Identification of a Group of Tetracarboxylate Porphyrins, Containing One Acetate and Three Propionate β -Substituents, in Faeces from Patients with Symptomatic Cutaneous Hepatic Porphyrria and from Rats with Porphyrria due to Hexachlorobenzene

By G. H. ELDER

*Department of Chemical Pathology, King's College Hospital Medical School,
Denmark Hill, London S.E.5, U.K.*

(Received 13 September 1971)

1. Three tetracarboxylate porphyrins, apart from coproporphyrin, were isolated from the faeces of patients with symptomatic cutaneous hepatic porphyria and of rats with porphyria caused by hexachlorobenzene poisoning. The porphyrins were not present in the urine of the porphyric rats or in the faeces of control rats. 2. Two of the porphyrins were identified as tris-(2-carboxyethyl)-carboxymethyl ethyl trimethyl porphin (isocoproporphyrin) and tris-(2-carboxyethyl)-carboxymethyl trimethyl porphin (de-ethylisocoproporphyrin). 3. The third porphyrin, previously reported to be an analogue of coproporphyrin in which one propionate group is replaced by an α - or β -hydroxypropionate group, was shown to be tris-(2-carboxyethyl)-carboxymethyl-(1-hydroxyethyl)-trimethyl porphin (hydroxyisocoproporphyrin). 4. The order of β -substituents around the porphin ring could not be determined for any of the compounds. 5. Evidence for the existence of a fourth porphyrin, tris-(2-carboxyethyl)-carboxymethyl trimethyl vinyl porphin (dehydroisocoproporphyrin), a postulated precursor of the others, is described. 6. It is suggested that dehydroisocoproporphyrinogen is produced by decarboxylation-dehydrogenation of one of the propionate groups of pentacarboxylate porphyrinogen III.

Patients with symptomatic cutaneous hepatic porphyria ('porphyria cutanea tarda'), patients with porphyria caused by hexachlorobenzene poisoning, and animals with experimental porphyria caused by hexachlorobenzene poisoning, have a similar disturbance of porphyrin metabolism, usually associated with evidence of liver damage (Ockner & Schmid, 1961; De Matteis *et al.*, 1961; Schmid, 1962; San Martin de Viale *et al.*, 1970). Symptomatic cutaneous hepatic porphyria is characterized biochemically by a marked increase in the urinary excretion of porphyrins, particularly uroporphyrin and heptacarboxylate porphyrin, whereas the concentrations of porphobilinogen and, usually, 5-aminolaevulinic acid in the urine are normal (Eales *et al.*, 1966; Taddeini & Watson, 1968). The faeces usually contain increased amounts of both ether-soluble and ether-insoluble porphyrins (Eales *et al.*, 1966; Taddeini & Watson, 1968; Eales *et al.*, 1971), most of the increase in the ether-soluble porphyrin fraction being due to a porphyrin reported to be coproporphyrin (Barnes, 1963; Sweeney, 1963; Herbert, 1966; Chu & Chu, 1967; Dowdle *et al.*, 1970). In a recent investigation the main porphyrin in the faeces of patients with symptomatic cutaneous hepatic porphyria was found to be a previously undescribed porphyrin, with properties similar to coproporphyrin, rather than

coproporphyrin itself (Elder, 1971*b*). This porphyrin was always accompanied by smaller amounts of an hydroxylated porphyrin, similar to one isolated from the urine of a patient with hepatic porphyria and infective hepatitis, and tentatively identified as a tris-(2-carboxyethyl)-mono-(1-hydroxy-2-carboxyethyl)-tetramethyl porphin, 'hydroxycoproporphyrin' (Elder & Chapman, 1970). Neither porphyrin was detected in faeces from normal subjects or patients with other types of porphyria (Elder, 1971*b*). The present paper reports that the same porphyrins are present in the faeces, but not in the urine, of rats with experimental porphyria produced by hexachlorobenzene poisoning, and describes further studies on their structure. The main faecal porphyrin has been shown to be a mixture of tetracarboxylate porphyrins, each having a tris-(2-carboxyethyl)-carboxymethyl trimethyl substituted porphin ring (see formulae I, II and IV), and the hydroxylated porphyrin has been identified as an hydroxyethyl analogue of the others (see formula III).

Materials

Protoporphyrin IX dimethyl ester (grade A) and coproporphyrin III tetramethyl ester were obtained from Sigma (London) Chemical Co. Ltd., London

(I) R = $-\text{CH}_2-\text{CH}_3$ tris-(2-carboxyethyl)-carboxymethyl ethyl trimethyl porphyrin, *isocoproporphyrin* (1a).

(II) R = $-\text{H}$ tris-(2-carboxyethyl)-carboxymethyl trimethyl porphyrin, *de-ethylisocoproporphyrin* (1b).

(III) R = $-\text{CHOH}-\text{CH}_3$ tris-(2-carboxyethyl)-carboxymethyl-mono-(1-hydroxyethyl)-trimethyl porphyrin, *hydroxyisocoproporphyrin* (2).

(IV) R = $-\text{CH}=\text{CH}_2$ tris-(2-carboxyethyl)-carboxymethyl trimethyl vinyl porphyrin, *dehydroisocoproporphyrin*.

S.W.6, U.K. Coproporphyrin I tetramethyl ester, penta-, hexa- and hepta-carboxylic porphyrin methyl esters and uroporphyrin octamethyl ester were prepared from porphyrin urine (Gray & Neuberger, 1950; Chu & Chu, 1959). Tris-(2-carboxyethyl)-mono-(2-carboxyvinyl)-tetramethyl porphyrin was isolated from calf meconium (French & Thonger, 1966; French *et al.*, 1970). Synthetic coproporphyrin III tetramethyl ester and harderoporphyrin trimethyl ester were gifts from Professor A. H. Jackson, Department of Organic Chemistry, University College, Cardiff, U.K. Bis-(trimethylsilyl)-acetamide was obtained from Ralph N. Emanuel Ltd., London S.E.1, U.K., and 10% palladium on charcoal (catalyst grade) from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.

Other chemicals were of analytical-reagent grade, except for kerosene (white), butan-2-one and cyclohexane (both special for chromatography) and talc (acid-washed) from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.; methyl acetate and methyl propionate, both of general-purpose reagent grade, and hexachlorobenzene (technical grade) from British Drug Houses Ltd., Poole, Dorset, U.K. Chloroform was washed three times with water, shaken with anhydrous Na_2SO_4 and filtered through three layers of chloroform-moistened filter paper (Whatman no. 1) before use.

