Erythrocyte Uroporphyrinogen I Synthase Activity in Diagnosis of Acute Intermittent Porphyria

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Measurement of the activity of uroporphyrinogen I synthase provides an excellent laboratory aid in the diagnosis of acute intermittent porphyria, particularly in those patients who are asymptomatic or in whom the disease is not biochemically manifested by porphyrin precursor excretion. We describe here a simplified fluorometric method for measuring the activity of this enzyme in whole blood. The assay is based upon a coupled‐enzyme procedure in which added δ‐aminolevulinic acid and the dehydratase that is present in erythrocytes are used to generate porphobilinogen as substrate for uroporphyrinogen synthase. After appropriate incubation the protein is removed with trichloroacetic acid, and the porphyrins formed are measured fluorometrically. The sensitivity, specificity, and precision of the assay compare well with previously described procedures. Activity in nonporphyrin male subjects was 31 (SD, 6.0) nmol of porphyrin formed per milliliter of erythrocytes per hour at 37 °C. Application of the method for identifying gene carriers of acute intermittent porphyria is demonstrated in three generations of an affected family.

Additional Keyphrases: inherited disorders • diagnostic aid • fluorometry • normal values • lead poisoning • three-generation pedigree • δ-aminolevulinate dehydratase

Acute intermittent porphyria (AIP) is a rare inherited disease characterized by three identified enzyme abnormalities: increased activity of δ-aminolevulinate synthase (EC 2.3.1.37), decreased activity of uroporphyrinogen synthase (URO-S), and decreased activity of steroid Δ4-5α-reductase (1-3). The usual laboratory findings include abnormally high urinary excretion of δ-aminolevulinic acid (ALA), porphobilinogen (PBG), and uro- and coproporphyrin (1). These metabolic changes may be accompanied by the clinical symptoms of intermittent abdominal pain, psychosis, constipation, nausea, hypertension, and varied additional signs and neurological symptoms (1). The true incidence of gene carriers for AIP has been difficult to assess because family studies have revealed patients with clinically latent forms who have normal urinary PBG and ALA (1, 2, 4, 5), which have until recently been the bases for diagnosing AIP.

Since the clinical expression of this potentially fatal disease may be precipitated by drugs, liver toxins, hormonal changes, diet, and other factors, it is important to identify the latent as well as the overt forms of AIP (1-3). All patients with AIP (overt or latent) have decreased erythrocyte URO-S activity, which accounts for the several laboratory assays that have been developed to detect this biochemical defect (3-8). However, most previously described methods have required the use of costly, labile porphobilinogen as substrate and have incorporated technical details that we have found unnecessary (4, 7, 8). A method originally described for measuring aminolevulinate dehydratase (EC 4.2.1.24) activity (9) has been modified into a fluorometric micro-assay for URO-S in whole blood, with ALA as substrate, a procedure generally suitable for clinical laboratories.

Materials and Methods

Reagents

Blood diluent: Dissolve 0.2 ml of "Triton X-100," a non-ionic surfactant, in 100 ml of water (we used deionized water in this study).

ALA substrate: Prepare 0.25 mol/liter Na2HPO4·7H2O by dissolving 6.70 g in 100 ml of de-ionized water. Titrate this to pH 7.5 with 0.25 mol/liter citric acid solution, prepared by dissolving 5.25 g of the dihydrate in 100 ml of water. Dissolve 67.04 g of δ-aminolevulinate acid hydrochloride (Sigma Chemical Co., St. Louis, Mo. 63178) in 100 ml of this buffer, yielding a 4 mmol/liter
solution of ALA. A stock solution of this buffer without added ALA is also required. The ALA solution is stable for at least six weeks when stored frozen.

