ISOTOPIC STUDIES OF THE ERYTHROPOIETIC AND
HEPATIC COMPONENTS OF CONGENITAL PORPHYRIA
AND 'ERYTHROPOIETIC' PROTOPORPHYRIA

D. C. NICHOLSON, M. L. COWGER,* J. KALIVAS,†
R. P. H. THOMPSON AND C. H. GRAY

Department of Chemical Pathology, King's College Hospital Medical School,
London

(Received 30 August 1972)

SUMMARY

1. Labelled glycine and/or δ-aminolaevulinic acid (ALA) were administered to a
child with congenital erythropoietic porphyria (Günther's disease), to three normal
children and to three patients with erythropoietic protoporphyria.
2. The utilization of [15N]ALA for the synthesis of faecal 'urobilin' in the con-
genital erythropoietic patient was normal.
3. This suggests there is no significant increase of hepatic bile-pigment formation
in congenital erythropoietic porphyria.
4. The utilization of glycine for synthesis of faecal 'urobilin' and protoporphyrin
in all three patients with erythropoietic protoporphyria was increased. There was a
similarly high utilization of [4-14C]ALA administered either orally or intravenously
to one of the patients.
5. The utilization of [4-14C]ALA was not affected by phlebotomy.
6. Utilizations of both ALA and glycine for free erythrocyte and plasma proto-
porphyrins were low.
7. This study provides further evidence that in erythropoietic protoporphyria
there is a greatly increased hepatic contribution to the early labelled fraction of bile
pigment and that in this disease the excessive protoporphyrin is formed mainly in
the liver.

Key words: aminolaevolinic acid, haem, porphyria, urobilin.

Porphyrias are classified as erythropoietic or hepatic according to the apparent site of the
primary defect of porphyrin metabolism. In congenital erythropoietic porphyria (CEP;
Günther's disease) there is excess uroporphyrin I in urine, skin, bone, erythrocytes and normoblasts, severe photosensitivity, and anaemia due both to ineffective erythropoiesis and reduced erythrocyte survival (Tschudy, 1969). By contrast, in erythropoietic protoporphyria (EPP) there is increased erythrocyte protoporphyrin and faecal copro- and proto-porphyrins, with less severe photosensitivity.

Normally 80–90% of the bile pigment is produced as the product of the breakdown of the haemoglobin haem of erythrocytes at the end of their life span, as is shown by the excretion of labelled bile pigment 90–120 days after the administration of isotopically labelled glycine. Labelled bile pigment is also excreted during the first 8 days after administration of labelled glycine because the remaining 10–20% (the early labelled fraction) of the pigment is derived from haems of short half-life in both erythropoietic and hepatic tissues (Yamamoto, Skanderbeg, Zipursky & Israels, 1965). After the administration of radioactively labelled δ-aminolaevulinic acid (ALA) which, unlike glycine, preferentially labels the hepatic component of the early labelled fraction, labelled bile pigment is excreted mainly in the first 8 days (Schwartz, Ibrahim & Watson, 1964).

There is increased incorporation of \([^{15}\text{N}]\text{glycine}\) into early labelled bile pigment in CEP (Gray, Neuberger & Sneath, 1950) and possibly also in EPP (Gray, Kulczycka, Nicholson, Magnus & Rimington, 1964). In CEP an increased turnover of marrow haems adequately explains this, although an increased contribution from the breakdown of hepatic haem compounds to the early labelled fraction has not been excluded. In EPP, however, the synthesis of erythropoietic haem does not appear to be increased (Holti, Magnus & Rimington, 1963) but enhanced catabolism of hepatic haems may increase the early labelled fraction (Gray et al., 1964; Scholnick, Marver & Schmid, 1971).

We have tried to determine, in EPP and CEP, the relative contributions of the hepatic and erythropoietic components to the early labelled fraction of bile pigment. \([^{15}\text{N}]\text{ALA}\) was administered orally to a child with CEP, and, since the normal incorporation of ALA at this age is unknown, the same dose was given to each of three normal children. \([^{15}\text{N}]\text{Glycine}\) and \([^{14}\text{C}]\text{ALA}\) were administered intravenously or orally to an adult with EPP and \([^{15}\text{N}]\text{glycine}\) alone to two other patients with the same disease. The utilizations of \(^{15}\text{N}\) and \(^{14}\text{C}\) for synthesis of faecal 'urobilin', and, in some subjects, of erythrocyte haem and erythrocyte and faecal porphyrins, were determined.