Methods

Chromatography

Porphyrin methyl esters, dissolved in chloroform, were applied to silica gel (Camag D5; Camag,

Chemie-Erzeugnisse und Adsorptionstechnik A.-G., Muttenz, Switzerland) plates, prepared as described by Elder (1971a), either as spots from a glass capillary or, for preparative-scale t.l.c. on $20\text{cm} \times 20\text{cm}$ plates, as streaks (up to about 0.3 mg of porphyrin ester/cm) from a 0.1 ml Hamilton syringe mounted on a Chromatocharger (Camag). Development was carried out in tanks, previously equilibrated with solvent vapour, in the dark. The following solvent systems were used: benzene-butan-2-one (40:3, v/v) followed, after drying, by chloroform-kerosene-methanol (40:20:1, by vol.) (solvent system A); cyclohexane-acetone (3:1, v/v) (solvent system B); carbon tetrachloride - dichloromethane - methyl propionate - methyl acetate (2:2:1:1, by vol.) (solvent system C) (Stoll & Gray, 1970); cyclohexane-acetone (4:1, v/v) (solvent system D) and chloroform-kerosene-methanol (100:200:7, by vol.) (solvent system E). Porphyrin esters were removed from the plates by sucking the porphyrin-containing zones of silica gel into glass eluters (Aimer Products Ltd., London N.W.1, U.K.), washing with light petroleum (b.p. $40-60^\circ\text{C}$) (about $4 \times 4\text{ml}$) to remove kerosene if necessary, and eluting with chloroform-methanol (4:1, v/v). Eluates were evaporated under N_2 at $40-50^\circ\text{C}$ and stored at -20°C in the dark.

The method of Jensen (1963) was used for t.l.c. of porphyrin free acids.

Electrophoresis

Porphyrin free acids were dissolved in ammonia solution (about aq. 25% NH_3) for electrophoresis by the method of Lockwood & Davies (1962).

Preparation and hydrolysis of methyl esters (Falk, 1964)

Porphyrin free acids were esterified with methanol- H_2SO_4 (19:1, v/v; 20h at room temperature in the dark). Esters were transferred to chloroform (Falk, 1964) which was washed once with 2M- NH_3 and three times with water, dried by filtration and evaporated to dryness under N_2 at $40-50^\circ\text{C}$. Methyl esters were hydrolysed by keeping them in 7M-HCl for 48h at room temperature in the dark.

Preparation of porphyrin derivatives

Decarboxylation (Edmondson & Schwartz, 1953). Porphyrin methyl esters were dissolved in 7M-HCl (0.05 ml), water was added (1.1 ml), and the solution sealed in an evacuated Carius tube and heated at $175 \pm 3^\circ\text{C}$ for 3h. After cooling, the solution was transferred to a small beaker, dried *in vacuo* over KOH pellets and esterified. The reaction products were separated by t.l.c. in solvent system D, the plates being developed three times in sequence. Porphyrin methyl esters were recovered from the plates and dissolved in chloroform for determination by

spectrophotometry. Coproporphyrin was not decarboxylated under these conditions.

Acetylation (Barrett, 1959). Porphyrin methyl esters were dissolved in pyridine–acetic anhydride (2:1, v/v; 0.3 ml), left overnight at room temperature in the dark, and the reagents were removed under N_2 at 50°C.

Formation of trimethylsilyl ether. Bis-(trimethylsilyl)-acetamide (0.05 ml) was added to the porphyrin methyl ester (10–50 µg) in a 2.5 ml glass vial (Johnsen Jorgensen Ltd., London E.C.4, U.K.), mixed, tightly stoppered and left at room temperature for 1 h, after which pyridine was added to complete solution of the porphyrin ester (5–10 µl). The tightly stoppered vial was kept at room temperature in the dark overnight, after which the reagents were removed in a stream of N_2 at room temperature and drying was completed over KOH pellets *in vacuo*.

Dehydration (Clezy & Barrett, 1961). Porphyrin methyl ester (0.27 mg) was refluxed in benzene (5 ml) containing toluene-*p*-sulphonic acid (2 mg) for 6 h in the dark. After chloroform (5 ml) had been added to the cooled solution, the mixture was washed twice with 2M- NH_3 , four times with water, dried by filtration through paper and evaporated to dryness under N_2 at 40–50°C in the dark. T.l.c. of the reaction products in solvent system E revealed a single reaction product (yield 58%) and a trace of starting material.

Catalytic hydrogenation. Hydrogen was bubbled rapidly through a solution of porphyrin methyl ester (45 µg) in formic acid (98–100%) (2 ml), containing 0.5 mg of palladium–charcoal (1:9, w/w) catalyst, for 10 min at 50 ± 2°C. After transfer to chloroform, the reaction products were separated by t.l.c. in solvent system B, the plates being developed four times in sequence. The recovery was 52%.

Spectrophotometry

Electronic spectra were determined with a Unicam SP.800 or SP.1800 recording spectrophotometer. Quantities of porphyrin methyl esters were measured by their extinctions in chloroform or chloroform–methanol (4:1, v/v) at the Soret maximum, by using E_{mM} as given by Dowdle *et al.* (1970) or, for protoporphyrin IX dimethyl ester, by Falk (1964) and correction factors as described by Falk (1964). Porphyrins 1a, 1b and 2 were measured by using the correction factor and E_{mM} for coproporphyrin tetramethyl ester in chloroform.

N.m.r. spectrometry

Proton magnetic resonance (p.m.r.) spectra of porphyrin methyl esters in deuterochloroform were recorded by Mr. P. N. Jenkins of Imperial College, London, with a Varian HA-100 spectrometer. Results are expressed in p.p.m. referred to tetramethyl-

silane as 10.00 (τ values) after Tiers (1958). A computer averaging technique was used to obtain the spectrum of porphyrin 2 tetramethyl ester.

Mass spectrometry

Mass spectra of porphyrin 1a and 1b methyl esters, and of the trimethylsilyl ether of porphyrin 2 methyl ester were recorded by Dr. J. R. Chapman, A.E.I. Scientific Instruments Ltd., Manchester, U.K., with an A.E.I. MS30 mass spectrometer, or by Mr. D. W. Carter of the University of London Mass Spectrometry service at the School of Pharmacy with an A.E.I. MS902 double-focusing instrument. Accurate masses were measured by Mr. J. Olliver, Department of Chemistry, Manchester University, with an A.E.I. MS902 spectrometer. Both instruments were operated at an ionizing energy of 70 eV and samples were inserted on a direct probe at source temperatures of 250–270°C.

Detection of porphyrins

Porphyrins were detected during extraction and separation procedures by observing their red fluorescence in the u.v. light transmitted by a Wood's filter.

Experimental

Animals

Norwegian brown rats, obtained from the Medical Research Council Cellular Immunology Research Unit, Sir William Dunn School of Pathology, Oxford, and housed at the Institute of Dermatology, London E.9, were used. One group of rats (porphyric) had been receiving a powdered diet 41B containing hexachlorobenzene (0.25%, w/w) (De Matteis *et al.*, 1961) for 13 months, the other group (controls) were fed on cube diet 41B alone. Both groups received food and water *ad libitum*.

Comparison of the excretion of haem precursors by porphyric and control rats

Four rats from each group were placed in individual metabolic cages and separate collections of urine and faeces were made for 24 h. Urine and faeces were stored at –20°C until analysed.

Faecal and urinary porphyrin excretion rates were measured as follows. The combined 24 h collections of faeces from two rats (about 1–2.5 g wet wt.) were extracted by grinding in methanol– H_2SO_4 (19:1, v/v) (15–20 ml). The suspension was kept for 20 h at room temperature in the dark, then filtered, and the residue was washed with methanol until the washings were colourless. Porphyrin esters in the combined filtrate

and washings were transferred to chloroform, which was washed and evaporated under N_2 at 40–50°C to leave a tarry residue, which was dried *in vacuo* over KOH pellets. Each residue was dissolved in chloroform (1 ml), of which 0.2 ml was applied as a streak to a silica-gel plate for t.l.c. After development in solvent system A, the porphyrin-containing zones corresponding in position to known porphyrins with two to eight carboxyl groups and porphyrins 1 and 2 (see below) were eluted with chloroform-methanol (4:1, v/v). The volume of eluate was made up to 3 ml and the porphyrin content was determined spectrophotometrically, after dilution if necessary. The results were expressed as nmol of haem precursor/24 h. No correction was made for the low recovery of protoporphyrin dimethyl ester obtained with solvent system A (Elder, 1971a).

