Congenital Erythropoietic Protoporphyria

II. An Experimental Study

By F. Stanley Porter

Two brothers with erythropoietic protoporphyria were described in the preceding report.1 The findings in these cases and others2-5 indicate that this disease is an inborn error of metabolism characterized by increased bone marrow synthesis of what appears to be protoporphyrin. This certainly suggests some abnormality of porphyrin or heme metabolism. In an attempt to define this abnormality three possibilities were considered: (1) the protoporphyrin synthesized was not protoporphyrin IX but some other porphyrin incapable of forming heme; (2) there was a defect in heme synthesis per se; (3) a defect in the mechanism normally regulating protoporphyrin synthesis was present. This report is concerned with the investigation of these hypotheses utilizing material from the patients previously presented.1

Material and Methods

Porphyrin Isolation

G-Proto: The porphyrin from a portion of patient A. G.'s (Case #1) liver biopsy specimen obtained at the time of splenectomy was extracted into an ethyl acetate-glacial acetic acid mixture, esterified with a methanol-sulfuric acid solution and extracted into chloroform.6 The chloroform was evaporated in vacuo and the resulting material was applied to a Hyflo Super-Cell* chromatography column and the column developed with a 2 to 1 mixture of petroleum ether (B. P. 30-60 C.) and chloroform according to the method of Chu and Chu.7 Almost all of the porphyrin moved with the solvent front. This front-running band was cut from the column, the porphyrin eluted with chloroform, and rechromatographed as above, in this instance moving as a single band with the solvent front. This band was cut from the column and the porphyrin again eluted with chloroform and crystallized 3 times from a hot chloroform-methanol solution.8 Hereafter this crystalline material will be referred to as G-Proto.

Stool porphyrin: The porphyrin from an aliquot of A. G.'s stool collected prior to splenectomy was extracted, esterified, re-extracted into chloroform and dried as described above.

Urinary porphyrin: The porphyrin from an aliquot of A. G.'s urine collected prior to splenectomy was adsorbed on to talc.6 The talc was dried and the porphyrin extracted with a methanol sulfuric acid solution. After esterification the porphyrin was extracted into chloroform and the chloroform evaporated in vacuo.

N-Proto (protoporphyrin IX): This was prepared from human blood by the method...
of Grinstein,8 purified by chromatography and recrystallized to a constant melting point. Hereafter this purified normal protoporphyrin IX will be referred to as N-proto.

Coproporphyrin III: The methyl ester of coproporphyrin III* was purified by chromatography and recrystallized to a constant melting point.

Uroporphyrin: The porphyrin from the urine of a patient with acute intermittent porphyria was extracted and esterified as described above under urinary porphyrin. This porphyrin was purified by chromatography and crystallized. No attempt was made to identify the type of isomer present.

**Porphyrin Identification**

Melting points: The melting point of crystals of porphyrin methyl esters were determined with a Fisher-John's melting point apparatus.

Absorption spectra: Absorption spectra of free porphyrins in a 1.5N hydrochloric acid solution were determined in a Beckman DU spectrophotometer.

Fluorescence spectra: Fluorescence spectra of free porphyrins in a 1.5N hydrochloric acid solution were determined in a Farrand spectrophotofluorometer using a 405 mp wave length excitation light source.

Chromatography: Two-dimensional paper chromatograms of porphyrin methyl esters were performed according to the method of Chu, Green and Chu10 or by the method of Chu and Chu.11 The latter is recommended for the separation of dicarboxylic porphyrins. Chromatograms of the free porphyrins were obtained by the method of Nicholas and Rimington12 as modified by Chu and Chu.12

