A Study of the Porphyrins Excreted in the Urine by a Case of Congenital Porphyria

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Congenital porphyria is a rare disease. Most of our knowledge concerning it has been derived from investigations by Fischer and his collaborators upon the patient Petry and the study of the disease in cattle by Fourie, Rimington and their associates. Uroporphyrin I was first isolated from Petry's urine (Fischer, 1915*a*) and coproporphyrin I from his faeces (Fischer, 1915*b*). At the patient's death, a detailed chemical examination of his organs was carried out by Fischer, Hilmer, Lindner & Pützer (1925), while the pathological and fluorescence microscopical investigation was performed by Borst & Königsdörfer (1929). Examining some of the Petry urine uroporphyrin at a later date, Fischer & Hofmann (1937) obtained evidence of the presence of another porphyrin which they considered was uroporphyrin III. Rimington (1936) encountered a uroporphyrin, differing from uroporphyrin I, as a minor constituent of the South African bovine material.

Within recent years, the isotope-labelling technique has been applied both in England and in America (Gray & Neuberger, 1950; Gray, Neuberger & Sneath, 1950; Gray, Muir & Neuberger, 1950; London, Shemin, West & Rittenberg, 1949; Grinstein, Aldrich, Hawkinson & Watson, 1949) in an attempt to elucidate the relationship existing, in patients with congenital porphyria, between their urinary uroporphyrin and coproporphyrin, faecal urobilinogen and their blood haemin. No closer study has been made, however, of the types of porphyrin which they excrete.

The technique of paper chromatography applied to porphyrins (Nicholas & Rimington, 1949, 1951*a*) makes possible the recognition in very small amounts of material of pigments differing in the number of carboxyl groups they possess, and has revealed the presence in porphyrin mixtures from several natural sources of hitherto unknown porphyrins with seven, six, five or three carboxyl groups respectively. A careful study of porphyrin chromatography upon standardized adsorbents and using purified solvent mixtures (Nicholas, 1951) has greatly facilitated the separation and purification of individual constituents from crude mixtures.

The present paper describes the application of these techniques to a study of the porphyrins present in the urine of a case of congenital porphyria. Such urine provides a very convenient source of uroporphyrin I, the pure octamethyl ester of which may readily be obtained in quantity by following the procedures described.

EXPERIMENTAL

The patient was a male congenital porphyrinuric, first described by Mackey & Garrod (1926). He has also been the subject of some recent experiments with isotopically labelled glycine (Gray & Neuberger, 1950; Gray, Neuberger & Sneath, 1950; Gray, Muir & Neuberger, 1950). The urine examined (15·7 l.) was collected during August 1950, preserved with toluene, and sent to the laboratory. It contained 46·12 mg./l. of uroporphyrin, determined by the method of Rimington & Sveinsson (1950).

The urine was filtered, acidified with acetic acid (8 ml./l.) and left at 5° overnight. The precipitate was collected on the centrifuge. The supernatant retained a little porphyrin which was adsorbed upon kieselguhr then eluted by stirring with dilute ammonia. After precipitation and esterification followed by chromatography, there was recovered from this source 40 mg. of uroporphyrin octamethyl ester, m.p. 292°.

The main precipitate of porphyrin was suspended in 300 ml. of acetic acid and the mixture boiled under a reflux condenser for 5 hr.* When cold, it was filtered by suction through a layer of approx. 1 cm. of Celite 'Hyflo' kieselguhr supported by two Whatman no. 54 filter papers in a 7 cm. diameter Büchner funnel. The residue was washed, using minimal suction, by three lots of hot acetic acid, the last washing being colourless. These washings were added to the dark-brown original filtrate, the further examination of which is described below. The purified porphyrin was then eluted from the kieselguhr by drawing through it, under minimal suction, successive small quantities of N-NaOH. About 500 ml. was required to remove all the porphyrin. To the deep-red solution, sufficient concentrated HCl was added to bring the pH to 3·1, as determined by the glass electrode and after standing overnight at 5° the precipitated porphyrin was separated on the centrifuge from the pale straw-coloured supernatant and washed four times with N-acetic acid. It was then dried *in vacuo*. To the dry residue were

* We are greatly indebted to Dr J. Keilin for an outline of a method (Keilin, 1950) used by her for isolating uroporphyrin. From this method we have adopted the very convenient manoeuvre of boiling the crude porphyrin precipitate with acetic acid to remove coproporphyrin, but otherwise have followed our usual procedures which are here described in full.

added approx. 100 ml. of methanol, previously saturated with HCl gas, and the mixture left at room temperature for 24 hr. An equal volume of chloroform was then added and the mixture passed through a no. 3 sintered-glass filter. It was placed in a 1 l. separatory funnel and saturated aqueous sodium acetate solution added rapidly, mixing being achieved by swirling, until the colour change from purplish to ruby-red indicated neutralization of the mineral acid (pH > 3). About 500 ml. of distilled water was then added and the mixture was shaken thoroughly. The $CHCl_3$ layer was washed repeatedly with water, then by dilute ammonia, again with water, filtered and concentrated by distillation under reduced pressure.

