Porphyrin Synthesis and Heme Synthetase Activity in Pyridoxine-Responsive Anemia

By William Ralph Vogler and Elizabeth S. Mincioli

The rate of heme synthesis was found to be reduced in a patient with pyridoxine-responsive anemia (PRA). The incubation of reticulocytes with glycine-2-14C or δ-aminolevulinic acid-4-14C (ALA) resulted in labeling of heme derived from hemoglobin. PRA reticulocytes incorporated glycine into heme at 11-25 per cent of the normal rate. ALA incorporation varied from 46 to 81 per cent of normal. These findings suggested that the patient with PRA had a major enzymatic defect at an early stage of heme synthesis prior to the formation of ALA, probably in ALA synthetase. A second block was suggested by the small, but consistent, reduction in the rate of heme formation from ALA. The further investigation of this latter observation is the subject of this report.

Methods

Measurement of Porphyrin Synthesis

Preparation of hemolysates. All manipulations were carried out at 0-5 C. Erythrocytes were collected by phlebotomy into a chilled cylinder containing heparin, and the cells were washed twice in isotonic saline. The cells were filtered through glass wool to remove clumped leukocytes and resuspended to their original volume in 0.05 M sodium phosphate buffer pH 7.4 containing 0.734 per cent KCl (isotonic buffer). Aliquots were removed for erythrocyte counts, and a known volume of the remainder centrifuged. The packed cells were hemolyzed by the addition of 3 volumes of 5 mM MgCl₂ for 10 minutes at 0 C. To restore isotonicity, 1.5 M sucrose in 0.15 M KCl was added to equal one-fifth of the volume of the MgCl₂ solution. The total volume of hemolysate was measured, and an amount corresponding to 10-20 ml of washed cell suspension was used in each incubation flask.

Incubations. The incubation mixture contained hemolysate, 20 μg ferrous iron in the form of ferrous ammonium sulphate, 5 ml 0.05 M isotonic buffer and unless otherwise specified, a final concentration of 1.25 mM ALA. Incubations were carried out in 50 ml Erlenmeyer flasks at 37 C. in the dark with slow rotary shaking for up to 2 hours. To stop the reaction, the samples were chilled in ice and added to 140 ml 4:1 ethyl acetate:glacial acetic acid mixture. All ethyl acetate used here and below was saturated with dl-sodium ethylenediamine tetraacetate (EDTA).

Isolation and assay of porphyrins. All steps were conducted under subdued incandescent light. After standing overnight in the ethyl acetate:glacial acetic acid mixture, the protein was removed by filtration, and porphyrins were extracted from the filtrate. Uroporphyrin was isolated by the method of Dresel and Falk. Coproporphyrin and protoporphyrin were then isolated by the method of Wranne. Assay of porphyrins was carried out by measurements.

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of absorbance in the Soret region using a Beckman DU spectrophotometer. The correction factor of With⁴ was found to be inadequate and new factors were determined.⁵ Equations used were:

\[
\begin{align*}
\text{Uro } A_{405} &= 1.306 A_{405} - 0.511 A_{396} - 0.695 A_{380} \\
\text{Copro } A_{401} &= 1.70 A_{401} - 0.948 A_{396} - 0.897 A_{380} \\
\text{Proto } A_{417} &= 1.563 A_{417} - 0.685 A_{390} - 0.603 A_{380}
\end{align*}
\]

**Free Erythrocyte Porphyrins**

Free erythrocyte uroporphyrin, coproporphyrin and protoporphyrin were determined as described above. Volumes of 80–160 ml. of whole blood were necessary to obtain detectable amounts of porphyrin.

**Heme Synthetase Assay**

_Heme synthetase preparation._ All manipulations were carried out at 0–5 C. Approximately 200 ml. of blood were collected in a chilled vessel containing 10,000 units of heparin. The cells were washed twice in isotonic saline and then filtered through glass wool to remove clumped leukocytes. The filtered cell suspension was reconstituted to its original volume in saline. An aliquot was removed for erythrocyte and reticulocyte counts. The remaining 190–200 ml. of suspension was centrifuged, and the packed cells were hemolyzed with 190–200 ml. ice cold distilled water for 30 minutes with occasional agitation. Isotonicity was restored by the addition of 19–20 ml. (0.1 water volume) 11.5 per cent KCl. The hemolysate was centrifuged 30 minutes at 37,000 × g. The hemolysate supernatant was decanted, and the very lightly packed unwashed sediment was designated “particulate fraction.”