The pH of a measured sample (6–24 ml) of the combined 24 h urine collections from two rats was adjusted to about 3.5 with acetic acid and sufficient talc added to adsorb all porphyrin present (Grinstein *et al.*, 1945). The talc was recovered by filtration and the moist talc was washed with methanol- H_2SO_4 (19:1, v/v) until all porphyrin was eluted. The eluate was kept at room temperature in the dark for 20 h, after which the porphyrin methyl esters were trans-

ferred to chloroform, fractionated by t.l.c. and measured as described above.

Porphobilinogen and 5-aminolaevulinate were determined by the method of Mauzerall & Granick (1956).

Isolation of porphyrins 1a, 1b and 2 from faeces

Faeces were collected from four patients with symptomatic cutaneous hepatic porphyria, previously shown to have similar porphyrin excretion patterns (Elder, 1971b), and from eight male and two female porphyric rats that continued to receive hexachlorobenzene in their diet during the period of collection (about 2 months). Daily collections of faeces were either extracted when fresh or after storage at -20°C for up to 3 months. Rat faeces were pulverized in an Ato-Mix Emulsifier (MSE) before extraction.

Extraction of porphyrins. Sufficient acetic acid was added to samples of faeces (32–800 g) to form a thick paste after mixing. Peroxide-free ether (5 vol.) was added and the mixture was stirred vigorously with a glass rod. After solid material had been allowed to sediment, or centrifugation, the supernatant was decanted. Extraction was repeated (usually five or

Fig. 1. Fractionation of porphyrin methyl ester preparations by preparative t.l.c. in solvent system A followed, after recovery from the plate, by solvent system B or C

For experimental details, see the text. 1, 1a, 1b, 2, porphyrin fractions described in the text; a, protoporphyrin IX dimethyl ester; b, tricarboxylic porphyrin trimethyl ester; c, coproporphyrin tetramethyl ester; d, e and f, penta-, hexa-, and hepta-carboxylic porphyrin methyl esters respectively. Porphyrin preparations from the faeces of patients and rats with porphyria gave similar patterns of separation.

Table 1. *Yields of porphyrin methyl esters from faeces of a patient with symptomatic cutaneous hepatic porphyria and of rats with hexachlorobenzene porphyria*

Porphyrin esters were determined by using E_{mm} and correction factor for coproporphyrin tetramethyl ester in chloroform.

	Wet wt. of faeces (g)	Total porphyrin (mg)	Porphyrin 1 (mg)	Porphyrin 2 (mg)
Patient with porphyria	279	10.40	3.75	0.48
Porphyric rats	59	3.47	0.47	0.17

six times) until only traces of porphyrin remained in the supernatant. The combined supernatants were extracted in a separatory funnel with successive 0.02 vol. portions of 5% (w/v) HCl until no further porphyrin appeared in the aqueous phase. The pH of the combined acid extracts was adjusted to 3.5 with saturated aq. sodium acetate and talc was added in batches until all porphyrin was adsorbed. The talc was recovered by filtration, washed with water and partially dried by suction. Porphyrins were eluted from the moist talc with methanol- H_2SO_4 (19:1, v/v), and the eluate was kept at room temperature in the dark for 20 h. The porphyrin methyl esters were then transferred to chloroform, which was washed, dried by filtration through paper and removed by distillation at 40–50°C under reduced pressure. The residue was dried *in vacuo* over KOH pellets and stored at –20°C in the dark.

Fractionation of porphyrin methyl ester preparations.

The crude porphyrin ester preparations were fractionated by preparative t.l.c. in solvent systems A, B and C. Initial development in solvent system A gave six bands in positions corresponding to known porphyrins and two unidentified porphyrin bands, porphyrins 1 and 2 (Fig. 1). Porphyrins 1 and 2 were recovered from the plates; typical yields of porphyrin methyl esters from representative samples of human and rat faeces are given in Table 1. Before further purification, all samples of porphyrin 1 obtained from patients were combined, as were those from rats. Porphyrin 2 preparations were combined similarly. Porphyrin 1 was then separated into two porphyrin bands, porphyrins 1a and 1b, by t.l.c. in solvent system B, the plates being developed four times in sequence (Fig. 1). Samples of porphyrin 2, particularly those from human faeces, contained large quantities of bile pigment, shown by the methods of Stoll & Gray (1970) to be a mixture of violins. The porphyrin was separated from bile pigment by t.l.c. in solvent system C, recovered from the plates, acetylated, re-chromatographed in solvent system C (Fig. 1), eluted, hydrolysed and re-esterified. The total amounts of porphyrins 1a, 1b and 2 obtained were 1.2, 4.6 and 0.74 mg respectively from patients with porphyria, and 9.2, 3.1 and 0.67 mg respectively from porphyric rats.

The structure of porphyrins 1a, 1b and 2 was investigated by physical and chemical techniques without further purification or crystallization. Porphyrin esters were stored at –20°C in the dark.

Homogeneity of porphyrins 1a, 1b and 2. Porphyrin esters isolated by t.l.c. are likely to be contaminated by other porphyrins owing to chromatographic entrainment (Dowdle *et al.*, 1970). The purity of porphyrins 1a, 1b and 2 methyl esters was assessed by t.l.c. in the solvent systems used for their isolation after hydrolysis and re-esterification, by t.l.c. and electrophoresis of the porphyrin free acids obtained by hydrolysis, and by mass spectrometry. Separation of porphyrins 1a and 1b was not complete, each containing traces of the other. Both were also contaminated by small amounts of a porphyrin identified from its electronic spectrum and chromatographic and electrophoretic behaviour as coproporphyrin. In addition, the samples of porphyrin 1b, particularly that from porphyric rats, contained a fourth tetracarboxylate porphyrin (see below). Porphyrin 2 was not contaminated by other porphyrins.

Of the t.l.c. methods used for isolation and investigation of homogeneity only one, that of Jensen (1963), resolves some porphyrins differing in the order of β -substituents around the porphin ring (position-isomers). Thus, although porphyrins 1a, 1b and 2 appeared to be single substances each may be a mixture of position-isomers, a possibility that cannot be excluded since reference porphyrins of established position-isomerism are not available.

Results

Effect of prolonged hexachlorobenzene intoxication on the excretion of haem precursors

Amounts of haem precursors in the urine and faeces of porphyric and control rats are shown in Table 2. Porphyrins 1 and 2 were present in the faeces, but not the urine, of the porphyric rats, but were not detected in the urine or faeces of rats in the control group. Porphyrin 1 was isolated from faeces collected from ten porphyric rats and shown to be a mixture of porphyrins 1a and 1b in the ratio of 3:1 (w/w, estimated as coproporphyrin tetramethyl ester). Ratios were not determined for individual rats.

