Radiochemical Microassay of \( \delta \)-Aminolevulinic Acid Dehydratase Activity in Whole Blood and Bone Marrow

Masao Kondo and Gumpel Urata

We describe an assay for \( \delta \)-aminolevulinic acid dehydratase (EC 4.2.1.24) activity. Radioactive \(^{14} \text{C}\)porphobilinogen, formed by action of this enzyme on \(^{14} \text{C}\)\( \delta \)-aminolevulinic acid, is purified by passage through an ion-exchange chromatographic column before measurement with a liquid scintillation counter. The radioactive substance in the final solution was identified as solely \(^{14} \text{C}\)porphobilinogen by paperchromatographic analysis. The present assay procedure requires only a 0.1-\( \mu \)L sample of blood and is about 100-fold more sensitive than the conventional colorimetric methods involving Ehrlich’s reagent. Using this method, we found that activity of this enzyme in the bone marrow of rats decreases abruptly and sharply two weeks after birth.

Additional Keyphrases: enzyme activity · bone-marrow cells · lead · rats · porphobilinogen

\( \delta \)-Aminolevulinic acid (ALA) dehydratase [porphobilinogen synthase; 5-aminolevulinic acid hydro-lyase (adding 5-aminolevulinate and cyclizing), EC 4.2.1.24] catalyzes the condensation of two molecules of ALA with loss of two molecules of water to form the monopyrrole porphobilinogen (PBG), the second intermediate in the pathway leading to the synthesis of porphyrin (1). The activity of ALA dehydratase in the blood of humans changes in various hematological diseases (2), hereditary tyrosinemia (3), porphyrias (4, 5), liver cirrhosis (6), alcoholism (5, 7), and poisoning with heavy metals such as lead (8, 9). Determination of ALA dehydratase activity in erythrocytes is sensitive, and is widely used as a biological indicator of exposure to environmental lead. Among the assay methods developed to correlate ALA dehydratase activity with lead exposure (10–13), the "European standardized method" (10) is the most widely accepted. Microassays for estimating ALA dehydratase activity in as little as 5 \( \mu \)L (14) or 10 \( \mu \)L (15) of whole blood have also been reported. All of these methods (10–15) are based on spectrophotometry of the color produced by reaction with Ehrlich’s reagent (16), which reflects the rate of PBG formation from ALA, catalyzed by the enzyme. These methods are suitable for determinations with blood samples but are not feasible for use with (e.g.) biopsy specimens of peripheral nerves or skin. Given our recent findings (17) that ALA dehydratase activity in hemopoietic tissues diminishes markedly after birth, becoming practically undetectable by colorimetric analysis in the bone-marrow cells of rats by the second to third week after birth, we have devised a new radiochemical microassay for determination of ALA dehydratase activity. Quantifying the \(^{14} \text{C}\)PBG formed from \(^{14} \text{C}\)ALA substrate serves to measure serial changes in ALA dehydratase activity in the bone marrow of growing rats, measurements that are not possible with the conventional spectrophotometric assay.

Department of Nutrition and Biochemistry, The Institute of Public Health, 6-1, Shirokanedai 4 chome, Minato-Ku, Tokyo 108, Japan.

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Here we report details of the new radiochemical microassay method and illustrate its application by measuring the serial changes in ALA dehydratase activity in the bone marrow of growing rats.

Materials and Methods

Chemicals. \(^{14} \text{C}\)\( \delta \)-Aminolevulinic acid (ALA) was obtained from New England Nuclear, Boston, MA; ALA and p-dimethylaminobenzaldehyde from Daichi Pure Chemical Co., Tokyo, Japan; reduced glutathione from Sigma Chemical Co., St. Louis, MO; sodium heparinate (166.5 USP units/mg) from Wako Pure Chemical Co., Tokyo, Japan; Sephadex G-10 from Pharmacia Fine Chemicals, Uppsala, Sweden; and Dowex 1-X8 (200–400 mesh, Cl⁻ form) from Dow Chemical Co., Midland, MI. Other chemicals were standard products of reagent grade.