_Incubation._ Unless otherwise specified, the 20 ml. reaction mixture contained 1 ml. 1 M tris buffer pH 7.6, 1 ml. 120 mM reduced glutathione (GSH), 0.5 ml. 1 per cent sodium deoxycholate, 0.2 μmoles protoporphyrin IX prepared by the method of Grinstein⁶ and Schwartz,⁷ 3–4 μcuries of ⁵⁹Fe ferrous citrate (Ferrutope, Squibb) plus enough unlabeled ferrous ammonium sulphate to equal 0.5 μmoles ferrous iron, particulate fraction corresponding to 20 ml. cell suspension, and 1.15 per cent KCl to bring to volume. Final substrate concentration was 2.5 × 10⁻⁴ M ferrous iron and 1.0 × 10⁻⁵ M protoporphyrin. Incubations were carried out in 50 ml. Erlenmeyer flasks at 37 C. in air in the dark with slow rotary agitation. After 30 to 60 minutes, the reaction was stopped by chilling, and enough cold hemolysate supernatant was added, if necessary, to achieve a total hemoglobin content of 0.8 to 1.2 Gm.

_Isolation and purification of hemin._ After incubation, the chilled mixture was added to 200 ml. of 4:1 ethyl acetate: glacial acetic acid mixture. Porphyrins and iron were repeatedly extracted by the methods mentioned above.²⁻³ The hemin remaining in the ethyl acetate layer was crystallized by evaporation to a small volume in vacuo, after the addition of a few crystals of sodium chloride. Hemin and sodium chloride crystals were centrifuged from the residue and washed twice with 5 ml. glacial acetic acid. Hemin was recrystallized once by the method of Shemin.⁸ Further recrystallization of the product resulted in no change in specific activity.

_Determination of specific activity of ⁵⁹Fe hemin._ Approximately half of the hemin isolated was dissolved in 2.1 ml. pyridine. For assay, 0.1 ml. was removed and determined as previously described.¹ The remaining 2 ml. was counted in a well type scintillation counter, and the specific activity (counts per minute per μmole of hemin) was corrected for the amount of carrier hemoglobin, the specific activity of the ⁵⁹Fe substrate counted at the same time and, except when the effect of reticulocytes was being measured, the number of reticulocytes per reaction mixture.

\[
SA = \frac{\text{CPM/μmole hemin} \times \text{mg. hemoglobin}}{\text{CPM}/10^{-15} \text{μmole Fe}^{2+} \times \text{no. reticulocytes} \times 10^{-15}}
\]
PORPHYRIN SYNTHESIS

**Measurement of Hemoglobin, Reticulocytes and Erythrocytes**

Hemoglobin was determined by the cyanmethemoglobin method of Crosby et al.\(^9\) Erythrocytes were counted in a Coulter counter, and reticulocytes were counted as previously reported.\(^1\)

**CLINICAL SUMMARY**

The patient under investigation is a white male born in 1929 and briefly reported elsewhere.\(^1\) He had been anemic since infancy. Two older brothers died of complications of iron overload secondary to pyridoxine-responsive anemia. The patient had been treated with pyridoxine since 1949. With treatment hemoglobin concentration averaged 11 Gm. per cent. Despite therapy, his red cells remained hypochromic and microcytic. Serum iron was elevated and liver biopsy in 1962 showed hemochromatosis. Phlebotomies were initiated in March 1962 and continued at intervals of every two to four weeks until October 1967. Deferoxamine B,\(^*\) 500 mg. daily, was given from May 1964 to December 1966. By October 1967 his serum iron fell to normal levels. It was calculated that an excess of 30 Gm. of iron were removed over the interval. Only during the last year when the serum iron began to fall was there an increase in hemoglobin concentration to 12 Gm. per cent despite continued phlebotomies. After phlebotomies were stopped the patient’s hemoglobin rose to 13 Gm. per cent.