**Liver Heme Synthesis**

Using a modification of a method described by Lochhead and Goldberg,13 liver slices were incubated with normal human serum, saturated with Fe59 and free proto- porphyrin in the presence of a 15 mM concentration of reduced glutathione at pH 7.4 for 3 hours at 37 C. in a shaking water bath. After incubation, carrier heme in the form of a solution of human hemoglobin was added, the total hemin was isolated by the method of Fischer14 and recrystallized once. The crystals were weighed, their radioactivity determined and the specific activity in counts per minute per mg. of hemin calculated. Further recrystallization did not appreciably change the specific activity. The source of the livers used in the various experiments were: (1) the liver biopsy from patient A. C. obtained at the time of splenectomy (C-liver); (2) liver obtained at autopsy from a 2 year old child dying of pneumonia which is referred to as normal liver (N-liver); (3) liver obtained at surgery in a 6 year old child undergoing splenectomy which is referred to as fresh normal liver.

**Bone Marrow Porphyrin and Heme Synthesis**

Granick15 has described an in vitro system for measuring protoporphyrin synthesis in avian erythrocytes, and has determined the optimum concentrations of glycine and α-ketoglutaric acid as well as optimum pH, temperature and time of incubation for synthesis. With some modifications, this system was found to be adaptable to the measurement of porphyrin and heme synthesis in human bone marrow.

The following is a description of the method devised for incubating bone marrow aspirates for porphyrin and heme synthesis in vitro:

**Reagents:**

Substrate solution. Nine hundred and eighty one mg. of glycine and 272.8 mg. of α-ketoglutaric acid were dissolved in 10 ml. of .07 M phosphate buffer, pH 7.5, and neutralized carefully with 2 per cent NaOH and then made up to 20 ml. with the phosphate buffer. Six tenths ml. of this solution gave a final concentration of .07 M glycine and .01 M α-ketoglutaric acid in the final incubation mixture.

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*Obtained from the Mann Research Laboratories, New York, N. Y.
Sucrose-phosphate buffer. Twenty-five hundredths M sucrose in .07 M phosphate buffer, pH 7.5, with 0.4 mg. of Streptomycin/ml. added.

Reduced glutathione (G-SH). Seventy-three and five tenths mg. of G-SH were dissolved in 10 ml. of sucrose-phosphate buffer so that 1.4 ml. of this solution would give a G-SH concentration of 6 mM in the final incubation mixture.

Fe^{59} serum. The latent iron binding capacity of a quantity of normal human serum was determined. Sufficient Fe^{59} citrate was added to 5 ml. of this serum to saturate it and the mixture incubated for 30 minutes at 37 C. The total iron content was then determined and by measuring the radioactivity of an appropriate dilution, the specific activity of the Fe in cpm/moles Fe was calculated.

Bone marrow: Approximately 1.5 ml. of bone marrow was obtained from an iliac crest aspiration, and placed immediately into a graduated centrifuge tube containing 5 ml. of a 100 mg. per cent solution of CaCl_2 in 0.25 M sucrose and 5 mg. of heparin. The contents were thoroughly mixed, the cells separated in a refrigerated centrifuge, and washed twice with 10 ml. of cold 0.25 M sucrose. After the last wash, the supernatant was decanted leaving approximately 1.25 ml. of cellular material. This was thoroughly mixed and the total number of nucleated cells were counted using a red cell counting pipette. A smear of the material was also obtained and stained with Wright's stain and the percentage of nucleated erythroid cells determined. The bone marrow was then aspirated into a small incubation flask with a pasteur pipette, the volume being controlled by the graduations on the centrifuge tube. The residual marrow was washed out of the pipette with buffer.

Incubation. After adding the appropriate substrates to the bone marrow in the flasks, the volume was brought to 5.6 ml. with sucrose-buffer and the flasks incubated in a shaking water bath for 15 hours at 37 C.

Porphyрин determination. After incubation, the porphyrin content of each flask was determined in the same manner as erythrocyte porphyrin determinations except no attempt was made to differentiate the type of porphyrin present and the 3N hydrochloric acid extract of the ethyl-acetate layer was diluted 1 to 1 with water and the total porphyrin concentration determined fluorometrically. Knowing this concentration and the volume of marrow in the flask, the porphyrin concentration in g. per ml. of marrow could be calculated.