Many results of isotopic studies of pyrrole pigment metabolism have been reported as the labelling of the pigments. This, however, takes no account of the quantity of pigment excreted and so does not reflect the true incorporation of precursor. The term 'incorporation' is often used, but is unsatisfactory because there is loss of four amino residues per eight moles of precursor. The term 'utilization' is better since it takes into account precursor not actually incorporated into pigment.

SUBJECTS AND METHODS

Sources of isotopically-labelled aminolaevulinic acid and glycine

\([4-{^{14}}\text{C}]\text{ALA}\) (15 mCi/mmole) and \([^{15}\text{N}]\text{ALA}\) (30-0 atom % excess \(^{15}\text{N}\)) were supplied by Commissariat a l'Énergie Atomique, Gif-sur-Yvette, France. \([^{15}\text{N}]\text{Glycine}\), containing either 94-6 or 31-6 atom % excess \(^{15}\text{N}\), was prepared from \([^{15}\text{N}]\text{ammonium nitrate}\).
Isotopic studies in porphyrias

Patients

M.D. Male aged 2.5 years, weight 12.6 kg, height 84 cm. CEP was diagnosed at 6 months. The urine contained excess of uroporphyrin, 7.6 mg/day (range 2.6–10.4) and coproporphyrin 1.50 mg/day (range 0.8–3.0), and the faeces excess copro- and proto-porphyrins (Table 1). Persistent photosensitivity with blistering developed later. He is now hirsute, with brown teeth which fluoresce in ultraviolet light. Haemoglobin 11.8–13.6 g/100 ml. Spleen impalpable. Measurement of erythrocyte survival was considered unjustifiable in a child of this age. Fifteen months later the spleen became palpable, with anaemia, and transfusion has since been required.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Faeces (mg/day)</th>
<th>Erythrocytes (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Copro-</td>
<td>Proto-</td>
</tr>
<tr>
<td></td>
<td>porphyrin</td>
<td>porphyrin</td>
</tr>
<tr>
<td>M.D.</td>
<td>5.50</td>
<td>1.32</td>
</tr>
<tr>
<td></td>
<td>(0.5–21)</td>
<td>(0.14–4.3)</td>
</tr>
<tr>
<td>H.C. (before bleeding)</td>
<td>2.2</td>
<td>9.2</td>
</tr>
<tr>
<td></td>
<td>(1.0–4.9)</td>
<td>(2.2–14.9)</td>
</tr>
<tr>
<td>H.C. (after bleeding)</td>
<td>2.4</td>
<td>11.6</td>
</tr>
<tr>
<td></td>
<td>(0.7–3.4)</td>
<td>(4.7–17.7)</td>
</tr>
<tr>
<td>G.M.</td>
<td>2.6</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td>(1.7–4.0)</td>
<td>(4.2–9.5)</td>
</tr>
<tr>
<td>M.P.</td>
<td>2.9</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td>(0.56–4.3)</td>
<td>(1.9–12.7)</td>
</tr>
</tbody>
</table>

Normal values (adults) 0.012–0.8 0–2.1 40–280 0–0.003 0.02–0.07

H.C. Male aged 48, weight 68 kg, height 172 cm. He has constant, mild photosensitivity and excess faecal copro- and proto-porphyrins and erythrocyte porphyrins (Table 1). The means and ranges of plasma protoporphyrin concentrations before and after phlebotomy were 21 (0–78) µg/100 ml and 93 (19–232) µg/100 ml respectively. EPP was diagnosed by Magnus, Jarrett, Prankerd & Rimington (1961).

Haemoglobin 12.2 g/100 ml. Ferrokinetics previously studied were normal as was the peak of stercobilin labelling at 120 days after administration of labelled glycine (for details see Gray et al., 1964).

G.M. Female aged 47, weight 47.7 kg, height 145 cm. She had EPP with photosensitivity and excess faecal and erythrocyte copro- and proto-porphyrins (Table 1).

Haemoglobin 11.0 g/100 ml. Labelling of haemin was steady at 50 days after administration of labelled glycine, indicating the absence of gross haemolysis.
M.P. Female aged 19, weight 58 kg, height 153 cm. She had EPP with severe photosensitivity and excess faecal protoporphyrins and coproporphyrins (Table 1). Haemoglobin 13.0 g/100 ml. Normal peak of stercobilin labelling at 120 days after administration of labelled glycine. The labelling of haemin began to decrease at 109 days.

Normal children. Three normal (male) children: W.E., aged 2, weight 17.8 kg; W.D., aged 2.5 years, weight 15.9 kg, and H.U., aged 2, weight 12.8 kg, were used as controls in the study of congenital porphyria. The mean daily faecal coproporphyrin and protoporphyrin for these controls were within the limits 0.04-0.2 and 0.25-0.38 mg respectively.