Table 2. *Comparison of the excretion of haem precursors by porphyric and control rats*

Results given are the average for four porphyric male rats (wt. range 250–325 g) and for three male and one female control rats (wt. range 400–475 g). Experimental details are described in the text. The range for each porphyrin was $\leq 30\%$ of the average value.

	5-Aminolaevulinic acid ($\mu\text{mol}/24\text{h}$)	Porphobilinogen ($\mu\text{mol}/24\text{h}$)	Porphyrins (nmol/24h) No. of carboxyl groups						Porphyrin 1 (nmol/24h)	Porphyrin 2 (nmol/24h)
			8	7	6	5	4	2		
Control rats										
Urine	0.2	0.1	Trace	Trace	Trace	Trace	7.0		0.0	0.0
Faeces						Trace	3.5	80.0	0.0	0.0
Porphyric rats										
Urine	18.0	7.5	38.0	13.1	6.1	18.1	70.0		0.0	0.0
Faeces			24.2	23.9	14.3	29.5	26.2	144.0	24.8	7.7

Comparison of porphyrins 1a, 1b and 2 from different sources

No differences between porphyrins 1a, 1b and 2 from human porphyric faeces and the corresponding porphyrins from rat porphyric faeces were revealed by comparison of the electronic and mass spectra of their methyl esters, by t.l.c. of the porphyrin free acids and their methyl esters and, for porphyrin 2, acetyl derivatives, and by electrophoresis. In addition the respective decarboxylation products of porphyrins 1a and 1b had identical electronic spectra and chromatographic behaviour. Similarly, porphyrin 2 from faeces was identical with a hydroxylated porphyrin isolated from the urine of a patient with hepatic porphyria and infective hepatitis and tentatively identified as tris-(2-carboxyethyl)-mono-(1-hydroxy-2-carboxyethyl)-tetramethyl porphin (Elder & Chapman, 1970).

Structure of porphyrins 1a, 1b and 2

Structures I, II and III are proposed for porphyrin 1a, 1b and 2 respectively. The structures are shown as type III isomers, but no chemical evidence was obtained for this order of β -substituents. The electronic spectra of their methyl ester derivatives are recorded in Table 3. All have aetio-type spectra with Soret (band V) maxima from 401.5–403 nm, in keeping with structures with either eight saturated β -substituents, or seven saturated β -substituents and one unsubstituted β -position (Falk, 1964). The difference of 1.5 nm between the Soret maxima of porphyrins 1a and 1b methyl esters, which is greater than the 0.5 nm reported for mesoporphyrin and deuteroporphyrin IX dimethyl esters (Falk, 1964), may be due to the presence of a small amount of a porphyrin with a Soret maximum at 405 nm in samples of porphyrin 1b (see below). There is reasonable agreement between the electronic spectrum of porphyrin 2 and that previously reported (Elder & Chapman, 1970), the differences probably being due to differences in purity and variation between instruments.

Number and nature of acidic β -substituents. Porphyrins 1a, 1b and 2 had the same mobility as coproporphyrin I on t.l.c. in a 2,6-dimethylpyridine-water-NH₃ solvent system, indicating four ionizable carboxyl groups/molecule (Nicholas & Rimington, 1949; Jensen, 1963). Decarboxylation of porphyrins 1a and 1b under conditions known to decarboxylate acetic acid, but not propionic acid side chains gave one porphyrin from each, both with three carboxyl groups/molecule. The mass spectra of the methyl esters of the decarboxylation products showed molecular ions at m/e 652 and m/e 624 respectively, 58 mass units (corresponding to CO₂-CH₂) lower than those of the original methyl esters, confirming that only one decarboxylation had occurred. Frag-

Table 3. Absorption maxima and minima of porphyrin methyl esters in chloroform

Values in parentheses are the ratios of band intensities calculated relative to band IV. The spectrum of authentic coproporphyrin III tetramethyl ester is included for comparison.

Band no. ...	Maxima (λ_{nm})					Minima (λ_{nm})		
	I	II	III	IV	V			
Porphyrin 1a 'Isocoproporphyrin'	623 (0.32)	569 (0.46)	534 (0.68)	499 (1.0)	401.5	603 (0.06)	552 (0.10)	519 (0.24)
Porphyrin 1b 'de-ethylisocoproporphyrin'	622.5 (0.24)	570 (0.51)	537 (0.71)	502.5 (1.0)	403	605 (0.07)	555 (0.19)	520 (0.30)
Porphyrin 2 'hydroxisocoproporphyrin'	623 (0.25)	568.5 (0.51)	538 (0.77)	501 (1.0)	403	605.5 (0.07)	554 (0.17)	520.5 (0.16)
Decarboxylation product of 1a	622 (0.35)	568 (0.47)	534 (0.70)	498 (1.0)	400	603 (0.06)	551 (0.11)	518 (0.25)
Decarboxylation product of 1b	620 (0.32)	567 (0.46)	531 (0.61)	497 (1.0)	400	602 (0.07)	549 (0.12)	517 (0.25)
Dehydration product of 2	626 (0.24)	571 (0.56)	541 (0.94)	503.5 (1.0)	405	607 (0.08)	557 (0.21)	522.5 (0.32)
Coproporphyrin III	622 (0.35)	568 (0.47)	533 (0.68)	498 (1.0)	401	603 (0.07)	551 (0.12)	518 (0.25)
					—			458 (0.15)

ment ions representing three successive losses of 73 mass units ($\text{CH}_2\text{--CO}_2\text{--CH}_3$) from the molecular ions with corresponding metastable ions were seen in both spectra, confirming the presence of three methoxycarbonyl ethyl side chains, which characteristically undergo fission at the bond β to the porphin nucleus (Jackson *et al.*, 1965), in each decarboxylation product. Similarly only one of the four acidic β -substituents of porphyrin 2 was susceptible to decarboxylation (Elder & Chapman, 1970). The electronic spectra of the methyl esters of the decarboxylation products of porphyrins 1a and 1b are recorded in Table 3.

The electrophoretic mobilities of porphyrins in alkaline media depend on the number of ionized carboxyl groups/molecule (Papastamatis & Kench, 1952; Lockwood & Davies, 1962). Table 4, which records the electrophoretic mobilities of porphyrins 1a, 1b and 2, and their tricarboxylate decarboxylation products, shows that an acetate group exerts a greater influence on mobility than a propionate group, an effect which may partly explain the reported non-linear relation between number of carboxyl groups and electrophoretic mobility (Lockwood & Davies, 1962).

Thus porphyrins 1a, 1b and 2 probably each contain one acetate and three propionate β -substituents.

Nature of the four remaining β -substituents. (a) Porphyrin 1a (isocoproporphyrin). The mass spectrum of porphyrin 1a tetramethyl ester is shown in Fig. 2(a). The molecular ion at m/e 710 corresponds to a molecular formula of $\text{C}_{40}\text{H}_{46}\text{O}_8\text{N}_4$ (m/e calc. 710.3316, m/e obs. 710.3240, difference -11 p.p.m.). Thus porphyrin 1a is a structural isomer of coproporphyrin. The fragmentation pattern is very similar to that of coproporphyrin tetramethyl ester, though a metastable peak of comparable prominence to that at m/e 597 (m/e 710 $\rightarrow$ m/e 651, $\text{CO}_2\text{--CH}_3$) was not seen in a spectrum of synthetic coproporphyrin III tetramethyl ester obtained under identical conditions. The prominence of this metastable ion suggests that the ion at m/e 651 is partly formed by fission of a bond β to the porphin ring (Jackson *et al.*, 1965), additional evidence for an acetate side chain in porphyrin 1a.

