A Red Cell Enzyme Method for the Diagnosis of Acute Intermittent Porphyria

By C. Richard Magnussen, Joel B. Levine, Joyce M. Doherty, Judy O. Cheesman, and Donald P. Tschudy

A method has been devised for the measurement of uroporphyrinogen I synthetase in red cells. By using trichloroacetic acid as a protein precipitant, heme is removed from the final solution, allowing accurate measurement of porphyrins. The method is highly reproducible and adaptable to varying incubation volumes and enzyme preparations. It is of great value as an enzyme diagnostic method for acute intermittent porphyria and appears capable of detecting patients with the latent disease who have normal urinary δ-aminolevulinic acid and porphobilinogen excretion. It also appears to distinguish other types of porphyria from acute intermittent porphyria. The mean value of the enzyme in red cells of patients with acute intermittent porphyria was approximately 50% that of normals, indicating that the mutation causes complete lack of catalytic activity in the mutant enzyme.

ACUTE INTERMITTENT porphyria is an inborn error of metabolism which causes increased excretion in the urine of the porphyrin precursors, δ-aminolevulinic acid (ALA), and porphobilinogen (PBG). The excesses of these substances are known to originate in the liver, where it has been shown that there is a decrease in the third enzyme (uroporphyrinogen I synthetase) and an increase in the first enzyme (ALA synthetase) of the heme biosynthetic pathway. In the past, the diagnosis of this disease was based on the demonstration of increased amounts of PBG in the urine, at first by means of the Watson-Schwarz test, and later by quantitative chromatographic methods. However, it is now known that these methods do not detect all individuals bearing the defect. It has recently been shown that the decreased level of uroporphyrinogen I synthetase also occurs in tissue outside the liver, such as red blood cells and fibroblasts. These studies have indicated that an enzymatic method of diagnosis is feasible for this disease, and three methods employing red cells for this purpose have recently been developed. All methods involve certain complexities, i.e., the use of minute volumes in microcuvettes or use of highly sophisticated equipment. For reasons which are not yet completely elucidated, it is apparent that it is not possible to reliably scale up in volume certain and perhaps all of these micro methods. This difficulty may relate to the failure of the protein precipitants to adequately remove heme and thereby prevent quenching caused by heme. The present method utilized a precipitant which circumvents this problem and allows its application to a wide range of volumes and dilutions which would be encountered in both research and clinical laboratories. The method has been applied to a...
number of patients with acute intermittent porphyria and some of their relatives.

MATERIALS AND METHODS

Porphobilinogen was purchased from Sigma Chemical Co., St. Louis, Mo. Protein concentrations of incubated hemolysate were determined by the method of Lowry et al. using an albumin standard.

Porphyrins were measured quantitatively in a Turner Model 430 spectrophotofluorimeter with an excitation peak at 405 m and emission peak at 596 m. Half-power slit widths of 15 m were used and samples were read in glass tubes of 10-mm diameter. Coproporphyrin and uroporphyrin standards were prepared by hydrolysis of the corresponding esters in 6 N HCl overnight. After hydrolysis, the porphyrins were then made up in the appropriate concentration in trichloroacetic acid, perchloric acid–ethanol or HCl, for use as standards as indicated later. All standards were maintained in the frozen state protected from light.

Blood was drawn into heparinized syringes, centrifuged, and after removal of the plasma and buffy coat, the red cells were washed twice in cold isotonic saline. After packing by centrifugation and freezing in dry ice-acetone, the effect of thawing on observed enzyme activity was studied in two ways: fast and slow. Fast freeze-thawing was performed by freezing in dry ice-acetone followed by fast thawing in a Dubnoff incubator at 37°C. Slow freeze-thawing was performed by freezing in dry ice-acetone followed by slow thawing at room temperature. Osmotic lysis of the red cells in distilled water and homogenization of the red cells in a VirTis Macro Model 45 were also studied as a means to release the enzyme. Osmotic lysis was accomplished by addition of one volume of red cells to either two or five volumes of distilled water. Red cells were homogenized in a VirTis homogenizer two times for 30-sec periods in an ice-cooled chamber.

All incubations were performed in a total volume of 1.5 ml.