Hemin isolation and specific activity. After the porphyrins had been extracted from the ethyl-acetate layer, 100 mg. of crystalline hemin dissolved in pyridine was added as carrier. The hemin was then crystallized and recovered by the method recommended by Schwartz and coworkers. The crystals were washed successively with water, methanol and ether, re-dissolved in pyridine and chloroform and recrystallized from hot glacial acetic acid. The resulting crystalline hemin was washed again, dried, weighed and the radioactivity determined in a well-type sodium iodide crystal scintillation counter. The specific activity in cpm/mg. hemin was then calculated. Further recrystallization did not change the specific activity.

Avian Erythrocyte Porphyrin Synthesis

In the development of the method described above, experiments were carried out utilizing avian erythrocytes as a ready source of nucleated cells. In these experiments, washed avian erythrocytes were incubated with .07 M glycine and .01 M α-ketoglutaric acid in sufficient 0.25 M sucrose-.07 M phosphate buffer pH 7.5, to make a total volume of 5.6 ml. The incubation was carried out at 38 C. in a shaking water bath for 15 hours. The total porphyrin was then extracted and the concentration determined fluorometrically as above. When substances were added to the incubation mixture to determine their effect on porphyrin synthesis, the total volume was maintained at 5.6 ml. by appropriate reduction of the amount of sucrose-buffer solution added.
RESULTS

Porphyrin Identification

The crystals of the methyl esters of G-proto and N-proto had the same gross structure (fig. 1), and the same melting point of 223 °C. The free porphyrins had the same absorption spectra (fig. 2) and the same fluorescence spectra (fig. 3).

Paper chromatograms of various porphyrin methyl esters in different solvent systems are represented in figure 4. Figure 4A represents a chromatogram performed by the method of Chu, Green and Chu. It can be seen that the porphyrin extracted from the stool and the liver of patient A. G. had the same Rf value as did N-proto, and that from the urine the same as coproporphyrin III. A comparison of the behavior of N-proto and G-proto in the same solvent system is shown in figure 4B. A comparison of the same porphyrins in the

Fig. 1.—(A) Crystals of the methyl ester of G-proto. (B) Crystals of the methyl ester of N-proto.
Fig. 2.—Absorption spectrum; free porphyrin in 1.5N HCl.

Fig. 3.—Fluorescence spectrum.

solvent system recommended for the separation of dicarboxylic porphyrins\textsuperscript{10} is shown in figure 4C. In both these solvent systems, N-proto and G-proto have the same Rf value. An additional chromatogram was done that is not represented. This compared the free porphyrin isolated from the erythrocytes of patient M. C. (Case \#2\textsuperscript{1}) and free N-proto in the solvent system
Fig. 4.—Chromatography of porphyrin methyl esters. (A) Solvent system recommended by Chu, Green and Chu,¹⁰ 1, Coproporphyrin III; 2, N-proto (protoporphyrin IX); 3, uroporphyrin; 4, porphyrin isolated from patient A. G.'s urine; 5, porphyrin isolated from his stool; 6, porphyrin isolated from his liver. (B) Same solvent system as (A) comparing 1, N-proto, and 2, G-proto. (C) Solvent system recommended by Chu and Chu¹⁰ for the separation of dicarboxylic porphyrins comparing 1, N-proto, and 2, G-proto.

used for the separation of free porphyrins.¹¹,¹² The two porphyrins migrated as two single spots and had the same Rf value.

Liver Heme Synthesis

The purpose of these experiments was to determine (1) if G-proto were biologically active and could form heme, and (2) if patient A. G.'s liver could synthesize heme from iron and normal protoporphyrin IX.