The p.m.r. spectrum of porphyrin 1a tetramethyl ester is shown in Fig. 3(a). Assignments are based on data from published spectra of porphyrin esters in deuteriochloroform (Becker *et al.*, 1961; Caughey & Koski, 1962; Sano *et al.*, 1965). The signals at 6.51, 6.55 and 6.59 τ represent three methyl groups which occupy three of the four remaining β -positions. The triplet centred at 8.24 τ and the quartet at 6.04–6.26 τ , with J values of 7–7.5 Hz, represent the methyl and methylene protons of a ring-substituted ethyl group at the remaining β -position. Irradiation at 8.21 τ led to spin decoupling of the methylene quartet, which was replaced by a singlet at 6.13 τ (not shown in Fig. 3). Thus porphyrin 1a, in addition to one acetate

Table 4. *Electrophoretic mobilities of porphyrins*

Mobilities are expressed relative to coproporphyrin, for electrophoresis in 0.04M-Na₂CO₃-0.1mM-EDTA (disodium salt) pH10.6 at approx. 10V/cm at room temperature for 2h.

Porphyrin	Acidic β -substituents	Electrophoretic mobility
1a, 1b, 2	3 propionate, 1 acetate	2.1
1a, 1b, 2 after decarboxylation	3 propionate	0.5
Coproporphyrin	4 propionate	1.0
Pentacarboxylate	4 propionate, 1 acetate	2.7

Fig. 2. *Mass spectra of porphyrin tetramethyl esters*

All ions of relative intensity $\geq 2\%$ are shown. (a) Porphyrin 1a (isocoproporphyrin) from faeces of porphyric rats; (b) porphyrin 1b (de-ethylisocoproporphyrin) from faeces of patients with porphyria. M^* indicates positions of metastable peaks.

and three propionate groups, contains one ethyl and three methyl β -substituents.

The p.m.r. spectrum confirms the presence of one methoxycarbonylmethyl and three methoxycarbonyl-ethyl groups. The methylene protons of the methoxycarbonyl methyl group give rise to a singlet at 5.05 τ . The proton numbers of this group and of the triplet

at 8.24 τ are in the ratio 1.7:3.0 (calc. 2:3). The multiplets centred at 5.76 and 6.84 τ , with J values of 6.9–7.3 Hz, represent the methylene protons of the methoxycarbonyl-ethyl side chains, α and β to the ring respectively. The four narrow singlets at 6.32, 6.39, 6.42 and 6.45 τ arise from the four methoxycarbonyl groups, that at 6.32 τ probably representing the

methoxycarbonyl protons of the methoxycarbonylmethyl side chain, since a similar downfield shift (0.12τ) of the methoxycarbonyl signal has been observed for the methoxycarbonylmethyl groups of uroporphyrin I octamethyl ester (Becker *et al.*, 1961). Minor signals in the methoxycarbonyl and ring methyl regions are probably due to the small amount of coproporphyrin tetramethyl ester present in the sample.

(b) Porphyrin 1b (de-ethylisocoproporphyrin). The mass spectrum of porphyrin 1b tetramethyl ester (Fig. 2b) shows a molecular ion at m/e 682 ($C_{38}H_{42}O_8N_4$; m/e calc. 682.3003, m/e obs. 682.2990, difference $-2p.p.m.$). The fragment ions and metastable peaks correspond closely to those observed for porphyrin 1a, but are at 28 mass units (C_2H_4) lower, which suggests that porphyrin 1b has an unsubstituted β -position in place of the ethyl group of porphyrin 1a.

The p.m.r. spectrum of porphyrin 1b tetramethyl ester (Fig. 3b) shows no signals corresponding to those of the ring ethyl group in porphyrin 1a. The signal at 1.09τ probably represents a hydryl group attached directly to the porphyrin ring, that is porphyrin 1b contains a single unsubstituted β -position. Sano *et al.* (1965) reported values of 1.12 and 1.35τ for the quartets ($J = 1$ Hz) representing ring hydryl groups in deuteroporphyrin and pemptoporphyrin dimethyl esters respectively, and that the methyl group on the same pyrrolyl ring as the hydryl group gave rise to a doublet ($J = 1$ Hz). With porphyrin 1b, expansion of the $6-7\tau$ region of the spectrum failed to resolve the ring methyl signals, which were represented by a single broad peak. Expansion of the signal at 1.09τ showed it to be a multiplet, though it was not possible to determine whether it was a triplet, which would indicate a methylene group at the other β -position on the pyrrolyl ring, or a quartet, or the two superimposed. An attempt to simplify the hydryl signal by spin decoupling, by irradiation at the frequencies of the ring methyl and methoxycarbonylmethyl methylene protons, failed owing to the small amounts of material available. Thus no conclusion was reached about the relationship of the ring hydryl to the other β -substituents. The ratio of the integrated areas under the signals at 1.09 and 5.01τ was approx. $1:3$, suggesting that a methoxycarbonylmethyl-substituted porphyrin other than 1b might be present in the sample, which may partly explain the poor resolution of the ring methyl signals.

(c) Porphyrin 2 (hydroxyisocoproporphyrin). Mass spectrometry of the trimethyl silyl ether of the tetramethyl ester of porphyrin 2 confirmed that porphyrin 2 tetramethyl ester had mol.wt. 726, corresponding to a molecular formula of $C_{40}H_{46}O_9N_4$, and contained one hydroxyl group, as reported by Elder (1971b). This, together with the evidence that it contains one acetate and three propionate groups, sug-

gested that it has the same general structure as porphyrins 1a and 1b with either a 1-hydroxyethyl (see formula III) or 2-hydroxyethyl group in place of the ethyl and hydryl β -substituents, and is not an α - or β -hydroxypropionate substituted porphyrin as suggested previously (Elder & Chapman, 1970; Elder, 1971b).

