accordingly, it is possible to express ALA-D activity by using the expression unit of "per g of Hb" or "per ml of total blood", instead of "per volume of erythrocyte". Figure 6 shows the relationship between the three expression units of ALA-D activity, that is, per ml of RBC, per ml of blood, and per g of Hb by using the data of blood ALA-D activities obtained from lead-contaminated carp (Fig., 4). The relationship between these three expression units of activity to water lead concentrations showed the same tendency. Consequently, the three expression units for ALA-D activity were found to be related as follows:

nmol PBG/ml RBC/h = 3.6 x nmol PBG/ml blood/h = 0.256 x nmol PBG/g Hb/h

However, the coloring reagent using for the measurement of blood Hb concentration contains a high concentration of potassium cyanide and potassium ferricyanide. Moreover, it is troublesome to remove these cyanides from experimental waste water following Hb measurement. On the other hand, this will not be a problem if the ALA-D activity is expressed as "per ml of blood". Therefore, it is best to express the ALA-D activity by using the unit of "per ml of blood" when assessing water lead pollution in the field survey.

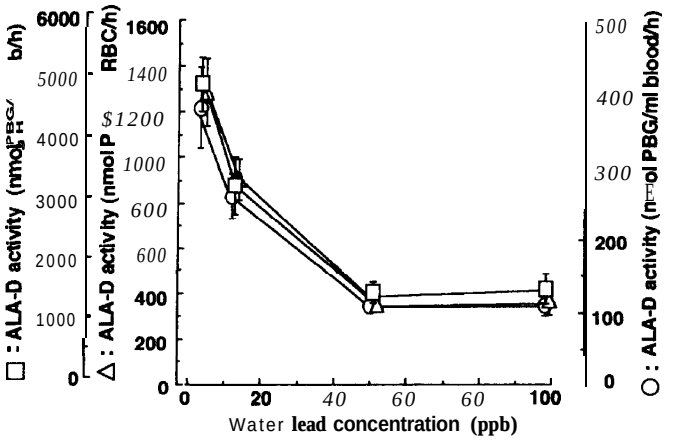

Fig. 6. Relationship between the three expression units of carp blood ALA-D activities. Values indicate the mean obtained from the measurements of five fish, while the vertical bars show the SE of the mean.

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