To determine whether G-proto could form heme, normal liver slices were used in each incubation and the type of protoporphyrin was varied. Two flasks contained 0.16 μg. of free G-proto, two flasks 0.16 μg. of free N-proto, and two flasks no protoporphyrin. The results of this experiment are shown in table 1. It is apparent that the de novo heme synthesis, which is proportional to the cpm/mg. heme, is the same with G-proto as with N-proto. Little heme was synthesized in the control flasks to which no protoporphyrin had been added.

To determine whether the patient's liver could form heme, free N-proto was used in each incubation and the type of liver slices was varied. Two flasks contained 100 mg. of G-liver (liver from patient A. G.) slices, two flasks 100 mg. of N-liver (normal liver) slices, and two flasks 100 mg. of heat-killed liver which was prepared by placing 100 mg. of N-liver in a boiling water bath for 5 minutes. As shown in table 2, the de novo heme synthesis with G-liver is equivalent to that with N-liver, again with little heme being synthesized in the control. At the time this experiment was performed, the patient's liver had been frozen for 18 months. The normal liver used had been frozen for the same period of time. To determine the effects of storage in the frozen state, the heme-synthesizing ability of the normal liver frozen for 18 months was compared to that of fresh normal liver frozen overnight and was found to be essentially the same, as is shown in table 3. Apparently, once the liver is frozen, this activity does not deteriorate appreciably with storage.
Table 1.—Heme Synthesis

<table>
<thead>
<tr>
<th>Cpm/mg. heme</th>
<th>0.16 µg. G-Proto*</th>
<th>0.16 µg. N-Proto†</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>96</td>
<td>114</td>
<td>86</td>
</tr>
</tbody>
</table>

Each incubation flask contained 200 mg. of normal liver slices, 0.5 ml. of normal serum saturated with Fe59, 1.5 ml. of 1.84 per cent solution of reduced glutathione in 0.45 M tris buffer, pH 7.4, plus the type of protoporphyrin indicated.

*G-Proto = free porphyrin isolated from the liver of patient A. G.
†N-Proto = free normal protoporphyrin IX.

Table 2.—Heme Synthesis

<table>
<thead>
<tr>
<th>Cpm/mg. heme</th>
<th>100 mg. G-Liver*</th>
<th>100 mg. N-Liver†</th>
<th>100 mg. H.K. Liver‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>105</td>
<td>102</td>
<td>108</td>
</tr>
</tbody>
</table>

Each incubation flask contained 1 ml. of a 10 mg. per cent solution of normal free protoporphyrin in 0.14 M Na2CO3, 0.5 ml. of normal serum saturated with Fe59, 1.5 ml. of 1.84 per cent solution of reduced glutathione in 0.45 M tris buffer, pH 7.4, plus the type of liver indicated.

*G-Liver = liver from patient A. G.
†N-Liver = normal liver.
‡H.K. Liver = heat-killed normal liver.

Table 3.—Heme Synthesis

<table>
<thead>
<tr>
<th>Cpm/mg. heme</th>
<th>100 mg. Old Frozen Liver*</th>
<th>100 mg. Fresh Frozen Liver*</th>
<th>100 mg. H.K. Liver†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>83</td>
<td>104</td>
<td>4.7</td>
</tr>
</tbody>
</table>

Each incubation flask contained 1 ml. of a 10 mg. per cent solution of normal free protoporphyrin in 0.14 M Na2CO3, 0.5 ml. of normal serum saturated with Fe59, 1.5 ml. of 1.84 per cent solution of reduced glutathione in 0.45 M tris buffer, pH 7.4, plus the type of liver indicated.

*See text for explanation.
‡H.K. Liver = heat-killed liver.

Bone Marrow Porphyrin and Heme Synthesis

Bone marrow aspirations were obtained from patient A. G. 2 years post-splenectomy and patient M. G. at 4 years of age and incubated as described above. The quantity of marrow, substrate solution, Fe59 serum and G-SH are shown in table 4, as are the results of the incubation.