Experimental design

M.D. and normal children W.E., W.D. and H.U. [15N]ALA hydrochloride (500 mg) was administered orally. Faeces were collected and analysed for 'urobilin' daily for 7 days. Urine was not collected.

H.C. In 1968 he was given 1-08 g of 31-6 atom % excess [15N]glycine and 2-78 μCi of [14C]-ALA hydrochloride intravenously. 'Urobilin' was isolated from faeces collected daily for 8 days, and on two separate occasions at 117 and 120 days.

In 1969 the patient was given 12.5 μCi of [4-14C]ALA hydrochloride and 0.75 g of glycine of 94-6 atom % excess 15N, both administered orally. Faeces were collected daily for 8 days. Three weeks later blood was removed on each of two successive days (total 1000 ml) and the administration of ALA and glycine was repeated. Faecal collection was resumed daily for 8 days. Protoporphyrin was isolated from the erythrocytes and the plasma obtained at the venesection, and 'urobilin' and protoporphyrin were isolated from the faecal specimens.

G.M. Glycine (5-0 g, 31-6 atom % excess 15N) was administered orally in one dose. Faeces were collected daily for 8 days. 'Urobilin' and copro- and proto-porphyrins were isolated from the specimens.

M.P. Glycine (3-0 g, 31-6 atom % excess 15N) was administered orally in three equal doses over 7-5 h. Faeces were collected for 8 days in two 4-day batches. 'Urobilin' was isolated from the specimens.

The objective of the studies was explained to the patients, or, in the case of the children, their parents, and full consent obtained.

Isolation and determination of pigments

Faecal 'urobilin'. This was isolated as the hydrochloride and purified by recrystallization (Gray et al., 1964). Purity was assessed by the specific extinction (in all specimens E1% above 1300) in chloroform at 495 nm, or the specific optical rotation in chloroform at 598 nm. The pigment from H.C., W.E. and W.D. was stercobilin of αD CHCl3 −3500°. That from M.D. had αD CHCl3 +2500° and was assumed to be a mixture of d-urobilin and other 'urobilins'. 'Urobilin' was determined by the method of Gray (1958).

Faecal protoporphyrin. This was removed into 1.37 M-HCl from the washed ethereal extract obtained during the 'urobilin' isolation. The acid extracts were neutralized to pH 3.8 (Congo Red) with sodium acetate and mixed porphyrins were re-extracted into peroxide-free diethyl ether. The ether extract was washed once with aqueous 3% sodium acetate and porphyrin removed by this was again taken into ether. From the ethereal solution, copro- and proto-porphyrins were re-extracted into 0-1 M- and 1.37 M-HCl respectively; these porphyrins were determined spectrophotometrically (Rimington, 1958).
For isolation of porphyrins the acid extracts were neutralized and the porphyrins returned to ether. After removal of ether the porphyrin was esterified with 5% (v/v) sulphuric acid in methanol. Porphyrin esters extracted from the diluted reaction mixtures were washed thrice with water and crystallized from chloroform–methanol.

**Determination of erythrocyte and plasma porphyrins.** Erythrocytes were separated by centrifugation and washed thrice with 1 vol. of 0.9% (w/v) NaCl. The cells were added slowly to 200 vol. of stirred ethyl acetate–acetic acid, 4:1 (v/v), and the mixture stored at 4°C in the dark. After separation of the liquid phase the residue was re-extracted with small amounts of extractant, and then with peroxide-free ether until the extracts were colourless. Pooled extracts were diluted with 3 vol. of ether and all porphyrins were extracted into 1.37 M-HCl and, after neutralization, into ether. They were fractionated into 0.1 M-HCl (coproporphyrin) and 1.37 M-HCl (protoporphyrin). Protoporphyrin was recrystallized as the dimethyl ester (m.p. 219°C).

**Plasma protoporphyrin.** This was similarly isolated (but not crystallized) and determined.

The methods for determination of faecal and erythrocyte porphyrins recovered between 80 and 90% of porphyrins added to normal specimens.