Control subjects were normal volunteers. Most of the studies were performed on samples from a white male volunteer of identical age as the patient who had normal blood counts, marrow and serum iron. Selected control experiments were repeated with other normal volunteers and were similar to the age-matched control.

**RESULTS**

**Free Erythrocyte Porphyrins**

Free erythrocyte porphyrins were measured on two occasions in the patient’s erythrocytes. Measurements, expressed as \(\mu\)g. per 100 ml. of packed red cells, were as follows: No uroporphyrin detected; coproporphyrin 0.685 and 3.12 (normal range 0.3 to 2.3)\(^8\); protoporphyrin 11.95 and 11.00 (normal range 16–52).\(^3\)

**Porphyrin Synthesis**

The effect of ALA concentration on the formation of porphyrins in normal and PRA red cell hemolysates was determined. Approximately 1.25 mM ALA is optimal for both, and this concentration was used in all subsequent experiments. Further studies indicated that porphyrin synthesis in the normal was only partly dependent on the number of reticulocytes present. After centrifuging washed cells, reticulocytes are known to be concentrated in the upper layer.\(^10\) Mixtures of middle and top fractions of cells gave varying concentrations of reticulocytes. As shown in Table 1, in the normal there was less than a two-fold increase in porphyrin synthesis with a three-fold increase

\(^*\)Kindly supplied by Ciba Pharmaceutical Company.
in the number of reticulocytes. In PRA no difference could be detected over a four-fold range of reticulocyte concentration. Therefore, the data were corrected for the number of erythrocytes from which each hemolysate was derived.

The slopes from two experiments with normal and PRA hemolysates are listed in Table 2. No consistently significant differences were observed between PRA and normal. Protoporphyrin synthesis apparently proceeded at approximately half the rate of uroporphyrin and coproporphyrin synthesis.

**Heme Synthetase Activity**

Early experiments (Fig. 1) indicated that normal particulate fractions from hemolysates incorporated radioiron into heme. Incorporation was linear for 60 minutes. As shown in Table 3, hemolysate supernatant (HS) had negligible activity. Omission of protoporphyrin resulted in essentially no incorporation.

PRA particulate preparations incorporated consistently less radioiron than the normal under identical conditions. Pyridoxal phosphate at concentrations of $5 \times 10^{-4}$M inhibited activity by 50 per cent. Deoxycholate increased activity by 50 per cent. Glutathione or cysteine enhanced activity by an additional

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**Table 1.—Effect of Reticulocyte Concentration on Porphyrin Synthesis by Red Cell Hemolysates**

<table>
<thead>
<tr>
<th></th>
<th>Reticulocytes</th>
<th>Erythrocytes</th>
<th>$\mu$moles</th>
<th>porphyrin/90 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.226 x $10^9$</td>
<td>4.89 x $10^{10}$</td>
<td>21.7</td>
<td>25.3</td>
</tr>
<tr>
<td></td>
<td>0.461 x $10^9$</td>
<td>4.71 x $10^{10}$</td>
<td>26.0</td>
<td>27.3</td>
</tr>
<tr>
<td></td>
<td>0.658 x $10^9$</td>
<td>4.52 x $10^{10}$</td>
<td>33.5</td>
<td>32.0</td>
</tr>
<tr>
<td></td>
<td>0.854 x $10^9$</td>
<td>4.34 x $10^{10}$</td>
<td>39.8</td>
<td>35.8</td>
</tr>
<tr>
<td>PRA</td>
<td>0.399 x $10^9$</td>
<td>4.01 x $10^{10}$</td>
<td>29.9</td>
<td>35.6</td>
</tr>
<tr>
<td></td>
<td>0.862 x $10^9$</td>
<td>4.35 x $10^{10}$</td>
<td>33.7</td>
<td>36.9</td>
</tr>
<tr>
<td></td>
<td>1.326 x $10^9$</td>
<td>4.70 x $10^{10}$</td>
<td>37.0</td>
<td>37.4</td>
</tr>
<tr>
<td></td>
<td>1.780 x $10^9$</td>
<td>5.04 x $10^{10}$</td>
<td>39.5</td>
<td>39.4</td>
</tr>
</tbody>
</table>