The p.m.r. spectrum of porphyrin 2 tetramethyl ester is shown in Fig. 3(c). The signals between 4.5 and 7.0τ , with calculation of their proton numbers from the integrating curve, confirm the presence of one methoxycarbonylmethyl, three 2-methoxycarbonylethyl and three methyl β -substituents. These signals, and those of the bridge methene protons, are all at a lower field ($0.07-0.18\tau$, average 0.13τ) than those in the spectrum of porphyrin 1a, as expected from the known concentration dependence of p.m.r. spectra of porphyrin esters in deuteriochloroform (Abraham *et al.*, 1966). When this effect is taken into account the one proton signal at 4.24τ can be assigned to the methene proton of a ring substituted secondary alcohol. Thus Sano *et al.* (1965) found a value of 4.5τ for this proton in haematoporphyrin IX dimethyl ester, whereas the signal for the corresponding proton in bis-(β -hydroxypropionic acid) deuteroporphyrin IX tetramethyl ester was at 3.85τ (Sano, 1966). The doublet at $7.57-7.64\tau$ ($J = 7$ Hz) represents three protons. If this signal is assigned to the methyl group of a ring-substituted secondary alcohol, it is at significantly lower field, even after allowing for the effect of dilution, than the corresponding doublets in the spectrum of haematoporphyrin IX dimethyl ester which are centred at 8.28 and 8.33τ ($J = 6$ Hz). This relative deshielding of the methyl group in porphyrin 2 might be due to the effect of a carbonyl function on a neighbouring substituent (Jackman, 1959). If this is correct it suggests that in porphyrin 2 the hydroxyethyl group is flanked by an acetate or propionate group either on the same or a neighbouring pyrrolyl ring, since the effect is not observed with haematoporphyrin IX dimethyl ester in which each hydroxyethyl group lies between two methyl groups. Since deshielding of the methyl protons of the ethyl groups of porphyrin 1a relative to those of mesoporphyrin IX dimethyl ester was not observed, porphyrins 1a and 2 either differ in the order of β -substituents around the porphyrin nucleus or some additional factor, such as hydrogen bonding between the hydroxyl and carbonyl groups, is responsible for holding the carbonyl group in porphyrin 2 in position. No signal in the spectrum could be assigned to the hydroxyl proton. Unfortunately the spectrum is obscured by impurities above 8.2τ but the close agreement between expected and determined proton numbers makes it unlikely that this region contains signals from porphyrin 2.

(d) Conversion of porphyrin 2 into porphyrin 1a. Dehydration of porphyrin 2 gave a porphyrin, de-

hydroisocoproporphyrin (see formula IV), the methyl ester of which had mol.wt. 708 (determined by mass spectrometry). Its electronic spectrum is given in

Table 3. The small shift in the position of the Soret maximum and the aetio-type spectrum with a relatively intense band III are in keeping with conversion

Fig. 3
1972

Fig. 3. *P.m.r. spectra of porphyrin tetramethyl esters in C²HCl₃*

The sample of porphyrin 1a (*a*; isocoproporphyrin, 9.2mg) was isolated from faeces of porphyric rats, those of porphyrins 1b (*b*; de-ethylisocoproporphyrin; 4.6mg) and 2 (*c*; hydroxyisocoproporphyrin; 0.35mg) from faeces of patients with porphyria.

of a 1-hydroxyethyl group into a vinyl group, rather than with dehydration of an hydroxypropionate group to an acrylate group, which would give a porphyrin with a Soret maximum at 413nm and a rhodo-type spectrum (French *et al.*, 1970). The product of hydrogenation of this porphyrin was identical with porphyrin 1a in its electronic spectrum and behaviour on t.l.c. in solvent system B.

Since vinylic porphyrins may be slowly hydrated by mineral acids (Falk *et al.*, 1956; Clezy & Barrett, 1961) the dehydration product was dissolved in 7M-HCl, kept in the dark at room temperature for 48h, dried and re-esterified with methanol-H₂SO₄. No hydroxylated porphyrin was detected on t.l.c. of the re-esterified material, which had the same electronic spectrum and chromatographic behaviour as the original porphyrin.

Evidence that dehydroisocoproporphyrin is present in faeces

Some evidence was obtained that small amounts of dehydroisocoproporphyrin tetramethyl ester were present in samples of de-ethylisocoproporphyrin pre-

pared from porphyric faeces. Thus the mass spectra of samples of this porphyrin from human and rat faeces showed an ion at *m/e* 708 (Fig. 2*b*), the molecular weight of dehydroisocoproporphyrin tetramethyl ester, in addition to the molecular ion of de-ethylisocoproporphyrin tetramethyl ester at *m/e* 682, the ion at *m/e* 708 being more prominent (47% of the intensity of the ion at *m/e* 682) in the spectrum of the sample from rat faeces. The p.m.r. spectrum of the combined 1b fractions (7.5mg) from human and rat faeces (Fig. 4) contains signals which were not apparent in the spectrum of the sample of de-ethylisocoproporphyrin (porphyrin 1b) isolated from human faeces, and which can be tentatively assigned to the protons of a ring-substituted vinyl group. Thus the multiplet with peaks at 3.74–4.02 τ is similar in shape and position to that given by the vinyl CH₂ protons of pemto- and proto-porphyrin IX dimethyl esters (Sano *et al.*, 1965), and the signals around 1.96 τ may be a poorly resolved multiplet representing the vinyl CH proton, which is at 2.37–1.77 τ for proto-porphyrin IX dimethyl ester. Dehydroisocoproporphyrin, prepared by dehydration of hydroxyisocoproporphyrin, and de-ethylisocoproporphyrin methyl

Fig. 4. Part of the p.m.r. spectrum in C^2HCl_3 of the combined porphyrin 1b fractions from human and rat porphyric faeces

esters were not separated by t.l.c. in solvent system A followed by solvent system B, which suggests that any naturally occurring dehydroisocoproporphyrin would be in fraction 1b. Another porphyrin of mol.wt. 708, the tetramethyl ester of tris-(2-carboxyethyl)-mono-(2-carboxyvinyl)-tetramethyl porphin, was only partially separated from dehydroisocoproporphyrin ester in these solvent systems. This porphyrin, which is present in normal human and calf bile and meconium (French & Thonger, 1966) has not been isolated from faeces. The electronic spectrum of its methyl ester is rhodo in type with a Soret maximum at 413 nm (French *et al.*, 1970), whereas that of fraction 1b from rat faeces, was aetio in type with a Soret maximum at 403.5 nm, which is more in keeping with contamination of de-ethylisocoproporphyrin by a porphyrin with a Soret maximum at 405 nm than by a rhodo-type porphyrin with a Soret maximum at 413 nm. The ion at m/e 708 might also represent an ion formed by loss of methanol from the methyl ether of hydroxyisocoproporphyrin tetramethyl ester (mol.wt. 740), which is formed as a by-product during esterification of hydroxyisocoproporphyrin and migrates on t.l.c. with pentacarboxylate porphyrin pentamethyl ester, though no appropriate parent ion was observed. However, the data from the electronic, mass and p.m.r. spectra in general support the identification of dehydroisocoproporphyrin methyl ester as a component of fraction 1b, particularly that from porphyric rat faeces.

Discussion

Tetracarboxylate porphyrins containing one acetate and three propionate groups have not been described previously, perhaps because, apart from hydroxyisocoproporphyrin, they behave similarly to coproporphyrin in several of the standard systems for

porphyrin fractionation. The structures proposed have not been confirmed by comparison with unambiguously synthesized compounds since, except for two position-isomers, hardero- and isohardero-porphyrin (Kennedy *et al.*, 1970), of the tripropionate decarboxylation product of dehydroisocoproporphyrin, these have not been prepared, and insufficient dehydroisocoproporphyrin was obtained to prepare enough derivative for comparison. The lack of suitable reference compounds has also prevented investigation of the order of β -substituents around the porphin ring, which is of particular importance since only type III isomers are intermediates in haem biosynthesis. De Matteis *et al.* (1961) and San Martin de Viale *et al.* (1970) have reported that porphyrins in the urine of hexachlorobenzene-treated rats are type III isomers and, if the same is true for the faecal porphyrins, it is likely that those described here are also type III isomers.