RESULTS

Linearity of Standards

Since trichloroacetic acid was used as a protein precipitant in the final procedure which evolved from the present studies, it was necessary to demonstrate a linear emission intensity as a function of concentration for both uro- and coproporphyrin in trichloroacetic acid. This linear response up to concentrations of 5 μg/100 ml in 12.5% trichloroacetic acid (the final trichloroacetic acid concentration in which the unknowns are read) is shown in Figs. 1A and 1B. In all assays, the actual concentrations of porphyrins in the unknown were below 5 μg/100 ml. It was also found that the fluorescence intensity of a fixed concentration of coproporphyrin (5 μg/100 ml) was constant in various concentrations of trichloroacetic acid ranging from 5% to 15%.

Preparation of Hemolysate

It was found that the method of rupturing red blood cells to release the enzymes into solution greatly influenced the activities observed. As seen in Table 1, a slow rate of freeze-thawing was much more effective than a fast freeze-thaw when this was performed twice (Expt. 1), or three times (Expt. 2). The apparent discrepancies between Expt. 1 and Expt. 2, where three slow freeze-thaws in Expt. 2 yield lower activity than two slow freeze-thaws in Expt. 1, is due to the fact that Expt. 2 utilized perchloric acid–ethanol as a protein
precipitant, whereas Expt. 1 utilized trichloroacetic acid. The superiority of the latter precipitant in this enzyme assay is demonstrated and discussed later. However, there was no difference in total enzyme activity observed between five fast or five slow freeze-thaws (Expt. 3). Furthermore, when the cells were homogenized in a VirTis homogenizer in addition to three fast freeze-thaws, activity was slightly decreased below that observed with three fast freeze-thaws alone (Expt. 2). Likewise, lysis alone in distilled water or lysis combined with three slow freeze-thaws produced approximately one-half the activity observed with five fast or five slow freeze-thaws (Expt. 3). Since repeated freeze-thawing appeared to produce optimal activity, the effect of the number of slow freeze-thaws on activity was studied as shown in Fig. 2.

Table 1. Effect of Various Methods of Red Cell Disruption on Porphyrin Production from Porphobilinogen

<table>
<thead>
<tr>
<th>Method of RBC Disruption</th>
<th>Observed Enzyme Activity (nmol porphyrin production/ml RBC/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. 1</td>
<td></td>
</tr>
<tr>
<td>2 fast freeze-thaws</td>
<td>23.9</td>
</tr>
<tr>
<td>2 slow freeze-thaws</td>
<td>34.8</td>
</tr>
<tr>
<td>Expt. 2</td>
<td></td>
</tr>
<tr>
<td>Homogenization +</td>
<td></td>
</tr>
<tr>
<td>3 fast freeze-thaws</td>
<td>13.6</td>
</tr>
<tr>
<td>3 fast freeze-thaws</td>
<td>17.7</td>
</tr>
<tr>
<td>3 slow freeze-thaws</td>
<td>24.3</td>
</tr>
<tr>
<td>Expt. 3</td>
<td></td>
</tr>
<tr>
<td>5 fast freeze-thaws</td>
<td>38.0</td>
</tr>
<tr>
<td>5 slow freeze-thaws</td>
<td>37.5</td>
</tr>
<tr>
<td>1 osmotic lysis</td>
<td>21.2</td>
</tr>
<tr>
<td>1 osmotic lysis +</td>
<td></td>
</tr>
<tr>
<td>3 slow freeze-thaws</td>
<td>21.2</td>
</tr>
</tbody>
</table>

Red cells from different normal individuals were used in each of the three experiments. All experiments were performed using a total incubation volume of 1.5 ml in Tris buffer (0.05 M, pH = 8.2) containing 10^{-4} M porphobilinogen. Incubations were performed at 37°C for 1 hr. In Expts. 1 and 3, the disrupted red cells were used at a final dilution of 1:40, and 1.5 ml of 25% trichloroacetic acid was used to precipitate protein. In Expt. 2, the disrupted red cells were used at a final dilution of 1:240 and 1.5 ml of 2 N perchloric acid-ethanol (1:1) was used to precipitate protein. The details of the fast and slow freeze-thaws, homogenization, and osmotic lysis are presented in the Materials and Methods section.
Fig. 2. The effect of the number of slow freeze-thaws on porphyrin production from porphobilinogen. A 1:540 dilution of disrupted red cells was incubated in a final volume of 1.5 ml Tris buffer (0.05 M, pH = 8.2) containing 10^{-4} M porphobilinogen for 1 hr at 37°C. The incubation was terminated by addition of 1.5 ml of 25% trichloroacetic acid, and the porphyrins were determined in a spectrophotofluorimeter as described in the Materials and Methods section.