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**Table 2.—Rate of Incorporation of ALA into Porphyrins by Red Cell Hemolysates**

<table>
<thead>
<tr>
<th>Expt. #</th>
<th>Porphyrin</th>
<th>$\mu$mole/10$^9$ RBC/hr.</th>
<th>Normal</th>
<th>Patient</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Uroporphyrin</td>
<td>3.15 ± 0.15*</td>
<td>2.23 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>3.55 ± 0.39</td>
<td>4.19 ± 0.30</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>3.84 ± 1.08</td>
<td>5.12 ± 0.22</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Coproporphyrin</td>
<td>4.53 ± 1.49</td>
<td>3.73 ± 0.40</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>4.44 ± 0.40</td>
<td>4.61 ± 0.16</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>4.15 ± 0.20</td>
<td>5.25 ± 0.37</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Protoporphyrin</td>
<td>1.26 ± 0.04</td>
<td>1.15 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>1.66 ± 0.24</td>
<td>1.49 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>2.59 ± 0.16</td>
<td>2.83 ± 0.21</td>
<td></td>
</tr>
</tbody>
</table>

*One standard deviation.
EFFECT OF TIME ON HEME SYNTHETASE ACTIVITY

![Graph showing the effect of time on heme synthetase activity.]

**Fig 1.**—Effect of time on heme synthetase activity. Conditions: Hemolysate particulate fractions corresponding to 20 ml. cell suspension were incubated at 37°C in 0.05 M tris buffer pH 8, 1 mM cysteine, 0.07 mM protoporphyrin and $4.9 \times 10^{-4}$m radioiron. Experiments carried out in duplicate.

10 to 20 per cent. The enzyme system had a broad pH optimum between 7.6 and 8.0 for both normal and PRA particulate fractions.

The effect of substrate concentration on heme synthesis activity was determined using the method of Lineweaver and Burk. The concentration of each substrate was varied from 0.2-15 $\times 10^{-5}$M. As shown in Table 4, the Michaelis constants for the patient and normal were similar. However, PRA preparations had 56 per cent of normal activity.

Enzyme activity was found to be linearly related to the number of reticulocytes and at similar reticulocyte concentrations the activity (under optimal conditions) of PRA preparations was significantly less than normal ($p < .01$) (Table 5). However, statistically significant improvement in heme synthetase activity was observed in PRA preparations. As shown in Table 5, the activity more than doubled over a 12 month period. During this interval the patient was continually under treatment with phlebotomies at intervals of every two to three weeks. Deferoxamine B 500 mg. daily was given until March 1967. Pyridoxine, 50 mg. daily, was continued throughout the study. Over a four and one-half year period preceding September 1966, 27.8 Gm. of

### Table 3.—Effect of Hemolysate Fractions and Protoporphyrin on Heme Synthetase Activity

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Date</th>
<th>Hemolysate Fraction</th>
<th>Omissions</th>
<th>Sp. Act. Hemin (SA$_v$/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Normal</td>
<td>PRA</td>
</tr>
<tr>
<td>1</td>
<td>June 1966</td>
<td>P</td>
<td>None</td>
<td>572</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P</td>
<td>Protoporphyrin</td>
<td>40</td>
</tr>
<tr>
<td>2</td>
<td>Sept. 1966</td>
<td>HS</td>
<td>None</td>
<td>648</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>None</td>
<td>34</td>
</tr>
</tbody>
</table>

*Conditions: Hemolysate fractions corresponding to 20 ml cell suspension were incubated 1 hr. in the presence of $1.0 \times 10^{-4}$M protoporphyrin, $1 \times 10^{-2}$M Fe, 0.05M tris pH 7.6, 0.025% sodium deoxycholate. Expt. #1 contained 1mM cysteine and Expt. #2 contained 6mM reduced glutathione.

*Abbreviations: P = particulate fraction, HS = hemolysate supernatant*
Incubated under standard conditions for 60 minutes.

Slopes obtained by varying the concentration of reticulocytes.