**Mass-spectrometric assay of 15N.** Quantities of pigment containing 100 μg of nitrogen were digested (Kjeldahl) in 2 ml of sulphuric acid M.A.G. (British Drug Houses Ltd, Poole, Dorset) to which 1.0 g of sodium sulphate–mercuric sulphate M.A.G., 20:1 (w/w) (British Drug Houses Ltd) was added. After digestion, dilution and alkalinization, the ammonia was distilled into 2 ml of 0.05 M-sulphuric acid and estimated by back titration with 0.05 M-NaOH. The solutions were reacidified, evaporated to 1–2 ml, and assayed in an AEI 900 mass spectrometer (Allied Electronic Industries) using reservoir bulbs of 50 ml capacity. Accuracy of determination of atom % 15N was within the limits ±1.0% of the observed value.

**Radioactivity measurements.** Radioactivities of erythrocyte and plasma protoporphyrin were determined on polythene planchettes in a gas-flow Geiger counter (Nuclear Chicago), of efficiency 22%, and also by scintillation counting after combustion as described below. Protoporphyrin was determined spectrophotometrically from the extinction coefficient in chloroform at 408 nm. Radioactivities of "urobilin" specimens were determined by combustion and collection of the 14CO2 into ethanolamine, followed by scintillation counting in a toluene PPO/POPOP phosphor using a Packard 'Tri-Carb' 3000 scintillation spectrometer. Counting was continued until an SD of 1.5% was achieved.

**Calculation of utilization of isotope into pigment**

The daily output of bile pigment was calculated from haemoglobin concentration, an assumed mean erythrocyte life span of 120 days, a 15% contribution of pigment from non-erythropoietic sources, and the blood volume, which for the adults, was derived from a weight–height nomogram (Strickland, 1967) and for the children from the data of Brines, Gibson & Kunkel (1940).

**Percentage utilization of precursor into bile pigment.** Utilization of [4-14C]ALA was calculated from the ratio of d.p.m. found to d.p.m. administered.

Calculations of utilization of 15N-labelled precursors were made using a formula derived as follows:

1 mmol (594 mg) of bile pigment requires $8 \times 75$ mg of glycine (molecular weight 75)
\[ P \text{ mg of bile pigment containing } Y \text{ atom } \% \text{ excess } ^{15}\text{N} \text{ must be derived from } \frac{8 \times 75 \times P}{594} \text{ mg glycine of } Y \text{ atom } \% \text{ excess } ^{15}\text{N}. \]

Percentage utilization of a dose of \( D \) mg of glycine containing \( X \) atom \% excess for formation of bile pigment:

\[
\frac{8 \times 75 \times PY \times 100}{594 \times X \times D}
\]

Similarly, for ALA hydrochloride (molecular weight 167.5) percentage utilization:

\[
\frac{8 \times 167.5 \times PY \times 100}{594 \times X \times D}
\]

In general percentage utilization:

\[
\frac{\text{mg of pigment produced/day} \times \text{atom } \% \text{ excess in pigment} \times 8 \times \text{molecular weight of the precursor} \times 100}{\text{molecular weight of the pigment} \times \text{dose of precursor (mg)} \times \text{atom } \% \text{ excess of } ^{15}\text{N} \text{ in the precursor}}
\]

A more rigid derivation would take account of the alteration in molecular weight caused by the introduction of isotopically labelled nitrogen, and would require more fundamental calculations involving Avogadro’s number.

In the absence of knowledge of the stable pool sizes of the precursors of tetrapyrroles, it would be difficult to determine the various contributions to faecal ‘urobilin’ from observations of the degree of labelling of ‘urobilin’ after administration of isotopically labelled precursors. However, the percentage utilization of labelled precursor for ‘urobilin’ should be independent of stable pool size provided that (a) ‘urobilin’ is collected until label has virtually vanished from the precursor pool, both in the porphyric and normal control subjects and (b) the quantity of the precursor pool which is utilized for purposes other than the formation of early labelled ‘urobilin’ is not different in the two groups. The results in the present study demonstrate that (a) is true for ALA and nearly so for glycine if collections are made for 8 days after administration of labelled precursor; (b) remains an assumption.

**RESULTS**

*Congenital erythropoietic porphyria and normal children*

Total utilizations over 8 days of \([^{15}\text{N}]\text{ALA}\) for faecal ‘urobilin’ by the normal children (W.E., H.U. and W.D.) were 4-8, 4-7 and 4-5\% of the administered dose respectively (Fig. 1). Utilization by the porphyric patient, M.D., was 2-0\% (Fig. 2). H.U. passed no stool on the third, fourth and sixth days. The maximum labelings of ‘urobilin’ occurred on the first or second day and were of similar magnitude in the three normal children and in M.D.