Precipitants and Dilution Curves

The effect of various protein precipitants on the fluorescence observed in the supernatants was studied at various dilutions of the freeze-thawed red cells used in the incubation (Fig. 3). The graphs represent specific activity of enzyme as a function of enzyme dilution for each precipitant. It is seen that 25% trichloroacetic acid is superior to 6 N HCl and 2 N perchloric acid-ethanol (1:1) at low enzyme dilutions. At very high dilutions, however, perchloric acid-ethanol is as good as trichloroacetic acid. From Fig. 3 it can be seen that, in
contrast to perchloric acid–ethanol, trichloroacetic acid can be used over a wide range of enzyme dilutions.

$pH$

Maximal activity in conversion of PBG to porphyrins occurs at $pH = 8.2$ as seen in Fig. 4.

Kinetics of Conversion of PBG to Porphyrin

The rate of conversion of PBG to porphyrins in a 1:540 dilution of both normal and acute intermittent porphyric red cells was studied at various concentrations of PBG. Lineweaver–Burke plots of these data, as determined by the method of least squares, are presented in Fig. 5. The $V_m$ of the porphyric patient (20.58 nmoles porphyrin production/ml RBC/hr) is very close to one-half that of the normal (38.79 nmoles porphyrin production/ml RBC/hr), whereas the $K_M$ values for the two are virtually identical ($1.37 \times 10^{-5} M$ and $1.39 \times 10^{-5} M$).

Linearity With Time

Using a dilution of disrupted red cells of 1:540 and a PBG concentration of $10^{-4} M$, porphyrin production was linear with time for over 3 hr (Fig. 6).

Method

Consideration of the above findings led to the development of the following simplified procedure for the measurement of conversion of PBG to porphyrins.

Blood is added to a heparinized tube, centrifuged, and after removal of the plasma and buffy coat, the packed red cells are washed twice with cold isotonic saline. The cells are then frozen and thawed five times, freezing in dry ice-acetone and thawing at room temperature. One-tenth milliliter of the disrupted red cells is then added to 17.9 ml of Tris buffer $0.05 M \text{pH } 8.2$. One-half milli-
Fig. 5. Lineweaver-Burke plots, as determined by the method of least squares, for the conversion of porphobilinogen to porphyrins by normal and acute intermittent porphyric red cell enzymes. Except for the varying substrate concentrations, incubations were performed as described in Fig. 2 with five slow freeze-thaws.

A liter of this enzyme preparation is added to 0.95 ml of Tris buffer (0.05 M pH = 8.2) and 0.05 ml of 3 mM porphobilinogen. This produces a final dilution of red cells of 1:540 and a final concentration of $10^{-4} M$ PBG in a final incubation volume of 1.5 ml. This mixture was incubated at 37°C for 1 hr in the dark in a Dubnoff incubator with shaking. At the end of the incubation, 1.5 ml of 25% trichloroacetic acid is added, the precipitate is removed by centrifugation, and the porphyrin content of the supernatant is determined fluorometrically against a coproporphyrin standard in 12.5% trichloroacetic acid. The data presented in this communication were obtained using a Turner Model 430 spectrophotofluorimeter as described in Materials and Methods. Tissue blanks were run in two ways: (1) boiled enzyme incubated to which trichloroacetic acid is added.
at the end of the incubation, and (2) boiled enzyme to which trichloroacetic acid is added prior to incubation. In both instances, blank values were usually less than 10% of "live" values. Although both types of blank gave similar values, the former were actually subtracted from "live" values for calculation of enzyme activity. All "live" determinations were performed in triplicate.

It was shown that the above method, using trichloroacetic acid as a precipitant, removes heme, and thus prevents quenching of fluorescence caused by heme. When heme was added at concentrations of $10^{-6} M$, $10^{-5} M$ and $5 \times 10^{-5} M$ at the end of the incubation but prior to addition of trichloroacetic acid, the readings in the trichloroacetic acid supernatant were almost identical to a sample to which no exogenous heme had been added. Recovery of coproporphyrin was 92%–95% and recovery of uroporphyrin was 90% when 0.085 μg of the respective porphyrins were added to the incubation mixture without PBG and the incubation performed as usual. Reproducibility on samples of the same blood which were run independently from the point of initial centrifugation was ±5% of the mean value. All results are expressed in terms of coproporphyrin. To express results in terms of uroporphyrin, the coproporphyrin values should be multiplied by the factor 1.09, which can be derived from the data of Figs. 1A and 1B.