### Table 4.—Heme Synthetase Kinetics in Particulate Fraction from Normal and PRA Red Cell Hemolysates

<table>
<thead>
<tr>
<th></th>
<th>Km (x 10^-6 M)</th>
<th>Vmax (S.A.μ/hr.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal Patient</td>
<td>Normal Patient</td>
</tr>
<tr>
<td>Protoporphyrin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.62</td>
<td>6.24</td>
<td>769</td>
</tr>
<tr>
<td>Ferrous iron</td>
<td>7.13</td>
<td>7.26</td>
</tr>
</tbody>
</table>

Protoporphyrin concentration was varied in the presence of 2.5 x 10^-2 M Fe++. Fe++ concentration was varied in the presence of 1.0 x 10^-3 M protoporphyrin.

Iron had been removed. A calculated 3.2 Gm. of iron were removed during the following year. Despite the phlebotomies, the hemoglobin value increased from 9.9 to 11.9 Gm. per cent and hematocrit from 34.5 to 39 per cent. Serum iron fell from 298 to 36 μg. per cent. After phlebotomies were stopped in October 1967, the hemoglobin increased to 13 Gm. per cent and hematocrit to 42 per cent. These values were higher than any previously obtained prior to phlebotomies.

### Heme Synthesis by Intact Reticulocytes

Since there was improvement in heme synthetase activity by PRA hemolysates after reduction of iron stores, it would be expected that incorporation of ALA-4-C would improve also. Therefore, the previously reported experiments with intact reticulocytes were repeated. In Table 6 experiments 1–3 were performed in 1964 when the patient's serum iron was high. Despite an unexplained variation in activity in these earlier studies, ALA incorporation in PRA was 46 to 81 per cent of normal. In two recent experiments following reduction in iron stores, the patient's ALA incorporation equalled the normal rate. Glycine incorporation was not significantly altered.

### Discussion

The level of free erythrocyte coproporphyrin (FEC) was within normal limits. That of free erythrocyte protoporphyrin (FEP) was below normal. Ret-

### Table 5.—Heme Synthetase Activity per billion reticulocytes in Normal and PRA Particulate Fractions. Correlation of Increasing Activity with Reduction in Iron Stores and Increasing Hemoglobin

<table>
<thead>
<tr>
<th>Date</th>
<th>Iron Store Reduction Gm.</th>
<th>Serum Iron μg.%</th>
<th>Hemoglobin Gm.%</th>
<th>SA/10^8 Reticulocytes (Regression coefficient)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRA</td>
<td>9-66</td>
<td>27.8</td>
<td>289</td>
<td>9.9</td>
</tr>
<tr>
<td></td>
<td>6-67</td>
<td>30.2</td>
<td>31</td>
<td>11.7</td>
</tr>
<tr>
<td></td>
<td>9-67</td>
<td>31</td>
<td>36</td>
<td>10.9</td>
</tr>
<tr>
<td></td>
<td>3-68</td>
<td>31.3</td>
<td>124</td>
<td>13.0</td>
</tr>
<tr>
<td>Normal</td>
<td>7-66</td>
<td>--</td>
<td>--</td>
<td>14.6</td>
</tr>
<tr>
<td></td>
<td>5-67</td>
<td>--</td>
<td>117</td>
<td>948 ± 27.5</td>
</tr>
</tbody>
</table>

In incubated under standard conditions for 60 minutes. Slopes obtained by varying the concentration of reticulocytes.
duced FEP has been observed in other patients with PRA.\textsuperscript{12} After administration of pyridoxine, a moderate increase in FEP has been noted in some patients. Our patient was receiving pyridoxine at the time the determinations were made. Despite this, FEP was below the normal range. This is consistent with a block in an early step of heme synthesis and is in contrast to a patient reported by Lee et al.\textsuperscript{13} with sideroachrestic anemia who had an increase in FEP from 300 to 2400 µg./100 ml. packed red cells following the administration of pyridoxine. In their patient FEP fell when pyridoxine was discontinued. This supports the idea that pyridoxal phosphate serves as a cofactor of ALA synthetase, resulting in a large increase in porphyrin formation.\textsuperscript{14} Failure to observe these high levels in our patient with PRA is further evidence for diminished ALA synthetase activity.

