Improved Method for Measurement of delta-Aminolevulinic Acid Dehydratase Activity of Human Erythrocytes

Helen B. Burch and Abraham L. Siegel

We have evaluated and modified a current method for estimating \( \delta \)-aminolevulinic acid dehydratase (5-aminolaevulinic acid hydro-lyase, EC 4.2.1.24) activity in blood, so as to adequately control the pH of the enzyme-substrate solution at the optimum throughout the incubation period. This has improved the sensitivity and reliability of the assay. Also, N-ethylmaleimide has been substituted for a potentially hazardous mercury salt, used to remove sulfhydryl groups before color development with a modified Ehrlich's reagent.

Additional Keyphrases  
N-ethylmaleimide as \(-SH\) binder  
phosphate-citrate buffer

The assay for blood ALA-D\(^1\) activity has been proposed as a practical screening test for unsuspected exposure to lead (1–9). Gibson et al. (10) showed that the activity of this enzyme, which catalyzes condensation of two moles of ALA to form one mole of PBG, is severely inhibited by lead.

In various recent studies on the activity of human erythrocyte ALA-D (2–8, 9), the authors used the aerobic method of Bonsignore et al. (1), who based their procedure on both the work of Gibson et al. (10) and of Granick and Mauzerall (11). In this method a hemolysate is incubated at a pH of 7.0 in a bicarbonate-buffered substrate. According to Gibson et al. and Granick and Mauzerall, the enzyme has a rather narrow pH optimum, at 6.7 to 6.8. For instance, the activity of the enzyme is decreased by half if the pH of the reaction mixture substrate is 7.5 instead of 6.7. When we performed the enzyme assay according to the method proposed by Bonsignore et al. (1), we discovered that the pH of the incubation mixture—composed of the hemolysate and the bicarbonate-buffered substrate—shifted from an initial value of 7.0 to about 8.3 at the end of the 1-h incubation. At the latter pH the enzyme would exhibit only 25% of the activity observed at the optimum pH, 6.7 (10). Obviously, inadequate control of substrate pH is a serious deficiency in this method.

We have reinvestigated the optimal conditions for the measurement of ALA-D activity in human erythrocytes, and incorporated these conditions into a reliable procedure that can readily be performed in the clinical laboratory.

Materials and Methods

Reagents

**Triton X-100 reagent.** 0.20 ml of Triton X-100 (Sigma Chemical Co., St. Louis, Mo. 63178) is diluted to 100 ml with distilled water.

**Buffered ALA substrate.** This contains 0.01 mol/liter of ALA. Prepare the buffer from a 0.25 mol/liter solution of dibasic sodium phosphate, Na\(_2\)HPO\(_4\)-7H\(_2\)O, made by dissolving 6.703 g in 100 ml of water. Make a 0.25 mol/liter citric acid solution by dissolving 5.254 g of the acid in 100
ml of water. Adjust the phosphate solution to pH 6.65 by adding a sufficient quantity of the citric acid solution. Dissolve 0.1676 g of ALA-HCl (Calbiochem, San Diego, Calif. 92112, or Sigma Chemical Co., St. Louis, Mo. 63178) in 100 ml of the phosphate-citrate buffer, pH 6.65.

TCA reagent containing 0.02 mol/liter of N-ethylmaleimide. To 0.250 g of N-ethylmaleimide (Sigma Chemical Co., St. Louis, Mo. 63178) dissolved in 30-40 ml of warm water, add 10 g of TCA and dilute to 100 ml.

Modified Ehrlich's reagent. This contains 2 mol of perchloric acid per liter. Dissolve 10 g of DMBA in 420 ml of glacial acetic acid. This may be stored at 4°C. If crystallization occurs, redissolve by warming before the reagent is used. On the day of the test, mix 8 ml of 70% perchloric acid with sufficient DMBA-acetic acid solution to make a final volume of 50 ml.

Anticoagulant. Heparin is the anticoagulant of choice. EDTA reportedly inhibits ALA-D activity (10). Blood samples should be iced when drawn and kept at 4°C, to retard loss of erythrocyte ALA-D activity.