*Erythropoietic protoporphyria*

*H.C. intravenous precursors.* The labelling of ‘urobilin’ from, and utilization of \([^{13}\text{N}]\text{glycine}\) for, this pigment over the first 8 days is shown in Fig. 3, together with that found after administration of oral \([^{15}\text{N}]\text{glycine}\) by Gray et al. (1964). Maximum labelling of ‘urobilin’
Isotopic studies in porphyrias

Fig. 1. Atom % excess of $^{15}$N in, and % utilization of, oral $[^{15}$N]ALA for the synthesis of faecal 'urobilin' in three normal children, W.E., W.D. and H.U. The results for H.U. are presented as a histogram because the patient produced no specimens on the third, fourth and sixth days.

Fig. 2. Atom % excess of $^{15}$N in, and % utilization of, oral $[^{15}$N]ALA for the synthesis of faecal 'urobilin' in subject M.D. with CEP. No correction has been made for increased erythropoiesis.
occurred on the third day and the total utilization was 0.19%; on both the 117th and 120th days utilization was 0.01%. In 1964 the total utilization was 0.23%.

Labelling of 'urobilin' from, and utilization of [14C]ALA is shown in Fig. 4. The faecal 'urobilin' was again labelled maximally on the third day, the total utilization over 8 days being 40%. At 117 and 120 days the utilization was 1.14 and 1.09% respectively.

**Fig. 3.** Atom % excess of $^{15}$N in, and % utilization of, $[^{13}$N]glycine for the synthesis of faecal 'urobilin' in subject H.C. with EPP: (●), calculated for oral glycine from the data of Gray et al. (1964); (■), present results for intravenous glycine.

**H.C. oral precursors.** The labelling and utilization of oral glycine for 'urobilin' over 8 days is shown in Fig. 5. The total utilization was 0.25%. The utilization for faecal protoporphyrin over 14 days (pooled in 3–4-day samples) was 0.09%. The corresponding labelling of the 'urobilin' from [14C]ALA is shown in Fig. 4. The maximum labelling again occurred on the fourth day, and the utilization was 5.9%. The utilization of ALA for the faecal protoporphyrin, determined in three pooled collections, over 10 days was 5.1%.

The apparent utilization of $[^{15}$N]glycine for erythrocyte protoporphyrin was 0.01%; for plasma protoporphyrin it was negligible. Utilization of [14C]ALA for erythrocyte protoporphyrin was 0.03% but no radioactivity was detected in the plasma protoporphyrin.

The labelling of faecal 'urobilin' from, and utilization of oral $[^{13}$N]glycine after bleeding is also shown in Fig. 5. Maximum labelling was on the third day and the utilization over 8 days was 0.33%. Maximum labelling of faecal protoporphyrin was on the third day and the utilization over 8 days was 0.03%, again from pooled collections. Simultaneous labelling of the
Fig. 4. Specific radioactivity of, and % utilization of, [14C]ALA for the synthesis of faecal 'urobilin' in subject H.C. with EPP: (●), ALA administered intravenously; (■), ALA administered orally before phlebotomy, and (○), oral ALA after phlebotomy (the ordinate scales of specific radioactivity differ because of the different doses of ALA administered).

Fig. 5. Atom % excess of 15N in, and % utilization of, oral [15N]glycine for the synthesis of faecal 'urobilin' in subject H.C. with EPP: (●), before phlebotomy; (■), after phlebotomy.
'urobilin' from and utilization of $[^{14}\text{C}]\text{ALA}$ is shown in Fig. 4. Maximum labelling occurred on the third day, and the total utilization was 7.1%. The utilization for faecal protoporphyrin over 8 days was 6.9% (analysed in three pooled collections). The maximum reticulocyte count achieved was 3%.

**G.M. oral precursor.** Labelling of 'urobilin' from, and utilization of $[^{15}\text{N}]\text{glycine}$ is shown in Fig. 6. Maximum labelling occurred on the fourth day and the utilization for 8 days was 0.17%.

**M.P. oral precursor.** The utilization of $[^{15}\text{N}]\text{glycine}$ for 'urobilin' for the first 8 days was 0.18% and for the 119th to the 124th day was 0.05%.

![Fig. 6. Atom % excess of $^{15}\text{N}$ in, and % utilization of, $[^{15}\text{N}]\text{glycine}$ for the synthesis of faecal 'urobilin' in subject G.M. with EPP.](image)

**DISCUSSION**

We have calculated percentage utilization of labelled precursor for tetrapyrrole formation during 8 days, rather than using values of peak labelling, since the former is unaffected by differences of pool size of precursor or of tetrapyrroles. Measurement of pool sizes in man would require constant infusion of labelled precursor to a steady state, and then measurement of the decay of labelling; this is impracticable (Scott & Gray, 1962), and a percentage utilization of ALA must therefore be used as an estimate of hepatic tetrapyrrole synthesis. The total hepatic utilization of ALA cannot be calculated without measuring the uptake of the administered ALA from the blood by the liver.