Synthesis of uroporphyrinogen and coproporphyrinogen from ALA is known to take place in the soluble portion of mammalian reticulocytes.\textsuperscript{15} Although the synthesis of protoporphyrin from coproporphyrinogen has been shown to take place in the particulate fraction of liver cells\textsuperscript{16} and avian erythrocytes,\textsuperscript{17} protoporphyrin synthesis has also been shown to take place in the supernatant from hemolysed duck erythrocytes.\textsuperscript{18} Our findings of a lack of close correlation with reticulocyte count suggest that protoporphyrin synthesis does occur at least in part in the soluble fraction from human erythrocytes. These studies indicate that synthesis of porphyrins by soluble enzymes from ALA through protoporphyrin is normal in PRA.

These findings show that in human blood heme synthesis from protoporphyrin and iron is an enzyme-dependent reaction requiring a particulate fraction from reticulocytes. Schwartz et al.\textsuperscript{19} demonstrated that the particulate fraction of avian erythroid cells contained enzymatic activity, and they designated the enzyme, "heme synthetase." In their experiments supernatant from the hemolysate was inactive. Yoshikawa and Yoneyama\textsuperscript{20} solubilized the enzyme by extracting the hemoglobin-free particulate fraction of duck erythrocytes with cholate-tris buffer. Enzyme activity in our studies was enhanced by the addition of sodium deoxycholate, although the extent to which the enzyme was solubilized was not studied.

Bottomley\textsuperscript{21} reported heme synthetase activity in human bone marrow crude lysates prepared by freezing and thawing. The enzyme was heat labile, had optimal pH of 7.4, and had Km's for iron of $1.7 \times 10^{-5}$M and protoporphyrin of $0.18 \times 10^{-5}$M. Activity was enhanced by ascorbic acid and glutathione and was inhibited 50 per cent by pyridoxal phosphate ($1 \times 10^{-5}$M). Langelaan et al.\textsuperscript{22} reported from human blood Km's for protoporphyrin of $4 \times 10^{-5}$M and iron of $10^{-5}$. In our studies the Km for iron was $0.7 \times 10^{-5}$ which is similar to those mentioned above. The Km for protoporphyrin was $0.6 \times 10^{-5}$M. This is at variance with those reported above, but similar to the Km for iron. One would expect a molar ratio of 1 for the two substrates. Further purification of these crude preparations should reduce these differences in kinetic data.

Our data indicate that heme synthetase activity is reduced in PRA. Despite some experimental variation, activity was consistently below normal. Even
though significant improvement in heme synthetase activity occurred over a 12 month period, it remained subnormal. It is unlikely that these findings can be explained by differences in age of reticulocytes. Throughout the investigation reticulocyte counts remained constant and phlebotomies were carried out at regularly spaced intervals. Thus, the number of mitochondria per reticulocyte would be constant. In the crude preparations used, no method for direct measurement of mitochondria seemed feasible. One would expect that in the absence of an abnormality the frequent phlebotomies would result in younger reticulocytes and increased heme synthetase activity.

Excessive aggregates of iron, producing morphologic abnormalities of mitochondria, have been reported in patients with sideroachrestic anemias by Bessis.²³ In some instances improvement in hemoglobin has occurred with deferoxamine.²⁴ Unfortunately, no observations have been made on the electron microscopic appearance of reticulocytes from this patient. It is likely that excess iron stores are responsible for a portion of the decrease in heme synthetase activity. The patient’s hemoglobin has increased with reduction in iron stores. At the same time heme synthetase activity has improved. Incorporation of radioactive ALA by intact reticulocytes now falls within the normal range.