**PBG substrate:** Dissolve porphobilinogen (Sigma) in buffer as for ALA.

**Trichloroacetic acid:** Dissolve 10 g of trichloroacetic acid in 100 ml of water. Stable indefinitely.

**Coproporphyrin standard:** Dissolve 5.0 μg of coproporphyrin (Sigma) in 5.0 ml of 1.5 mol/liter HCl, heat, then dilute to 100 ml with 1.5 mol/liter HCl to yield a coproporphyrin concentration of 76 nmol/liter (or 5.0 μg/dl), the unit commonly used in porphyrin analysis. This solution is stable for at least a year when kept in a tightly stopped tube.

**Procedures**

Blood may be obtained by venipuncture or by capillary puncture, with either ethylenediaminetetraacetate or heparin as anticoagulant; the latter is recommended if the enzyme assay cannot be done within 24 h. Sufficient blood to obtain a hematocrit, plus 0.15 ml for incubation, is required to perform the test in duplicate. The blood may be stored at 4 °C for 24–72 h, although unnecessary delay is not recommended. The buffered ALA substrate can be stored frozen at −20 °C in convenient-size aliquots, kept in airtight plastic tubes.

To do the assay, add 1.0 ml of Triton X-100 reagent to three different 15 × 75 mm test tubes for each specimen to be assayed. To each test tube then add 50 μl of whole blood. Prepare the blank tube by adding 1.0 ml of phosphate–citrate buffer without enzyme substrate (ALA). Add 1 ml of the buffered ALA substrate to each of the remaining two tubes. Vortex-mix the contents of the tubes immediately, cover them, and incubate them in the dark at 37 °C. After 1 h, add 2.5 ml of trichloroacetic acid, vortex-mix, and allow the tubes to stand at room temperature in the dark for about an hour to facilitate deproteinization. Remove the protein precipitate by centrifuging (15 min, 2400 rpm, or adequately to yield a clear supernatant solution). Measure the fluorescence of the supernatant solution in a Turner 100 (or equivalent fluorometer) fitted with a No. 405 (peak wavelength) primary filter and a No. 25 secondary filter transmitting light at wavelengths >595 nm. The sample blank usually has little if any fluorescence, although occasionally a significant reading may be obtained if increased endogenous porphyrins are present.

Use Oxford micro pipettes for measuring blood and the buffered ALA substrate and Oxford pipettes to dispense the Triton X-100 and trichloroacetic acid.

**Calculations**

URO-S activity expressed as coproporphyrin can be calculated from the following formula:

nanomoles of porphyrin formed per milliliter of erythrocytes per hour

\[
\frac{\text{nmol/mg}}{\text{h}} = \frac{(\text{fluor. of test}) (76.4) (4.55) (100)}{(\text{fluor. of std}) (1000) (0.05) \text{ (hematocrit)}}
\]

where:

- fluor. is the fluorometric reading, arb. units
- 76.4 = concn of coproporphyrin std. in nmol/liter
- 4.55 = final volume of TCA supernatant solution, ml
- 1000 = conversion factor, nmol/liter to nmol/ml
- 0.05 = volume of specimen, ml

For the quantities used as described, this expression simplifies to:

\[
\frac{\text{nmol/ml per hour}}{\text{fluor. of test}} = \frac{695}{\text{fluor. of std}} \text{ (hematocrit)}
\]

**Analytical Variables**

**Buffer.** Various buffers have been recommended for use in the assay of both ALA dehydratase and URO-S. For ALA dehydratase assay, phosphate, 2-(N-morpholino)ethanesulfonic acid, and tris(hydroxymethyl)aminomethane buffers have all been used (5, 9–13) and the recommended pH has ranged from 6.1 to 8.5. For assay of URO-S activity, pH 7.4 with a phosphate buffer (3, 7, 8, 11) and pH 8.2 with a tris(hydroxymethyl)aminomethane buffer (4) have been used. Because assay of URO-S with ALA as substrate depends on two enzymes, δ-aminolaevulinate dehydratase and URO-S, the pH optima of both must be considered. Doss (5) used tris(hydroxymethyl)aminomethane buffer at pH 7.2 in his assay for URO-S activity with ALA as substrate. We found pH 7.5 to be optimal in citrate/phosphate buffer (Figure 1), pH 8.0 optimal in tris(hydroxymethyl)aminomethane buffer. Because activity was greater in the citrate/phosphate buffer, we chose it for routine use. The pH does not change during the incubation.