Procedure

Draw blood by venipuncture into heparinized tubes. Anticoagulants such as EDTA, which chelate calcium, have been reported to inhibit ALA-D activity (10). Use an aliquot to determine the hematocrit, and run the ALA-D assays in duplicate. Prepare the incubation mixtures in disposable plastic tubes.

Regular laboratory glassware, rinsed in dilute nitric acid followed by thorough rinsing with distilled water, is suitable for the other steps in the procedure. Dichromate cleaning solutions should be avoided. Prepare the incubation mixture by adding 0.20 ml of blood to 1.30 ml of Triton X-100 reagent, which assures immediate hemolysis. Add 1 ml of the buffered ALA substrate and mix. Remove 1 ml immediately for the blank and promptly add it to a tube containing 1.0 ml of the TCA reagent. After mixing and centrifuging, remove 1.0 ml of the clear supernatant fluid for the color reaction. Cover the tubes containing the remainder of the incubation mixture (1.5 ml) and incubate them at 38°C for 1 h. At this time, add 1.5 ml of the TCA reagent to the incubating tubes. After mixing and centrifuging, take 1.0 ml of the supernatant fluid for the color reaction. To each of the aliquots of the blanks and test supernatant fluids add 1.0 ml of the modified Ehrlich's reagent. Mix the solutions and allow the color to develop for 13 min. Read the absorbances at 555 nm within the next 10 min in a spectrophotometer, with use of cuvets with a 1.0-cm light path. In our experience, absorbances of the blank are negligible compared to a method blank in which water is substituted for blood. Therefore, the specimen blank could be disregarded, especially if this procedure is used for screening purposes. The volume of blood required for the assay may be decreased to 0.10 or 0.05 ml by appropriately scaling down the volumes of the reagents.

Calculation

The corrected absorbance (A) is the A of the test less the A of the blank. The unit of enzyme activity is expressed as an increase in A at 555 nm of 0.100, with a 1.0-cm light path, per ml of erythrocytes per hour, at 38°C. Corrected

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\text{Corrected } A \times \frac{100}{\text{hematocrit}} \times 12.5 \times 10, \text{ or }
\]

Corrected

\[
\text{Corrected } A \times \frac{12,500}{\text{hematocrit}} = \text{units of ALA-D activity}
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where 12.5 is the dilution factor of the blood.

Experimental Variables

Selection of an appropriate buffer. The bicarbonate-buffered ALA substrate prepared according to Bonsignore et al. (1) is unstable. On standing for less than an hour, the pH increases to values as great as pH 8.0. When the hemolysate is added to fresh substrate at pH 7.0, the pH of the incubation mixtures increases to 7.3 to 7.6 before incubation. The postincubation pH values for 37 determinations varied from pH 7.6 to 8.3 (mean, 7.9). To avoid pH alterations during incubation and to establish optimum conditions for the measurement of ALA-D, we used several buffers, in various concentrations, to prepare the substrate. A precipitate formed with the Ehrlich's reagent when phosphate buffers (Na₂HPO₄ + KH₂PO₄) were used in concentrations sufficient to maintain optimum pH throughout incubation. Imidazole buffers inhibited ALA-D activity and had to be discarded. The greatest activity was found when a 0.25 mol/liter dibasic sodium phosphate-citrate buffer, pH 6.65, was used as the diluent for preparing the ALA substrate. This buffer maintains the pH of the incubation mixture at 6.70 ± 0.05, the optimum pH we found for the enzyme (Figure 1), in agreement with the optimum pH reported for the purified enzyme from ox liver by Gibson et al. (10) and for that from chicken erythrocytes by Granick and Mauzerall (11). In a comparison of the Bonsignore procedure and the one described in this study, 17 blood samples were run in duplicate with the buffer of the substrate as the only variable. The mean value was 98 ± 32 (sd) enzyme units with the bicarbonate-buffered substrate. We obtained a mean of 164 ± 63 units for the same samples when the phosphate-citrate buffer described in our proposed method was used.
Effect of storage on ALA-D activity. The ALA-D activity was determined for each of 22 freshly collected and iced blood samples and again after 24-h storage at 4°C. The mean for the fresh group was 156 ± 49 (SD) units, after 24-h storage 149 ± 46 units. The difference was significant at the 95% confidence level by the paired t test (12). The average decrease in activity was 4%, but a decrease of as much as 10% can be expected when specimens are stored at 4°C for 24 h. Bloods stored overnight at 23°C showed decreases of about 20%.