The µg. of porphyrin synthesized per 10⁶ erythroid cells in the marrow was calculated by subtracting the value obtained for µg. of porphyrin per ml. of marrow in the flask without substrates from that value obtained in the flask with substrates and dividing this number by the erythroid cells x 10⁶ per ml. of marrow. The value is given as µg. of porphyrin but was actually calculated as protoporphyrin (a correction factor of 1.25 was used in the fluorometric analysis⁸), and the molecular weight of protoporphyrin was used to convert µg. to moles.

The moles of heme synthesized x 10⁻¹² per 10⁶ erythroid cells was calculated by making the assumption that the weight of the heme synthesized is negligible in comparison to the weight of carrier hemin added during the
recovery procedure. Therefore, multiplying the specific activity of the hemin by the mg. of carrier hemin added and dividing by the specific activity of the Fe, the number of moles of Fe originating from the Fe59 serum solution that had been incorporated into heme then results. Since iron is incorporated into heme mole for mole, this is then equal to the moles of heme synthesized from the exogenous iron added to the incubation, and dividing this by the number of erythroid cells x \(10^6\) present in the incubation sample gives the final result. There is undoubtedly some additional heme synthesized from iron already present in the marrow cells, but this is not measured by this method.

From table 4 it can be seen that A. G.'s marrow synthesized \(228 \times 10^{-12}\) moles of porphyrin per \(10^6\) erythroid cells, and M. G.'s marrow \(420 \times 10^{-12}\). A. G.'s marrow synthesized \(0.702 \times 10^{-12}\) moles of heme per \(10^6\) erythroid cells in a single sample, and M. G.'s marrow \(3.67 \times 10^{-12}\) and \(1.47 \times 10^{-12}\) in two samples, respectively. For comparison, the results of incubating the marrow from a patient with sickle cell disease in an identical manner are \(24.6 \times 10^{-12}\) moles of porphyrin per \(10^6\) erythroid cells and \(3.36 \times 10^{-12}\) moles of heme per \(10^6\) erythroid cells. A. G.'s marrow synthesized 10 times the amount of porphyrin that the sickle cell marrow did, and M. G.'s marrow 20 times this amount. The heme synthesized by the three marrows are of the same order of magnitude.

**Peripheral Blood Porphyrin Synthesis**

To determine if some or all of the porphyrin synthesized might be due to synthesis in the mature erythrocytes present in the marrow sample, peripheral blood from each patient was obtained, washed, incubated, the porphyrins extracted, and quantitated in the same manner as the bone marrow. The results of these incubations are given in \(\mu g\). of porphyrin, calculated as protoporphyrin, per ml. of erythrocytes and are as follows: in patient A. G. without substrates 5.85, with substrates 4.90; in patient M. G. without substrates 8.40, with substrates 7.40. No porphyrin was therefore synthesized by the erythrocytes of either patient under these conditions.

**Avian Erythrocyte Porphyrin Synthesis**

The effect on porphyrin synthesis of varying the concentration of nucleated erythrocytes with a given concentration of substrates was investigated utilizing avian erythrocytes. As shown in table 5, with the erythrocyte concentration varying 25-fold, the porphyrin synthesized per ml. of erythrocytes was essentially the same with the exception of duplicate flasks 5 and 6.

In similar experiments it was found that the addition of 0.5 ml. of normal human serum saturated with Fe59 and a 6 mM concentration of reduced glutathione did not inhibit porphyrin synthesis. A concentration of reduced glutathione of 15 mM, however, did decrease porphyrin synthesis significantly. Two other buffer systems, Tris and imidazole, were compared to the phosphate buffer ordinarily used. Both, in concentrations sufficient to maintain pH at 7.4 throughout the incubation, were found to inhibit porphyrin synthesis.
Table 4.—Bone Marrow Porphyrin and Heme Synthesis