Isocoproporphyrin and de-ethylisocoproporphyrin are probably produced by the action of intestinal micro-organisms on dehydroisocoproporphyrin(o)gen (Scheme 1), for it is well established that the vinyl groups of protoporphyrin IX are converted into ethyl and hydryl groups in this way (Barnes, 1963; French *et al.*, 1964). This suggests that dehydroisocoproporphyrin, or a precursor of it, is excreted in the bile of patients with symptomatic cutaneous hepatic porphyria and of rats with hexachlorobenzene poisoning, since the other recognized sources of faecal porphyrins, such as the diet and synthesis by micro-organisms within the intestinal lumen, do not account for the absence of this group of tetracarboxylate porphyrins in the faeces of control rats or for their excretion being confined to a particular type of hepatic porphyria (Elder, 1971*b*). The proportions of the three porphyrins in faeces would be expected to vary, depending on the extent of bacterial modifica-

Scheme 1. *Postulated origin of faecal tetracarboxylate porphyrins from dehydroisocoproporphyrin(ogen)*

[P] = tris-(2-carboxyethyl)-trimethylporphyrin or tris-(2-carboxyethyl)-trimethyl hexahydroporphyrin. Formulae in parentheses represent additional β -substituents.

tion of dehydroisocoproporphyrin. Watson *et al.* (1969) have demonstrated differences in the metabolism of the vinyl groups of bile pigments by rat and human faecal flora, and this may explain the difference in the proportions of faecal porphyrins observed.

It is possible that hydroxyisocoproporphyrin is also derived from dehydroisocoproporphyrin, by hydration of the vinyl group (Scheme 1). Although bacterial hydration of porphyrin vinyl groups has not been reported, hydration of protoporphyrin IX (Falk *et al.*, 1956; Clezy & Barrett, 1961) and protoporphyrinogen IX (Sano *et al.*, 1964) by mineral acids occurs and a similar hydration of dehydroisocoproporphyrin, or its porphyrinogen, during the extraction procedure may lead to the formation of hydroxyisocoproporphyrin, though an attempt to hydrate pure dehydroisocoproporphyrin in this way was not successful. Rimington *et al.* (1972) have described a patient with Felty's syndrome and cutaneous hepatic porphyria whose urine contained porphyrins 1 and 2 and a hydrophilic porphyrin, similar to the porphyrin-peptide conjugates of variegate porphyria (Rimington *et al.*, 1968). A hydrophilic porphyrin with similar properties was also present, as well as hydroxyisocoproporphyrin, in the urine of the patient with cutaneous hepatic porphyria and infective hepatitis studied by Elder & Chapman (1970), and the same hydrophilic porphyrin may also occur in the faeces of some patients with symptomatic cutaneous hepatic porphyria (G. H. Elder, unpublished work). If this porphyrin is a conjugate of dehydroisocoproporphyrin, rather than of protoporphyrin as in variegate porphyria, hydroxyisocoproporphyrin may be formed from it

during extraction (Scheme 1), in the same way as haematoporphyrin IX arises during the isolation of porphyrin-peptide conjugates from the urine, faeces and bile of patients with variegate porphyria (Rimington *et al.*, 1968; Belcher *et al.*, 1969). It is clear from the investigation of these two patients that readily detectable amounts of hydroxyisocoproporphyrin and porphyrin 1 can be excreted in the urine. Although this does not seem to be a major route of excretion in most patients with symptomatic cutaneous hepatic porphyria or in rats with hexachlorobenzene poisoning, it may become important when biliary function is impaired.

Dehydroisocoproporphyrin and its derivatives account for a significant fraction of the difference between the amount of porphyrin excreted daily by the porphyric and control rats (Table 2). Although the rats were of a different strain and had been receiving hexachlorobenzene for much longer than reported in other studies, the abnormality of urinary haem-precursor excretion observed resembles that reported by others (Ockner & Schmid, 1961; De Matteis *et al.*, 1961; San Martin de Viale *et al.*, 1970), with the important difference that, even though no attempt was made to prevent conversion of porphobilinogen into uroporphyrin during extraction, the concentration of uroporphyrin was less than that of coproporphyrin, which has previously only been reported for rats in the initial stages of poisoning (De Matteis *et al.*, 1961). However, total daily excretion of uroporphyrin and heptacarboxylate porphyrin is about equal to that of coproporphyrin. Although the faecal porphyrins have not previously been investigated in detail, Ockner & Schmid (1961) reported slight to moderate increases in both coproporphyrin and

Scheme 2. Hypothetical pathway for formation and degradation of dehydroisocoproporphyrin(ogen)

Formulae are represented as in Scheme 1.

protoporphyrin. Thus it is probable that the excretion of dehydroisocoproporphyrin and its bacterial metabolites is a hitherto overlooked feature of this form of experimental porphyria. The same porphyrins are also excreted by patients with symptomatic cutaneous hepatic porphyria, which reinforces the view that hexachlorobenzene poisoning is an experimental model for this type of porphyria (De Matteis, 1967).

In both conditions increased amounts of porphyrin derived from all the porphyrinogens known to be precursors of protoporphyrinogen IX are excreted (San Martin de Viale *et al.*, 1970). Scheme 2 illustrates a possible way of relating dehydroisocoproporphyrinogen to the biosynthesis of protoporphyrinogen IX. At present there is no conclusive evidence that reaction (i) (Scheme 2) takes place in the liver, though this seems a more likely site than the intestinal lumen, since transformation of the propionate groups of excreted porphyrins and bile pigments by intestinal micro-organisms has not been reported. In the liver, the oxidative decarboxylation-dehydrogenation of the 2- and 4-propionate groups of coproporphyrinogen III is catalysed by a mitochondrial enzyme, coproporphyrinogenase, which has a high degree of substrate specificity (Batlle *et al.*, 1965), though, of porphyrinogens with acetate groups, only uroporphyrinogen III seems to have been excluded as a substrate (Sano & Granick, 1961). If dehydroisocoproporphyrinogen is produced by the action of coproporphyrinogenase on pentacarboxylate porphyrinogen III (reaction i, Scheme 2), the vinyl group may occupy the 2-position, since there is some evi-

dence that the 2-propionate group is more susceptible to enzymic attack than that at the 4-position (Kennedy *et al.*, 1970). The p.m.r. spectrum of hydroxyisocoproporphyrin provides evidence that the hydroxyethyl group of this porphyrin is adjacent to either an acetate or a propionate group. If the same is true for the vinyl group of dehydroisocoproporphyrinogen and if this is derived from the 2-propionate group of pentacarboxylate porphyrinogen III, the acetate group of dehydroisocoproporphyrinogen probably occupies either the 1- or 3-position, but further investigation of the position-isomerism of these porphyrins is required. If this interpretation of the origin and structure of dehydroisocoproporphyrinogen is correct, it implies that its precursor, pentacarboxylate porphyrinogen III, also has its acetate group in the same position and is not a random mixture of isomers, each with an acetate group at position 1, 3, 5 or 8.