The daily faecal bile pigment is not stoichiometric with the haem catabolized and determination of faecal 'urobilin' is inaccurate (Bloomer, Berk, Howe, Waggoner & Berlin, 1970).
Isotopic studies in porphyrias

We have therefore calculated daily 'urobilin' excretion from estimates of haemoglobin breakdown, and assumed a 15% contribution from other sources. Also, the peak labelling may not represent true utilization of isotopic precursors because the 24 h excretion of 'urobilin' is affected by bowel habits. We have therefore calculated the utilization over the period of early labelling and assumed a constant 'urobilin' output. The estimates of the daily 'urobilin' outputs of G.M. and M.P. assume that excretion of 'urobilin' was normal in spite of mildly abnormal tests of liver function.

Table 2. Percentage utilizations of oral and intravenous glycine and aminolaevulinic acid for the biosynthesis of early labelled 'urobilin' (8 days) by porphyric patients and normal subjects. Calculations of values from published data were calculated as in the text.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Reference or comment</th>
<th>Glycine</th>
<th>ALA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orally administered</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(H.C.) Gray et al. (1964)</td>
<td>0.23</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>(H.C.) 1969 before bleeding; present work</td>
<td>0.25</td>
<td>5.9</td>
<td></td>
</tr>
<tr>
<td>(H.C.) 1969 after bleeding; present work</td>
<td>0.33</td>
<td>7.1</td>
<td></td>
</tr>
<tr>
<td>(G.M.) Present work</td>
<td>0.17</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>(M.P.) Present work</td>
<td>0.18</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>CEP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(M.D.) Present work</td>
<td>—</td>
<td>2.0†</td>
<td></td>
</tr>
<tr>
<td>(G.L.) Gray et al. (1950)</td>
<td>0.5*</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>—</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gray et al. (1950)</td>
<td>0.14</td>
<td>—</td>
</tr>
<tr>
<td>(W.E.) Present work</td>
<td>—</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>(H.U.) Present work</td>
<td>—</td>
<td>4.7</td>
<td></td>
</tr>
<tr>
<td>(W.D.) Present work</td>
<td>—</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>—</td>
<td>Berlin et al. (1956)</td>
<td>—</td>
<td>2.7</td>
</tr>
<tr>
<td>—</td>
<td>Yamamoto et al. (1965)</td>
<td>0.06</td>
<td>—</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intravenously administered</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPP (H.C.) Present work</td>
<td>0.19</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>—</td>
<td>Yamamoto et al. (1969)</td>
<td>0.09</td>
<td>22</td>
</tr>
<tr>
<td>—</td>
<td>Yamamoto (1964)</td>
<td>—</td>
<td>29</td>
</tr>
<tr>
<td>—</td>
<td>Jones et al. (1972)</td>
<td>—</td>
<td>21</td>
</tr>
<tr>
<td>—</td>
<td>Jones et al. (1972)</td>
<td>—</td>
<td>25</td>
</tr>
</tbody>
</table>

* Calculated over 10 days; utilization over whole of the early labelling period was 1%.
† Uncorrected for increased daily 'urobilin' production (see text).

In Table 2 is listed previously published data concerning the incorporation of precursors into early labelled 'urobilin' in both normal subjects and those with congenital erythropoietic porphyria (CEP) and erythropoietic protoporphyria (EPP). The results of the present studies are included for comparison.

Congenital erythropoietic porphyria

In the normal children the utilization of oral ALA for the 'urobilin' was similar to that of a normal adult also given an oral dose of this precursor (Berlin, Neuberger & Scott, 1956), but was much lower than that in adults given the precursor intravenously (Yamamoto, Fujii,
The apparent utilization of ALA by the child M.D. with CEP, calculated assuming a normal daily ‘urobilin’ output for a child of his age, was lower than in normal children. In a similar patient (also not subjected to splenectomy) studied by Haining, Cowger, Shurtleff & Labbe (1968), the ‘urobilin’ production was increased but was calculated as being not more than twice normal. In our patient the measured daily production of ‘urobilin’ was similarly increased above the value calculated from his expected blood volume (Table 1), so the actual incorporation of isotope was probably normal rather than low. There is probably no increased hepatic contribution to the bile pigment in this disease, the increased utilization of glycine (Gray et al., 1950) being due to excessive haemopoiesis.