<table>
<thead>
<tr>
<th>Patient</th>
<th>No. of Nucleated Cells in Marrow/ cu.mm.</th>
<th>Erythroid Cells %</th>
<th>Erythroid Cells x 10⁶/ml Marrow</th>
<th>Marrow ml</th>
<th>Fe⁺⁺ Serum ml</th>
<th>G-SH Buffer ml</th>
<th>Sucrose-Buffer ml</th>
<th>Substrate Solution ml</th>
<th>µg. Porphyrin/ml Marrow</th>
<th>µg. Porphyrin/Synthesized/10⁶ Erythroid Cells</th>
<th>Moles Porphyrin Synthesized x 10⁻¹⁶/10⁶ Erythroid Cells</th>
<th>Specific Activity Hemin cpm/mg.</th>
<th>Moles Heme Synthesized x 10⁻¹⁶/10⁶ Erythroid Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. G.</td>
<td>153,000</td>
<td>14.9</td>
<td>22.8</td>
<td>0.5</td>
<td>0.5</td>
<td>1.4</td>
<td>3.2</td>
<td>0</td>
<td>6.0</td>
<td>0.128</td>
<td>228</td>
<td>—</td>
<td>1.7</td>
</tr>
<tr>
<td>(Case 11)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. G.</td>
<td>91,000</td>
<td>12.0</td>
<td>10.9</td>
<td>0.25</td>
<td>0.5</td>
<td>1.4</td>
<td>3.45</td>
<td>0</td>
<td>9.0</td>
<td>0.236</td>
<td>420</td>
<td>0.81</td>
<td>3.67</td>
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<tr>
<td>(Case 21)</td>
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<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
</tbody>
</table>

*The specific activity of the Fe⁺⁺ serum was 2.14 x 10¹³ cpm/M of Fe. The iron content of the Fe⁺⁺ serum was 1 µg. of Fe/ml. of solution.†Calculated on the basis of the molecular weight of protoporphyrin.
CONGENITAL ERYTHROPOIETIC PROTOPORPHYRIA. II

Table 5.—Porphyrin Synthesis with Varying Concentrations of Avian Erythrocytes

<table>
<thead>
<tr>
<th>Flask #</th>
<th>Erythrocytes ml.</th>
<th>Glycine Solution ml.</th>
<th>a-Ketoglutaric Acid Solution ml.</th>
<th>Sucrose-Po₄ Buffer ml.</th>
<th>Total Porphyrin μg.</th>
<th>Porphyrin/ml. Erythrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.5</td>
<td>0</td>
<td>0</td>
<td>3.1</td>
<td>2.64</td>
<td>1.05</td>
</tr>
<tr>
<td>2</td>
<td>2.5</td>
<td>0</td>
<td>0</td>
<td>3.1</td>
<td>2.9</td>
<td>1.16</td>
</tr>
<tr>
<td>3</td>
<td>2.5</td>
<td>0.3</td>
<td>0.3</td>
<td>2.5</td>
<td>32.1</td>
<td>12.8</td>
</tr>
<tr>
<td>4</td>
<td>2.5</td>
<td>0.3</td>
<td>0.3</td>
<td>2.5</td>
<td>33.3</td>
<td>13.3</td>
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<tr>
<td>5</td>
<td>1.0</td>
<td>0.3</td>
<td>0.3</td>
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<tr>
<td>6</td>
<td>1.0</td>
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<td>4.0</td>
<td>8.24</td>
<td>8.2</td>
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<td>7</td>
<td>0.5</td>
<td>0.3</td>
<td>0.3</td>
<td>4.5</td>
<td>6.11</td>
<td>12.2</td>
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<td>8</td>
<td>0.5</td>
<td>0.3</td>
<td>0.3</td>
<td>4.5</td>
<td>5.36</td>
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<tr>
<td>9</td>
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<td>0.3</td>
<td>0.3</td>
<td>4.8</td>
<td>2.94</td>
<td>14.7</td>
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<tr>
<td>10</td>
<td>0.2</td>
<td>0.3</td>
<td>0.3</td>
<td>4.8</td>
<td>2.77</td>
<td>13.8</td>
</tr>
<tr>
<td>11</td>
<td>0.1</td>
<td>0.3</td>
<td>0.3</td>
<td>4.9</td>
<td>1.64</td>
<td>16.4</td>
</tr>
<tr>
<td>12</td>
<td>0.1</td>
<td>0.3</td>
<td>0.3</td>
<td>4.9</td>
<td>1.89</td>
<td>18.9</td>
</tr>
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</table>