Modified Ehrlich’s reagent. Dissolve 8 g of p-dimethylaminobenzaldehyde in 356 mL of glacial acetic acid, then add 80 mL of 700 g/L (cond.) perchloric acid solution and 20 mL of 0.2 mol/L \( \text{HgCl}_2 \) solution. This solution can be used for at least six months when stored at 4 °C in a dark-brown bottle.

Pretreatment of Dowex resins. After removal of small particles by decantation, wash the Dowex 1-X8 first with 10 vol of 2 mol/L HCl, then with 10 vol of distilled water, and finally with 10 vol of 2 mol/L NaOH. Repeat this procedure three times. Then wash the resin suspension twice with 10 vol of distilled water, and change it to the acetate form by treatment with 3 mol/L sodium acetate. Wash the resin suspension three times with 10 vol of distilled water. The third washing should be free of \( \text{Na}^+ \), \( \text{Cl}^- \), and \( \text{CH}_3\text{COO}^- \) ions.

Preparation of authentic PBG. We isolated PBG from urine of patients with acute intermittent porphyria, according to the method of Westall (18), and recrystallized it as the monohydrate. This was used as the standard. The Ehrlich–PBG color salt has a molar absorptivity of 6.1 \( \times 10^4 \) at 555 nm and has a minor absorbance peak at 525 nm with a molar absorptivity of 5.0 \( \times 10^4 \) (16). The ratios of the absorbances of the two absorption bands, 525 nm/555 nm, was 0.85.

Preparation of the enzyme solution from rat bone-marrow cells. Bone-marrow cells were collected from femurs of male Donryu rats (Nippon Rat Co. Ltd., Tokyo, Japan), and suspended in cold 9 g/L NaCl solution. The cells were precipitated by centrifugation at 3000 \( \times g \) for 10 min at 4 °C, washed twice with cold 9 g/L NaCl solution, and hemolyzed by adding about 5 vol of ice-cold de-ionized water. Isotonicity was restored by adding 90 g/L NaCl solution containing sodium phosphate buffer (0.1 mol/L, pH 7.8). We centrifuged the hemolysate at 105 000 \( \times g \) for 30 min at 4 °C, and used the supernatant fluid for the assay of ALA dehydratase.

Radioassay of ALA dehydratase activity. The incubation mixture contained \(^{14} \text{C}\)ALA, 0.08 mmol/L (0.2 \( \mu \)Ci, 49.0 Ci/mmol); glutathione, 10 mmol/L; sodium phosphate buffer (pH 6.8, 50 mmol/L); and 0.1 to 20 \( \mu \)L of enzyme solution in
a final volume of 50 \mu L. After this mixture was incubated for 1 h at 37 °C, we terminated the reaction by adding 1.0 mL of 0.2 mol/L trichloroacetic acid. The precipitate was removed by centrifugation at 3000 \times g for 10 min, and all of the supernate was mixed with 0.2 mL of a 250 g/L solution of Na2CO3.

Isolation of [14C]PBG. The supernatant fluid containing Na2CO3 was then applied to the top of a 0.8 × 1 cm column of the ion-exchange resin. After the column was washed with 30 mL of 80 g/L Na2CO3 and 10 mL of water, [14C]PBG was eluted with 3.0 mL of 12 mol/L acetic acid. The [14C]ALA and [14C]porphyrin, which had appeared in the incubation mixture during the enzymic reaction, were removed from the column by the washing with 80 g/L Na2CO3. The analytical recovery of PBG was about 70%.

Counting with the liquid scintillation counter. The eluate from the resin column was mixed with 13 mL of dioxane scintillator containing 2,5-diphenyloxazole, 4 g/L, 1,4-bis(2-(5-phenyloxazoly))benzene, 0.4 g/L; and naphthalene, 60 g/L. The counting efficiency was approximately 60 to 70%.

Conventional colorimetric assay of ALA dehydratase. For comparison, we assayed ALA dehydratase colorimetrically as described previously (9).

Protein determination. Protein was determined by the method of Lowry et al. (19) with crystalline bovine serum albumin as the standard.