**Erythropoietic protoporphyria**

Because of the excessive free protoporphyrin in erythrocytes in this disease, it was originally assumed to be wholly erythropoietic (Magnus et al., 1961; Haeger-Aronsen, 1963), but there is no strong evidence for this. The amount of protoporphyrin in the erythrocytes of H.C. was small compared with that excreted daily in the faeces. This makes it unlikely that more than 10% of faecal protoporphyrin is derived from erythrocytes.

Cripps & MacEachern (1971) found no relation between the amount of protoporphyrin in erythrocytes and in stools. Schwartz, Johnson, Stephenson, Anderson, Edmondson & Fusaro (1971) found insufficient total label from glycine and ALA in the protoporphyrin of erythrocytes to account for the labelling of faecal protoporphyrin. Scholnick et al. (1971) gave labelled ALA to a patient with EPP and observed that faecal protoporphyrin and stercobilin were labelled similarly, with lower labelling of plasma and erythrocyte porphyrins. They also suggested that both erythropoietic and hepatic cells were affected in EPP, which they renamed erythrohepatic porphyria as had Gray et al. (1964).

The labelling from glycine in the early-labelled ‘urobilin’ has been determined in a few normal subjects, but only from some of these published data can the percentage utilization be calculated. Using the data of Gray et al. (1950) and Yamamoto et al. (1965, 1969), calculations similar to those described in the Subjects and Methods section show that 0.06-0.14% of oral glycine was utilized for the synthesis of ‘urobilin’. The utilization of oral glycine for ‘urobilin’ was therefore above the normal values in H.C., G.M. and M.P., all of whom had EPP. Similar results were obtained when H.C. was investigated in 1964, 1968 and 1969. The increased utilization in these patients must be due to increased breakdown of erythropoietic and/or hepatic haem.

Utilization of ALA for the early labelled ‘urobilin’ in normal subjects can be similarly calculated from published results. The subject of Berlin et al. (1956) utilized 2.7% of an oral dose of ALA, in contrast with two patients of Yamamoto et al. (1969) who utilized 22%, and a patient of Yamamoto (1964) who utilized 29% of an intravenous dose. The values of Yamamoto et al. (1969) agree closely with those of 21% and 25% found in normal people by Jones, Schrager, Bloomer, Berk, Howe & Berlin (1972). There was a comparable difference in the utilization of ALA administered by the two routes in our patient, H.C. These different results may reflect the different presentation of ALA to the hepatic tissues, but there is no reason to believe that the rate of absorption of ALA from the intestine should affect the percentage utilization. Urinary losses were not measured. The utilization of intravenous ALA by H.C. was twice, and of oral ALA two to three times, the normal utilization of ALA given by these routes (Table 2). Assuming that much more ALA is utilized for hepatic than for erythropoietic haem,
our results suggest that in EPP the increased utilization of glycine is also due to a much increased contribution from the catabolism of hepatic haem, rather than of erythropoietic haem, to early labelled bile pigment.

In EPP hepatic haem breakdown may be increased, and its biosynthesis secondarily increased due to reduced repression by a decreased pool of haem. Ferrochelatase can become rate-limiting in \textit{in vitro} porphyrin systems (Doss, 1968), suggesting that its substrate protoporphyrin, and even copro- and uro-porphyrin, could appear in increased amounts in EPP. This has been observed by Peterka, Fusaro, Runge, Jaffe & Watson (1965). The low labelling of erythrocyte protoporphyrin and plasma protoporphyrin by H.C. and by the patient of Scholnick \textit{et al.} (1971) might have resulted from the reflux into the plasma of porphyrins synthesized in the liver, and their uptake by erythrocytes. Such labelled protoporphyrin would be diluted by unlabelled porphyrin previously acquired. Bilirubin has recently been shown to undergo such a reflux (Berk, Howe, Bloomer & Berlin, 1969). This mechanism is compatible with the observation of fluorescent erythrocytes and normal plasma protoporphyrin concentrations in a few patients with EPP (Redeker & Bronow, 1964), and the findings in other patients of increased protoporphyrin amounts only in the faeces.

In man griseofulvin increases protoporphyrin in stools and erythrocytes (Rimington, Morgan, Nicholls, Everall & Davies, 1963; Ziprkowski, Szeinberg, Crispin, Krakowski & Zaidman, 1966) and produces in mice a condition similar to EPP. In these animals there is increased hepatic synthesis of haem \textit{in vitro}, but normal hepatic synthesis of haem \textit{in vivo} (de Matteis & Rimington, 1963).