**DISCUSSION**

A crucial question in regard to the excessive amounts of protoporphyrin present in patients with erythropoietic protoporphyria is whether this is normal protoporphyrin IX or some other abnormal porphyrin incapable of combining with iron and forming heme. By gross crystal structure, melting point determinations, absorption spectrum, fluorescence spectrum and chromatographic behavior, the porphyrin isolated from patient A. C. appeared to be identical to protoporphyrin IX. The heme synthesis experiments using normal human liver slices showed that this porphyrin could combine with iron and form heme as well as protoporphyrin IX under the conditions stated. Therefore, since this porphyrin is chemically and biologically identical to protoporphyrin IX, there is no reason to suppose that it is anything but protoporphyrin IX. Using somewhat different technics, Magnus and co-workers² came to the same conclusion with the protoporphyrin isolated from the erythrocytes of their case.

The liver of patient A. C. was shown to be capable of synthesizing heme from protoporphyrin IX and iron, in vitro, in amounts comparable to normal liver. Therefore, the excessive amount of protoporphyrin found was not due to the inability of the patient to synthesis heme, at least in the liver. This is supported by the absence of deposits of iron in the liver¹ which would be expected if a block in heme synthesis existed at the protoporphyrin stage.

The other possibility is that there might not have been iron available for heme synthesis in the liver. This is unlikely as there was iron available for hemoglobin synthesis in the bone marrow, and even in the severest iron deficiency anemia there has not been a report of this type of porphyria. This supports the clinical evidence, as discussed in the preceding report,¹ indicating that the liver was not the site of abnormal porphyrin synthesis.

In the development of the method for incubating human bone marrow for porphyrin and heme synthesis, several problems became apparent. In defining the conditions for optimum porphyrin synthesis, one of the variables...
not investigated by Granick was the concentration of nucleated erythrocytes with a given concentration of substrates. In using material from human bone marrow aspirations, this would be important since the number of nucleated erythrocytes would vary from aspiration to aspiration and from individual to individual. Using avian erythrocytes it was found that, within the range studied, the concentration of erythrocytes did not influence the porphyrin synthesized per ml. of erythrocytes.

The importance of a reducing agent in the in vitro synthesis of heme from protoporphyrin and iron has been pointed out by Lochhead and Goldberg and Goldberg. In order to use an incubation system for both porphyrin and heme synthesis, it was necessary to determine the effect on porphyrin synthesis of normal human serum saturated with Fe and a reducing agent such as reduced glutathione in the incubation mixture. In experiments using avian erythrocytes, it was found that 0.5 ml. of normal human serum saturated with Fe and a 6 mM concentration of reduced glutathione did not inhibit porphyrin synthesis. Because of the possible interference of phosphate buffer with iron utilization, two other buffers were tried, Tris and imidazole. Both were found to inhibit porphyrin synthesis, so despite the objection, phosphate buffer was used.

The greatest problem in incubating the bone marrow for porphyrin synthesis was the presence in the aspirate of a clear mucoid material which formed a gelatinous coagulum around the marrow cells. This prevented substrates from getting to the cells and little if any synthesis occurred. It also made enumeration of the marrow cells impossible. It was found, however, that washing the cells once with a 100 mg. per cent CaCl₂ solution in isotonic sucrose rid the specimen of this material. Apparently it was solubilized by the calcium and could be discarded in the wash.