**Decay Curve**

The stability of the enzyme system is indicated in Fig. 7 where the conversion of PBG to porphyrins was measured in red cells which were initially frozen in dry ice-acetone and then stored at -4°C for varying periods up to and beyond 1 mo. There is no significant decline in enzyme activity for periods of at least 1 mo, but some loss of activity is evident at 49 days of storage.

**Normal Versus Porphyric Patients**

The level of the enzyme activity for conversion of PBG to porphyrins was measured by the above method in a series of normals and patients with various types of porphyria, and is presented as enzyme activity per milliliter of red
cells and also per milligram of protein in Fig. 8. The measurement was also performed on ten children (aged 12-18) with a parent known to have acute intermittent porphyria. The values obtained in the nine patients with acute intermittent porphyria do not overlap with the 28 normal values or with the values obtained in a small group of patients with variegate porphyria, porphyria cutanea tarda, or erythropoietic protoporphyria. The mean value in the patients with acute intermittent porphyria was 68.0 pmoles porphyrin production/mg protein/hr (17.6 nmoles porphyrin production/ml RBC/hr), approximately one-half of the normal mean of 151.4 pmoles porphyrin production/mg protein/hr (40.7 nmoles porphyrin production/ml RBC/hr). In the ten offspring of patients with known acute intermittent porphyria, four were in the normal range, five were in the acute intermittent porphyric range, and one was in the "gray area," i.e., the zone between the acute intermittent porphyric and normal ranges. In all of the offspring with a parent known to have acute intermittent porphyria, quantitative determination of urinary precursors had been performed, five at the time of the enzyme study and the other five previously. In no instances were elevated values of porphyrin precursors found in this group.
Effect of Heat on Enzyme Preparation

Two experiments were performed to determine the effect of heating the enzyme preparation at 60°C for periods of 3, 5, 7, 9, 11, 13, and 15 min, prior to performing the incubations for measurement of conversion of PBG to porphyrin as described above. In both experiments, the activity was measured using red cells from a normal and a patient with acute intermittent porphyria. A slight decline in total porphyrin production was evident in both experiments after 9 min of heating. Although there was some variation observed in the ratio of normal to porphyric activity with varying periods of heating in the second experiment, both experiments clearly show no progressive change in this ratio for periods of heating up to 15 min. In the first experiment, the ratio remained essentially constant through 13 min of heating.

DISCUSSION

Because acute intermittent porphyria in its most active form is so devastating and because many attacks of the disease can be prevented by avoidance of known precipitating factors, the early diagnosis of this disease is of great importance to afflicted individuals. In the past, it has been necessary to depend on the demonstration of increased amounts of urinary porphobilinogen, by either the Watson–Schwartz test or quantitative chromatographic methods. In surveys of individuals genetically related to patients with acute intermittent porphyria, quantitative measurements of urinary porphobilinogen demonstrated increased levels at very close to the expected frequency. These findings suggested that the quantitative measurement of urinary PBG would be highly efficient in detecting individuals bearing the defect of acute intermittent porphyria. However, recent studies have demonstrated that decreased conversion of PBG to porphyrins in red cells can exist with normal urinary PBG levels. Furthermore, one such individual experienced a classic attack of acute intermittent porphyria associated with excessive urinary PBG excretion. This means that even the quantitative urinary measurement of PBG is not infallible in detecting this disease in asymptomatic patients. The ability of the enzyme method utilizing red cells to detect the latent defect is obviously superior to the urine method, but also is not entirely infallible, as evidenced by the gray area of occasional overlap between normal and porphyric values. The present studies indicate, however, that the problem of the gray area is relatively minor since only one out of a total of 53 observations occurred in this zone. Thus, the present test completely separated the nine patients known to have acute intermittent porphyria from 28 normals with no overlap in the two groups (Fig. 8). Likewise, patients with other types of hepatic and erythropoietic porphyria were clearly separated from those with acute intermittent porphyria by the test. Furthermore, when ten offspring of patients with acute intermittent porphyria were tested, four were found to be in the normal range, five in the porphyric range, and one in the gray area between the above two ranges. That these values closely approach the predicted distribution for a mendelian dominant defect again indicates the value of the test, particularly in view of the fact that none of these ten individuals had increased porphobilinogen in the urine.