In EPP the concentration of erythrocyte protoporphyrin decreases with the age of the cell (Clark & Nicholson, 1971). This would be compatible with a hepatic source only if the young cells took up and retained protoporphyrin against a high concentration gradient. Erythrocytes from normal and protoporphyric patients do take up protoporphyrin \textit{in vitro} (G. Rehm, personal communication) as do those of mice and dogs \textit{in vivo} (Nakao, Wada, Takaku, Sassa, Yano & Urata, 1967) and may not then readily release it (Redeker & Bryan, 1964). It is uncertain whether this uptake is sufficient to account for the erythrocyte protoporphyrin concentrations.

Other evidence, however, supports an abnormal erythropoietic contribution in EPP. Thus patients have been described with raised erythrocyte protoporphyrin and yet normal faecal protoporphyrin amounts. A mild defect of hepatic porphyrin excretion could explain this observation (Redeker, Bronow & Sterling, 1963); and there have been recent reports of hepatic dysfunction in this disease (Barnes, Hurworth & Millar, 1968; Donaldson, McCall, Magnus, Simpson, Caldwell & Hargreaves, 1971; Scott, Ansford, Webster & Stringer, 1971) and G.M. and M.P. both died in hepatic failure. Further, Porter (1963) suggested that in EPP the bone marrow synthesizes protoporphyrin \textit{in vitro} at a rate greater than normal, while hepatic tissue is normal. Masuya (1969) found increased activity of ALA synthetase in liver and Miyagi (1967) in both bone marrow and liver. Scholnick \textit{et al.} (1971) have recently interpreted the pattern of labelling of plasma and erythrocyte protoporphyrin in a case of EPP given isotopically labelled glycine as indicating an erythropoietic origin for much of the erythrocyte protoporphyrin. They found a rapid fall of the specific activity of the erythrocyte protoporphyrin labelled from ALA. This could be attributed, however, to transient incorporation of ALA into hepatic protoporphyrin, reflux of this labelled protoporphyrin into plasma and reversible uptake into erythrocytes. Subsequently the protoporphyrin is excreted in the bile.
Equilibration between plasma and erythrocyte protoporphyrin was clearly slow in the patient of Scholnick et al. (1971), since the specific activity of erythrocyte protoporphyrin was always much smaller than that of the plasma protoporphyrin.

Phlebotomy should increase erythropoiesis and therefore also the utilization of glycine for erythrocyte protoporphyrin and thence for ‘urobilin’ (London, Yamasaki & Sabella, 1951), whereas the hepatic protoporphyrin and ‘urobilin’, which are preferentially labelled by ALA, should be unaffected. However, only a small increase of circulating reticulocytes followed the bleeding of H.C. so that the significance of the unchanged utilization of glycine and ALA for faecal protoporphyrin is uncertain.

The low labelling of erythrocyte and plasma protoporphyrin may result from making the analysis at an inappropriate time after administration of precursors. These results require comparison with those for a normal subject, but this is difficult owing to the small quantities of protoporphyrin in normal blood.

It is still impossible to define the relative size of the hepatic and erythropoietic contributions in EPP, although in H.C. that from the liver was clearly important. The similar utilization of isotope from ALA for faecal protoporphyrin and stercobilin suggests that equal amounts of labelled protoporphyrin were converted into ‘urobilins’ via haem, or excreted unchanged.

We are unable to explain the relatively high incorporation by H.C. of ALA into stercobilin excreted about 120 days after excretion of the early labelled fraction, unless in this condition ALA is anomalously incorporated into erythrocyte haem.

Schwartz et al. (1971), making certain basic but unproven assumptions, interpreted the observed pattern of faecal pigment labelling from isotopically labelled ALA in a patient with EPP as showing the conversion, in the liver, of protoporphyrin from normoblasts and reticulocytes into haem and thence bile pigment. Our own assumption of a constant 15% contribution to bile pigment from non-catabolic sources may be equally invalid but this would probably be most in error in considering CEP in which the early contribution is greater than 15%. This will increase still further the factor used in the calculation of percentage utilization of precursor for early labelled ‘urobilin’.

ACKNOWLEDGMENTS

R.P.H.T. was in receipt of a Medical Research Council Clinical Research Fellowship and J.K. of a Royal Society of Medicine Foundation Fellowship. We are grateful to our patients for their co-operation, to Dr D. I. Williams for allowing us to study his patients, to Dr C. W. Crane for $^{15}$N assays and to Dr S. Glascock and R. W. Smith for $^{14}$C assays.

REFERENCES

Isotopic studies in porphyrias 149