**δ-Aminolaevulinic acid as substrate.** The purpose of the assay is measurement of URO-S activity, so δ-aminolaevulinate dehydratase activity must not be rate limiting because it generates porphobilinogen as substrate. The reported (10) activity of this dehydratase at pH 7.3 in phosphate buffer at 37 °C is at least 18-fold that of URO-S under similar conditions (14). Although

![Fig. 1. Porphyrin formation (nmol/ml erythrocytes per h) in citrate/phosphate buffer as a function of pH](image-url)
eight molecules of ALA or four molecules of PBG are required to form one molecule of uroporphyrinogen, the activity of the dehydratase is sufficiently greater than that of URO-S that the latter becomes rate limiting for the assay. Almost maximum activity of URO-S was obtained with as little as 20 nmol of ALA in the incubation mixture. However, 4000 nmol of ALA was chosen as substrate concentration; this clearly saturates the enzyme but still is less than is sometimes used for the dehydratase assay (9).

Substituting ALA for PBG as substrate has three advantages: (a) ALA is about 3% as costly as PBG, (b) ALA solution is more stable during storage, and (c) ALA does not cyclize to porphyrins nonenzymically as can happen with PBG. Storage of ALA in buffer for up to six weeks, the longest time observed, had no effect on URO-S activity. Presumably, the ALA in this form could be stored for considerably longer if desired.

The activity of URO-S with 2000 nmol of PBG as substrate (32 nmol of porphyrin formed per milliliter per hour) was almost identical to that found with 4000 nmol of ALA as substrate (33 nmol of porphyrin formed per milliliter per hour) after corrections were made for the nonenzymic cyclization of PBG to porphyrins. This is similar to the activity reported by Doss (5). Uroporphyrin comprised 60–70% of the porphyrins formed; most of the remainder (20–30%) was coproporphyrin (15). Sassa and colleagues (3, 11) also found uroporphyrin to be the principal reaction product. Doss (5) reported a difference in the uroporphyrin/coproporphyrin ratio with PBG or ALA as substrate, but we found no such difference. URO-S actually produces uroporphyrinogen I, but this reduced form is rapidly oxidized in strong acid to the fluorescent porphyrin.

Reaction conditions. Various quantities of enzyme (i.e., volumes of erythrocytes) were plotted vs. porphyrins formed (Figure 2); the relationship was linear up to 40 µl of cells per incubation mixture, after which the slope begins to decrease. Thus, 50 µl of whole blood was chosen as a convenient and suitable volume for assay. The activity of URO-S was linear with time at 37 °C for 4.5 h (Figure 3), the longest period observed. The activity was also linear with time at room temperature (24 °C), although the reaction rate was about 25% of that at 37 °C; other authors have reported somewhat greater activity of URO-S at 60 °C (7), but we chose the 37 °C incubation temperature for convenience. The error resulting from pipetting delays between samples should be negligible at room temperature, because less than 1 nmol of porphyrin is formed during 10 min at this temperature. Therefore, keeping the tubes in an ice bath during preparation is not necessary unless a long delay is likely after mixing enzyme and substrate.

Specimen. Some URO-S assays called for washing erythrocytes in isotonic saline. However, we found this to have no effect on URO-S activity. Therefore, heparinized whole blood can be used as indicated previously by Collier (10), who measured erythrocyte δ-aminolaevulate dehydratase activity, and by Sassa et al. (3), who measured URO-S activity in whole blood.