The kinetic data indicate that sufficient exogenous iron has been added in these studies to exclude the possibility that an increased amount of intracellular or intramitochondrial iron is responsible for a dilution effect on the isotope. If a difference existed in accessibility of exogenous iron to the enzyme, this would be reflected in a higher Fe⁺⁺ Km for PRA particles than normal. This was not the case. Furthermore, this defect was observed in intact red cells using ¹⁴C-ALA as substrate.¹

Since both ALA synthetase and heme synthetase are confined to particulate fractions of maturing red cells, primarily mitochondria, one might speculate that a secondary or coexisting mitochondrial abnormality is present. This can be partially overcome by the administration of pyridoxine which stimulates what little ALA synthetase is present. Although one report²⁵ has appeared indicating that pyridoxal phosphate is a necessary cofactor for the release of heme from mitochondria, to our knowledge this has not been confirmed. We can demonstrate no stimulatory effect. Our findings are similar to Bottomley’s²¹ in showing an apparent inhibitory effect of pyridoxal phosphate on heme synthetase activity. Furthermore, in the study by Lee et al.¹⁵ despite a very marked increase in free erythrocyte protoporphyrin after pyridoxine therapy, the patient with refractory anemia showed no improvement in hemoglobin levels.

The finding of improvement in heme synthetase activity with reduction of iron stores suggests that excess iron bound to mitochondria inhibits heme synthetase. This may explain why some patients with PRA respond more completely to pyridoxine than others.

**Summary**

Previous in vitro studies had shown that reticulocytes from a patient with
pyridoxine-responsive anemia (PRA) incorporated glycine-2-¹⁴C into heme at 11-25 per cent of the normal rate. Incorporation of the intermediate compound, δ-aminolevulinic acid-4-¹⁴C (ALA), varied from 46 to 81 per cent of normal. These findings suggested two defects in the synthesis of heme: a major one prior to formation of ALA, probably ALA synthetase, and a minor one somewhere between ALA and heme. This minor defect was investigated in a patient with PRA during a period of repeated phlebotomies resulting in a 30 gram reduction in iron stores. Porphyrin synthesis from ALA was measured in vitro and found to be normal. Heme synthetase activity in particular fractions of reticulocytes measured by radioiron incorporation into heme was 50 per cent less than normal when the iron stores were high and significantly improved when the iron stores were reduced to normal. Repeat studies showed that incorporation of ALA-4-¹⁴C into heme by intact reticulocytes equaled the normal rate. Glycine incorporation was still reduced. Concomitant with the improvement in heme synthetase activity was an improvement in anemia. These results suggest that excess iron is a significant cause of the reduced heme synthetase activity observed in this patient with pyridoxine-responsive anemia.

SUMMARİO IN INTERLINGUA
Previe studios in vitro ha demonstrate que le reticulocytos ab un patiente con anemia pyridoxino-responsive (APR) incorporava glycina-2-¹⁴C ad in heme a solo 11 a 25 pro cento del intensitate normal. Le incorporation del composito intermedie, acid-δ-aminolevulínico-4-¹⁴C (ALA), variava inter 46 e 81 pro cento del norma. Iste constatationes suggesionava duo defectos in le synthese de heme: Le un, de importantia major, es active ante le formation de ALA (e concern probablemente synthetase de ALA) e un altere, de significacion minor, es situate alicubi inter ALA e heme. Iste secunde defecto, i.e., le defecto minor, esseva investigate in un patiente con APR durante un periodo de repetite phlebotomias resultante in un reduction del reservas de ferro per 30 g. Le synthese de porphyrina ab ALA esseva mesurate in vitro, con le constatation que illo esseva normal. Le activitate de synthetase in fractiones particulate de reticulocytos mesurate a base del incorporation de radioferro ad in heme esseva 50 pro cento minus que le activitate normal quando le reservas de ferro esseva alte e se meliorava significativamente quando le reservas de ferro esseva reduce a nivels normal. Repetite studios demonstrava que le incorporation de ALA-4-¹⁴C ad in heme per reticulocytos intacte occurreva con un intensitate normal. Le incorporation de glycina remaneva reduce. In concomitantia con le melioration del activitate de synthetase in heme occurreva un melioration del anemia. Iste resultatos suggestiona que excessos de ferro es un causa significative del reduce activitate de synthetase in heme que es observate in le presente paciente con anemia pyridoxino-responsive.

REFERENCES


Porphyrin Synthesis and Heme Synthetase Activity in Pyridoxine-Responsive Anemia

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