Enzyme activity vs. dilution. Serial aqueous dilutions were prepared from a hemolysate of normal human erythrocytes. The ALA-D activity measured was directly proportional to the fraction of hemolysate in the incubation mixture (Figure 2).

Substrate concentration. Optimum substrate concentration was determined from a plot of enzyme activity vs. substrate concentration (Figure 3). A concentration of 0.01 mol/liter before the addition of the hemolysate is optimal, and agrees with the concentration used by Bonsignore et al. (1).

Incubation temperature. A plot of erythrocyte ALA-D activity vs. incubation temperature is shown in Figure 4. Of those temperatures we investigated, the enzyme activity is greatest at 38°C, which was the temperature selected for this procedure. (Although it possibly is not the optimum temperature).

Erythrocyte hemolysis. The addition of Triton X-100 to the water used for the hemolysate assures immediate and complete hemolysis of the cells and does not alter the pH of the incubation mixture.

Other methods suggest the use of a heavy metal such as mercury to overcome sulfhydryl interference with the color reaction for PBG, because the pink salt formed with the Ehrlich's reagent is decolorized by sulfhydryl compounds (13). Because heavy metals are serious potential contaminants in the laboratory, an effective sulfhydryl-binding reagent, N-ethylmaleimide, has
been substituted for mercuric chloride in the proposed procedure.

A modified Ehrlich’s reagent containing perchloric acid (4 mol/liter) was recommended by Bonsignore et al. (1). This reagent gives a rapidly developed color, which must be read at 555 nm 5 min after mixing, because it fades readily (13). Mauzerall and Granick recommend reading 15 min after mixing when using a modified Ehrlich’s containing perchloric acid (2 mol/liter). This color remains stable for an additional 10–15 min. When a series of absorbances is being read, this allows time for determining the absorbances before the color fades. This modification of Ehrlich’s reagent is the one included in the proposed method.

Results

Variability and Normal Values

Duplicates of 50 consecutive estimations of erythrocyte ALA-D activity, as measured by the proposed method, had a mean value for the differences of 0.3 ± 4.9 (sd) units, an insignificant difference by the paired t test (12).

Reproducibility of the method was checked by estimating the erythrocyte ALA-D activity in the same subject 15 times during a 6-month period. The mean was 116 ± 4 (sd) units, and the relative standard deviation (cv) 3.5% (range, 111–124 units). This suggests that an individual’s ALA-D activity is rather constant over a considerable time.

Erythrocyte ALA-D enzyme activity for 35 healthy children (2–1/2 to 17 years old) averaged 175 ± 36 (sd) units, ranging from 124 to 260 units, with the proposed method. For 18 healthy adults the mean was 176 ± 43 (sd) units, with a range of 108 to 299 units. The normal mean for adults given by Bonsignore et al. (1) is 100 ± 20, that by de Bruin and Hoolboom (2) is 113 ± 13. The increased enzyme activities found in the proposed method may largely be attributed to the fact that optimum pH conditions were maintained throughout the incubation period.

Lead Intoxication

Lead intoxication was verified in a group of 17 patients in whom urinary lead excretions of 0.81–13.2 mg/24 h were demonstrated after stimulation by 1 g of the calcium salt of EDTA as described by Morgan (14). The erythrocyte ALA-D activities of these patients ranged from 10 to 58 units with a mean of 26 ± 13 (sp) units. This supports other published studies in which the ALA-D activities were found to be lowered only in erythrocytes of individuals who have been exposed to lead (7, 8). In our experience, no evidence of excessive lead absorption as determined by a stimulated urinary lead excretion, hematocrit, and clinical history has been noted for patients showing erythrocyte ALA-D activities of 100 units or more.

References