The dehydratase (16, 17) and URO-S (7) activity are reported to increase with increasing reticulocyte count. We have not measured dehydratase activity, but we have compared URO-S activity in more than 100 specimens containing abnormally large numbers of reticulocytes. There was significant correlation (P < 0.001) between reticulocyte count and URO-S activity, but this was true only after specimens having a very high (>5%) proportion of reticulocytes were included. Although ethylenediaminetetraacetate does not inhibit URO-S activity (11), it reportedly decreases the activity of the dehydratase (10, 18, 19). We added various amounts of ethylenediaminetetraacetate to whole blood to determine whether the dehydratase would be sufficiently inhibited to cause this enzyme to be rate-limiting. We saw no effect with ethylenediaminetetraacetate concentrations up to 10-fold those normally encountered with use of evacuated blood-drawing (Vacutainer) tubes. If blood is to be stored, however, heparinized
blood should be used because of the more rapid deterioration of URO-S (or of the dehydratase) activity in ethylenediaminetetraacetate.

The activity of URO-S in heparinized whole blood is stable for as long as 72 h at room temperature. It is stable for 120 h at 4 °C. The activity of URO-S in blood anticoagulated with ethylenediaminetetraacetate and stored at −20 °C decreases 30% in 24 h, 46% in 48 h. URO-S activity in frozen heparinized blood decreases gradually, with a half-life of about 14 days. Because URO-S activity is stable for weeks at 4 °C (4, 11), the decrease in activity noted here may be due to a loss in δ-aminolaevulinate dehydratase activity, making the conversion of ALA to PBG rate-limiting. Although this dehydratase is reportedly stable in liquid nitrogen, its activity during storage at 4 °C decreases by 15% by day 5, 30% by day 12 (11). After storing blood for 20 h, Haeger-Aronsen et al. (13) found a 7% decrease in the dehydratase activity at 4 °C and a 24% decrease at room temperature. The difference in URO-S activity between frozen heparinized and ethylenediaminetetraacetate-treated blood is not understood, but perhaps relates to the chelation of intracellular cations by ethylenediaminetetraacetate.

Protein precipitation. A potential error with the measurement of porphyrin fluorescence in the type of system we describe is the possibility of some hemin remaining in solution, causing quenching of porphyrin fluorescence. Trichloroacetic acid has been shown to remove the hemin that could otherwise create a problem, while leaving 90% of the uroporphyrin and 92–95% of the coproporphyrin in solution (4).

Results

URO-S activity in 89 healthy young adult males (Figure 4) averaged 31 (SD, 6.0) nmol of porphyrin formed per milliliter of erythrocytes per hour (range, 16–43). Thirteen unselected hospitalized children (ages 2–18) had a mean URO-S activity of 29 (SD, 6.0). Sixteen women had a mean activity of 34 (SD, 7.0). Thus, a sex- or age-related difference is not apparent. The normal values reported by Sassa et al. (3) were 35.7 ± 8.4, with a range of 20.7–62.5; Magnussen et al. (4) reported a mean of 40.7 nmol/ml per hour. Our test results gave within-day coefficients of variation ranging from 3.9 to 4.3%.

The clinical application of the URO-S assay we describe is illustrated in Figure 5 and Table 1. Figure 5 shows the pedigree of a family of Scandinavian ancestry in which two members (III-2 and III-7) had died at early ages with documented AIP. Unfortunately, the diagnosis of AIP was not entertained in either individual until after the onset of irreversible neurological complications. Table 1 records the urinary porphyrin and porphyrin precursor excretions of eight family members and the URO-S activity of seven members and one spouse. Affected family member II-4 has had episodic abdominal pain and distention for 25 years without serious complications and his 34-year-old son (III-5) has had unexplained episodic low back pain for 15 years. Family members IV-5 and IV-7 are asymptomatic and have normal physical examinations, yet they, too, have inherited the gene for AIP according to the results of the URO-S assay. Of diagnostic significance was the finding that neither III-5 nor his daughter, IV-7, would have been considered gene carriers on the basis of their urinary porphyrin or precursor excretion alone.