The present studies show that while the final method is relatively simple, a
number of details must be elucidated before optimal activity can be obtained. For example, five freeze-thaws of the red cells were found to be superior to other methods of red cell disruption (Table 1 and Fig. 2). Also of considerable importance is the method utilized to remove heme from the final porphyrin solution, since heme is known to quench porphyrin fluorescence.\textsuperscript{12,16} This can be done in two ways: (1) dilution of the enzyme preparation to the point where heme concentrations no longer quench, or (2) use of a protein precipitant which also removes heme. In a previously published micro method,\textsuperscript{12} a total incubation volume of 0.15 ml and a red cell dilution of 1:60 along with perchloric acid–ethanol as a protein precipitant were used. When that procedure is scaled up to a total incubation volume of 1.5 ml, the present data (Fig. 3) show that this combination of a 1:60 red cell dilution and perchloric acid–ethanol protein precipitant gives very low (sometimes undetectable) values of enzyme activity. This presumably results from quenching caused by heme which is not removed in sufficient amount. However, with increasing dilution, the activity per unit enzyme rises progressively until it finally equals the activity seen at various enzyme dilutions with trichloroacetic acid as a precipitant (Fig. 3). The progressive rise in specific activity with dilution of the system employing perchloric acid–ethanol as the precipitant results from the fact that quenching produced by heme (initial concentration $10^{-4} \text{M}$ or greater) declines more rapidly with dilution than does enzyme activity. The fact that activity per unit enzyme is essentially constant at various dilutions of red cells when trichloroacetic acid is used as a precipitant indicates that this agent is removing heme and thereby preventing quenching from the heme. This was further substantiated by addition of various concentrations of heme to incubation mixtures prior to addition of trichloroacetic acid. Since heme quenching of porphyrin fluorescence was not observed in any of the samples to which heme was added, it appears that trichloroacetic acid precipitates all or most of the heme. Furthermore, these conclusions are supported by direct visual observation that supernatants of trichloroacetic acid precipitants were completely clear, whereas those derived from perchloric acid–ethanol were colored.

As expected, comparison of a Lineweaver–Burke plot of the enzyme activity of a normal and porphyric individual indicated identical $K_M$’s, and $V_m$’s which differed by a factor close to 2 (Fig. 5). The $K_M$’s of this system are close to those previously reported in fowl erythrocytes for over-all conversion of porphobilinogen to porphyrins.\textsuperscript{20} Likewise, the $V_m$ values obtained in the present study (mean normal = 151.4 pmoles porphyrin production/mg protein/hr or 40.7 nmoles porphyrin production/ml RBC/hr) are close to those reported by Sassa et al.\textsuperscript{13} (mean normal, 36.4 nmoles porphyrin production/ml RBC/hr) and by a new method of Meyer\textsuperscript{14} (mean normal, 134.2 pmoles porphyrin production/mg protein/hr). However, the presently reported $K_M$ of $1.39 \times 10^{-5} \text{M}$ is more than twice that reported in the method of Strand et al. for the human red cell system.\textsuperscript{12} And the $V_m$ for normal red cells in the present study is almost five times the level reported by Strand et al.\textsuperscript{12} for normal red cells (34 pmoles porphyrin production/mg protein/hr).

The diagnostic value of the enzymatic method is even further enhanced by the fact that the enzyme system is quite stable in red cells stored in the frozen
state (Fig. 7). There appears to be little loss of enzyme activity even up to 1 mo of storage, but significant decreases in activity (16%) are evident at 49 days of storage (Fig. 7).

Although there are two enzymes involved in the overall conversion of PBG to porphyrins (uroporphyrinogen I synthetase and uroporphyrinogen III cosynthetase), there are multiple pieces of evidence which indicate that the observed decreases in the overall rate in patients with acute intermittent porphyria result from a decrease in the level of uroporphyrinogen I synthetase. First is the fact that a decrease in the cosynthetase would lead to greatly increased excretion of uroporphyrin I, a finding which is not seen in acute intermittent porphyria, and which is characteristic of congenital erythropoietic porphyria, where diminished levels of the cosynthetase have been implicated. Second, as the present results indicate, is the fact that the difference in rate of conversion of PBG to porphyrin in porphyric versus normal red cells is demonstrable in heated preparations in which the cosynthetase is known to be inactivated, and the uroporphyrinogen I synthetase is essentially intact. In these preparations heated for varying periods of time, there was no significant change in the ratios of normal to porphyric activity. Furthermore, Strand et al. have presented evidence indicating that the method measures the level of uroporphyrinogen I synthetase even in the presence of uroporphyrinogen III cosynthetase and uroporphyrinogen decarboxylase. The present studies show that for the diagnosis of acute intermittent porphyria, there is no advantage gained by attempting to measure the uroporphyrinogen I synthetase alone, obtained by heat inactivation of the cosynthetase.

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