The increased excretion of dehydroisocoproporphyrin and related porphyrins by the porphyric rats is of the same order as that of the other porphyrins, including protoporphyrin (Table 2). This suggests that dehydroisocoproporphyrinogen may not be a side product of haem biosynthesis but can be metabolized further, possibly by enzymic decarboxylation of its acetate group to give harderoporphyrinogen (reaction ii, Scheme 2), a probable precursor of protoporphyrinogen IX (Sano & Granick, 1961; Kennedy *et al.*, 1970). One difficulty in accepting that this hypothetical alternative pathway functions within the liver cell lies in the intracellular distribution of the enzymes involved. Thus uroporphyrinogen decarboxylase, which catalyses the decarboxylation of porphyrinogen acetate groups, is present in the soluble fraction (Mauzerall & Granick, 1958; San Martin de Viale & Grinstein, 1968), whereas coproporphyrinogenase is a mitochondrial enzyme. Possibly the liver damage common to both symptomatic cutaneous porphyria and hexachlorobenzene poisoning enables substrates to reach enzymes that are not normally accessible.

The mechanism of the disturbances of porphyrin metabolism in both types of porphyria is unknown. The significance of the excretion of dehydroisocoproporphyrin in both conditions, and the possible existence of the pathway shown in Scheme 2, require further investigation.

I thank Dr. I. A. Magnus and Mr. J. E. Wilson for their collaboration with the animal experiments which were done at the Institute of Dermatology, Homerton Grove, London E.9. I also thank Professor C. H. Gray for help and advice; Dr. J. R. Chapman, Dr. D. C. Nicholson and Dr. M. S. Stoll for valuable discussion; and Dr. P. F. Borrie, Dr. D. D. Munro and Dr. Roger Williams for allowing me to study patients under their care. The work was carried out while holding a Medical Research Council Clinical Research Fellowship.

References

- Abraham, R. J., Burbridge, P. A., Jackson, A. H. & MacDonald, D. B. (1966) *J. Chem. Soc. B* 620
- Barnes, H. D. (1963) *S. Afr. J. Lab. Clin. Med.* **9**, 177
- Barrett, J. (1959) *Nature (London)* **183**, 1185
- Battle, A. M. del C., Benson, A. & Rimington, C. (1965) *Biochem. J.* **97**, 731
- Becker, E. D., Bradley, R. B. & Watson, C. J. (1961) *J. Amer. Chem. Soc.* **83**, 3743
- Belcher, R. V., Smith, S. G. & Mahler, R. (1969) *Clin. Chim. Acta* **25**, 45
- Caughey, W. S. & Koski, W. S. (1962) *Biochemistry* **1**, 923
- Chu, T. C. & Chu, E. J.-H. (1959) *J. Biol. Chem.* **234**, 2741
- Chu, T. C. & Chu, E. J.-H. (1967) *Clin. Chem.* **13**, 371
- Clezy, P. S. & Barrett, J. (1961) *Biochem. J.* **78**, 798
- De Matteis, F. (1967) *Pharmacol. Rev.* **19**, 523
- De Matteis, F., Prior, B. E. & Rimington, C. (1961) *Nature (London)* **191**, 363
- Dowdle, E., Goldswain, P., Spong, N. & Eales, L. (1970) *Clin. Sci.* **39**, 147
- Eales, L., Grosser, Y. & Sano, S. (1971) *S. Afr. J. Lab. Clin. Med.* **17**, 160
- Eales, L., Levey, M. J. & Sweeney, G. D. (1966) *S. Afr. Med. J.* **40**, 63
- Edmondson, P. R. & Schwartz, S. (1953) *J. Biol. Chem.* **205**, 605
- Elder, G. H. (1971a) *J. Chromatogr.* **59**, 234
- Elder, G. H. (1971b) *S. Afr. J. Lab. Clin. Med.* **17**, 45
- Elder, G. H. & Chapman, J. R. (1970) *Biochim. Biophys. Acta* **208**, 535
- Falk, J. E. (1964) *Porphyryns and Metalloporphyryns*, pp. 124, 125, 171, 232, Elsevier Publishing Co., Amsterdam, London and New York
- Falk, J. E., Dresel, E. I. B., Benson, A. & Knight, B. C. (1956) *Biochem. J.* **63**, 87
- French, J. M. & Thonger, E. (1966) *Clin. Sci.* **31**, 337
- French, J. M., England, M. T., Lines, J. & Thonger, E. (1964) *Arch. Biochem. Biophys.* **107**, 404
- French, J. M., Nicholson, D. C. & Rimington, C. (1970) *Biochem. J.* **120**, 393
- Gray, C. H. & Neuberger, A. (1950) *Biochem. J.* **47**, 81
- Grinstein, M., Schwartz, S. & Watson, C. J. (1945) *J. Biol. Chem.* **157**, 323
- Herbert, F. K. (1966) *Clin. Chim. Acta* **13**, 19
- Jackman, L. M. (1959) *Applications of NMR Spectroscopy in Organic Chemistry*, p. 122, Pergamon Press Ltd., London
- Jackson, A. H., Kenner, G. W., Smith, K. M., Aplin, R. T., Budzikiewicz, H. & Djerassi, C. (1965) *Tetrahedron* **21**, 2913
- Jensen, J. (1963) *J. Chromatogr.* **10**, 236
- Kennedy, G. Y., Jackson, A. H., Kenner, G. W. & Suckling, C. J. (1970) *FEBS Lett.* **6**, 9
- Lockwood, W. H. & Davies, J. L. (1962) *Clin. Chim. Acta* **7**, 301
- Mauzerall, D. & Granick, S. (1956) *J. Biol. Chem.* **219**, 435
- Mauzerall, D. & Granick, S. (1958) *J. Biol. Chem.* **232**, 1141
- Nicholas, R. E. H. & Rimington, C. (1949) *Scand. J. Clin. Lab. Invest.* **1**, 12
- Ockner, R. K. & Schmid, R. (1961) *Nature (London)* **189**, 499
- Papastamatis, S. C. & Kench, J. E. (1952) *Nature (London)* **170**, 33
- Rimington, C., Lockwood, W. H. & Belcher, R. V. (1968) *Clin. Sci.* **35**, 211
- Rimington, C., Sears, W. G. & Eales, L. (1972) *Clin. Chem.* in the press
- San Martin de Viale, L. C. & Grinstein, M. (1968) *Biochim. Biophys. Acta* **158**, 79
- San Martin de Viale, L. C., Viale, A. A., Nacht, S. & Grinstein, M. (1970) *Clin. Chim. Acta* **28**, 13
- Sano, S. (1966) *J. Biol. Chem.* **241**, 5276
- Sano, S. & Granick, S. (1961) *J. Biol. Chem.* **236**, 1173
- Sano, S., Nanzyo, N. & Rimington, C. (1964) *Biochem. J.* **93**, 270
- Sano, S., Shingu, T., French, J. M. & Thonger, E. (1965) *Biochem. J.* **97**, 250
- Schmid, R. (1962) *Les Maladies due Metabolisme des Porphyrines*, p. 33, Presses Universitaires de France, Paris
- Stoll, M. S. & Gray, C. H. (1970) *Biochem. J.* **117**, 271
- Sweeney, G. D. (1963) *S. Afr. J. Lab. Clin. Med.* **9**, 182
- Taddeini, L. & Watson, C. J. (1968) *Semin. Hematol.* **5**, 335
- Tiers, G. V. D. (1958) *J. Phys. Chem.* **62**, 1151
- Watson, C. J., Moscovitz, A., Lightner, D. A., Petryka, Z. J. & Davis, E. (1969) *Biochem. Med.* **2**, 485