Not enough bone marrow specimens from normal individuals have been incubated in a standard manner to give a valid normal value for porphyrin and heme synthesis to compare with the results obtained from the patients with erythropoietic protoporphyria. However, 15 marrows from patients with a variety of diseases have been incubated for porphyrin synthesis during the development of this procedure and the most porphyrin synthesized so far, other than in these cases, was with bone marrow from a child with sickle cell disease. For this reason the results from this patient are given for comparison. As stated above, patient A. G.’s marrow synthesized 10 times the amount of porphyrin that the marrow from the sickle cell disease patient did, and patient M. G.’s marrow 20 times this amount. Even though absolute normals are not available, this certainly represents an abnormal increase in porphyrin synthesis.

The possibility that this does not represent an actual increase in synthesis, but a failure of degradation of protoporphyrin, is unlikely. There is no evidence that the erythrocyte or erythroblast degrades protoporphyrin and it is known that free protoporphyrin occurs normally in mature erythrocytes. The liver is the only organ that has been suggested as a site of protoporphyrin degradation. The moles of porphyrin were calculated on the basis of the molecular weight of protoporphyrin. No differential analysis was done and
it is possible that the porphyrin extracted from the marrow was not predominantly protoporphyrin. However, in two other marrow incubations, differential analyses were done and about 90 per cent of the porphyrin present was protoporphyrin. Also, the porphyrin extracted from the marrow of patient M. G. after incubation was esterified and chromatographed on paper and moved as a single spot corresponding to protoporphyrin IX.

That the increased protoporphyrin synthesis did not occur in the mature erythrocytes was demonstrated by incubating the patients' peripheral blood in the same manner as the marrow samples, and subsequent failure to observe increased concentration of porphyrin in the sample to which substrates were added. Magnus and co-workers came to the same conclusion by incubating the erythrocytes of their patient with δ-aminolevulinic acid as substrate.

The marrow heme synthesis observed in these patients indicated, as in the liver, that there was no abnormality in this function. Again, without normal values available, no absolute statement can be made, but it is apparent that both of these marrows are capable of synthesizing heme and within the same order of magnitude as the marrow of a patient with sickle cell disease. Also, there is no clinical evidence for a defect in hemoglobin synthesis in these patients. Their erythrocytes were not hypochromic and microcytic and they were not anemic except in the instance of patient A. G. when he had hypersplenism. Therefore, the increased protoporphyrin synthesis observed was not the result of a block in hemoglobin formation.

These findings and the findings presented in the preceding report indicate that the basic defect in erythropoietic protoporphyria is an abnormal increase in the synthesis of protoporphyrin IX by the erythroid precursors in the bone marrow. This increased production is not due to a failure or decreased rate of synthesis of heme. This disorder appears to present a unique inborn error of metabolism due to the overproduction of a normal substance not in response to a block in the synthetic pathway in which this substance is involved. It is tempting to speculate that this may be due to an absence or malfunction of a mechanism normally regulating protoporphyrin synthesis. This, of course, remains to be confirmed.

**Summary**

Investigations into the pathophysiology of erythropoietic protoporphyria suggest that the basic defect is an inborn error of metabolism resulting in the overproduction of protoporphyrin IX by the bone marrow not in response to a failure of heme synthesis. A method is described for evaluating the in vitro synthesis of protoporphyrin and heme by human bone marrow.

**Summario in Interlingua**

Investigationes in le pathophysiologia de protoporphyria erythropoietic suggere que le defecto fundamental es un innate error del metabolismo resultante in le hyperproduction de protoporphyrina IX per le medulla ossee e que le excesso de protoporphyrina non es un effecto de dysfunction del synthesse de hem. Es describite un metodo pro evalutar le synthesse in vitro de protoporphyrina e de hem per le human medulla ossee.
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