Results and Discussion

Identification of PBG

The following two sets of experiments were performed to ascertain that the eluate from resin column (Figure 1) was indeed PBG. In the first experiment (Figure 2) we eluted and collected the acetate eluate from a column of Sephadex G-10. As can be seen, approximately 95% of the total 14C radioactivity eluted completely coincided with the color produced by the PBG standard after the Ehrlich reaction. From data as illustrated in Figure 2, we calculated that the [14C]PBG in the final solution was about 95% pure. The small peak of 14C radioactivity appearing a little before the main peak in Figure 2 remains to be identified. Secondly, the eluate from the Sephadex G-10 column containing [14C]PBG was concentrated by evaporation under reduced pressure at about 40 °C and analyzed by paper chromatography (Whatman 51A paper, 2 × 30 cm, developed in n-}

![Fig. 1. Chromatographic profile of the eluate from the Dowex 1-X8 column](image)

After zero (A) or 2 h (C) of incubation of the reaction mixture at 37 °C, the reaction was terminated by adding 2.0 mL of trichloroacetic acid, the precipitate was removed by centrifugation, and all of the supernate was mixed with 0.4 mL of 250 g/L Na2CO3 solution before application (total volume: 2.46 mL) to the column. □, standard PBG. Arrows indicate the fraction number at which the eluted was changed.

![Fig. 2. Chromatographic profile of the PBG fraction (2 mL, concentrated from fractions 31–33 in Fig. 1) eluted from a 1.5 × 80 cm column of Sephadex G-10 equilibrated with 1 mol/L acetic acid](image)

Symbo as in Fig. 1

butanol/acetic acid/water, 4/1/5 by vol) to confirm the finding. In the chromatograms the main radioactive spot (Rf 0.5) exactly coincided with the color spot produced by the PBG standard, which was developed on a separate paper and sprayed with Ehrlich's reagent.

Determination of Optimum Conditions

To determine the optimum conditions for [14C]PBG formation, we examined the effects of substrate concentration, incubation interval, and whole-blood constituents on the enzyme activity. [14C]PBG formation became constant in the range 40 to 80 \mu mol of [14C]ALA per tube.

We examined the effect of the incubation interval by both the conventional colorimetric method and the new radiochemical method. Results by the two procedures were linearly correlated for incubation times up to 180 min. At 1 h of incubation, the measured value in 1 \mu L of whole blood was 10 000 dpm in the radiochemical assay, compared with 0.006 A in the colorimetric assay.

To evaluate the effect of whole-blood constituents, we used mouse blood. The results of the conventional colorimetric method were linear with sample size in the range 1.0 to 5 \mu L; for the radiochemical method the range extended from 0.1 to 5 \mu L. ALA dehydratase activity was practically undetectable for samples less than 1.0 \mu L by the colorimetric method, while in the radiochemical method as much as 3000 dpm of [14C] radioactivity was detected in as little as 0.2 \mu L of blood.

Changes in ALA Dehydratase Activity in Bone-Marrow Cells of Growing Rats

The ALA dehydratase activity in bone-marrow specimens from rats one to six weeks old was determined by both the colorimetric method and the radiochemical method. We also attempted to ascertain whether bone-marrow ALA dehydratase activity might differ between a group of rats nourished with maternal breast feeding and another maintained on a diet containing casein (180 mg/g). Activity of ALA dehydratase in bone marrow decreased abruptly and sharply at two weeks of age, and was no longer detectable by the conventional colorimetric assay at age three weeks. During this period, however, the radiochemical assay was sensitive enough to demonstrate the declining enzyme activity in the bone-marrow cells of the growing rats. We found no significant difference in bone-marrow ALA dehydratase activity between the group fed on mother's milk and the group maintained on a synthetic diet at any period during the course.

Our recent studies (17) have demonstrated the presence in
rat bone marrow of a protein factor specifically inhibiting ALA dehydratase activity, and this is the apparent cause for this depression of ALA dehydratase activity. The present data suggest the potential usefulness of our assay technique in studies such as these as well as in examinations of biotic specimens of peripheral nerves and specimens containing extremely low enzyme activity that is undetectable by assay methods currently in use.

References