Discussion

Sometimes values for PBG and porphyrins in urine do not reveal carriers of the gene for AIP (Table 1). This is especially a problem in asymptomatic children. Early detection of the genetic lesion for AIP is important, so that known precipitating factors can be avoided or at least minimized. These considerations are the basis for assaying for URO-S, abnormal values for which are a biochemical defect that is consistent and unrelated to symptomatology or age—even antenatal diagnosis is possible by assaying URO-S in cultured amniotic fluid cells (7).

Typically, URO-S activity in carriers of the gene for AIP is about half the normal value (3, 4). Even though the family reported in Table 1 showed considerable

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**Fig. 4.** Frequency distribution of enzyme activity in erythrocytes of healthy men
Mean and 3 SD range is indicated below

<table>
<thead>
<tr>
<th>Number</th>
<th>Uroporphyrinogen Synthase Activity</th>
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<tr>
<td>10</td>
<td>31</td>
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<td>15</td>
<td>32</td>
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<td>40</td>
<td>37</td>
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<td>45</td>
<td>38</td>
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**Fig. 5.** Pedigree of a family in which acute intermittent porphyria has been established in three generations and in which the use of uroporphyrinogen 1 synthase as a diagnostic aid is illustrative
Numbers under each individual's symbol represent that person's present age or age at time of death

<table>
<thead>
<tr>
<th>I</th>
<th>II</th>
<th>III</th>
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**Table 1.** Urinary porphyrin and porphyrin precursor excretions of eight family members and the URO-S activity of seven members and one spouse

<table>
<thead>
<tr>
<th>Member</th>
<th>Urinary Porphyrins</th>
<th>URO-S Activity</th>
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<tbody>
<tr>
<td>II-4</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>III-5</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>IV-5</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>IV-7</td>
<td>Normal</td>
<td>Normal</td>
</tr>
</tbody>
</table>

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variability in URO-S, those bearing the genetic defect were identifiable. Further studies may help us to understand the very low values found in two family members (III-5 and IV-7). Other reports have indicated some overlap between normal and porphyric URO-S activities, a problem that may be resolved by family studies (1-3). Children of a parent with diagnosed AIP can probably be excluded as having inherited the genetic defect if the child has normal URO-S activity (3). In attempting a very early diagnosis—as with cord or fetal blood—one should remember that the normal range of URO-S activity in such specimens is several-fold higher than in blood from adults (20). Cultured fibroblasts may allow a more precise diagnostic test (7) than do circulating erythrocytes, but culturing cells in order to perform an uncommon test is not so practical for a routine clinical laboratory.

Because lead is a potent inhibitor of δ-aminolaevulinate dehydratase activity (9, 10, 13, 17), its toxic reaction should be considered when a marked decrease in measured URO-S activity is found. Although moderate intoxication with lead would be expected to create no problem, more severe lead intake could markedly inhibit the dehydratase activity, resulting in decreased URO-S activity and leading in turn to a false interpretation of the results. The lowest URO-S activity that we have found (6 nmol/ml per hour) was in a patient who subsequently was diagnosed as having chronic lead poisoning. An activity this low suggests that lead may inhibit not only the dehydratase but also URO-S, which could explain the occasional finding of increased porphobilinogen excretion in patients with lead toxicity. Lead interference can be resolved in two ways: (a) test the specimen for increased protoporphyrin as an indication of lead intoxication (21), or (b) perform a repeat assay with dithiothreitol added to chelate any lead present (22).

The method for measuring URO-S activity we describe here is suitable for use in a general hospital laboratory, because no special equipment or exotic procedures are needed. The time required to perform the test has been minimized by eliminating extraction procedures (3) and by use of reagents that can be made in advance and stored. The use of ALA simplifies handling of the substrate and substantially reduces its cost. ALA further eliminates the error introduced by nonenzymic conversion of PBG to porphyrins, which can be 5% or more (5, 11). The major current limitations of the assay consist of the lack of a suitable control to assay simultaneously with the test specimen and the potential for interference from lead.

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References