To the hot concentrated solution were added approx. 3 vol. boiling methanol which caused immediate crystallization of the crude porphyrin ester. After chilling overnight, the crystals were collected on a no. 3 sintered-glass filter, washed with methanol, then ether and dried (wt. 712 mg.; m.p. 286°). The washings were added to the red mother liquor which was worked up separately (see below).

A paper chromatogram (Nicholas & Rimington, 1949, 1951*a*) on the crude uroporphyrin of m.p. 286° showed the presence of 8 carboxyl porphyrin (very strong), 7 carboxyl porphyrin (moderately strong) and approx. 6 carboxyl porphyrin (strong). Recrystallization from hot $CHCl_3$-methanol raised the melting point to 291°, but did not appreciably alter the paper chromatographic analysis.

Chromatographic purification of the crude uroporphyrins (see Fig. 1)

Isolation of uroporphyrin I. The material (712 mg.) was divided and chromatographed in two lots on MgO grade III (Nicholas, 1951) columns each of 17 × 5 cm., packed with $CHCl_3$. Development with 100 vol. $CHCl_3$/0·5 vol. methanol caused the greater part of the pigment to pass through the column as a compact band (*A* i). The pigment from this fraction was crystallized in typical hair-like crystals (634·4 mg., m.p. 293°) leaving an almost colourless mother liquor. The paper chromatogram revealed 8-carboxyl porphyrin only. (Micro-analysis: C, 61·4; H, 5·9; N, 5·4. Calc. for $C_{48}H_{54}O_{16}N_4$: C, 61·1; H, 5·8; N, 5·9%.) Decarboxylation afforded coproporphyrin I, m.p. 251–253°, unchanged by recrystallization (yield, 44% of theory). The material was thus pure uroporphyrin I ester. Continued development with a mixture of equal volumes of $CHCl_3$ and methanol caused the separation of two rather diffuse bands (*A* ii and *A* iii) which were collected together, leaving a deeply pigmented narrow layer (*A* iv) on the surface of the adsorbent. This could only be removed by methanol containing 1% (w/v) H_2SO_4. The pigment was transferred to $CHCl_3$ and crystallized by addition of boiling methanol, m.p. 289°, α-band 624·7 mμ. Rechromatography upon MgO afforded an ester crystallizing in the typical habit of uroporphyrin I (m.p. 292°). Experience has shown that

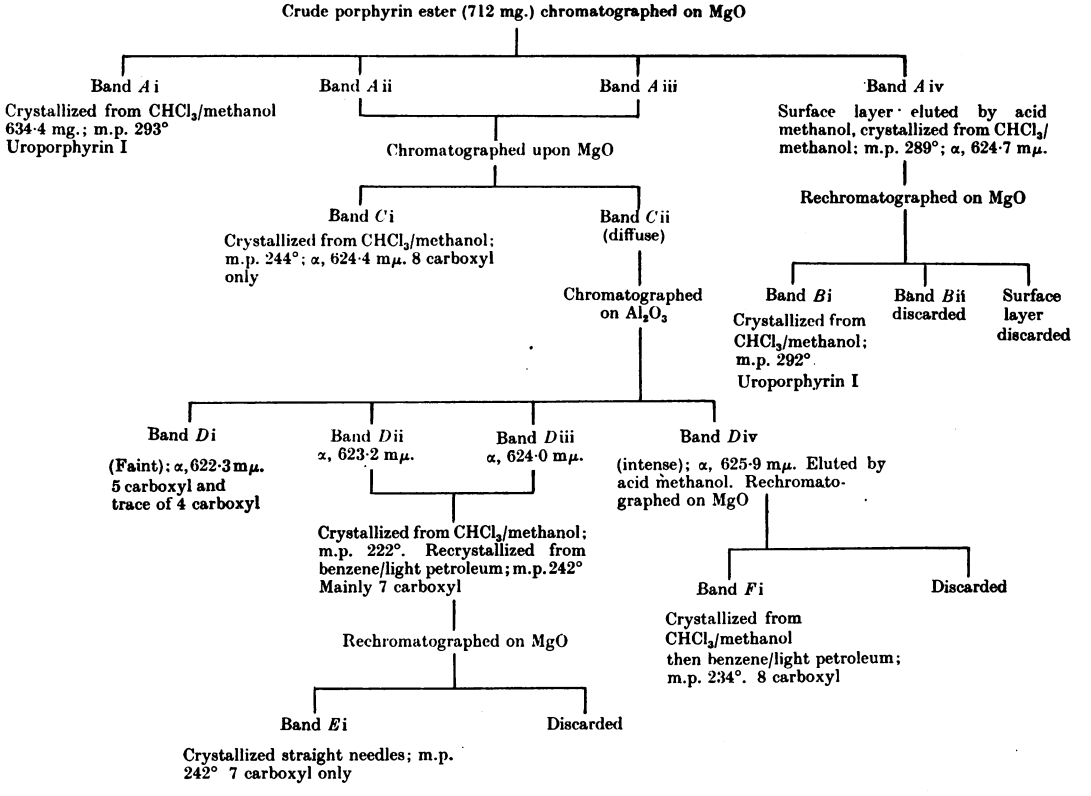

Fig. 1. Chromatographic separation of esters of uroporphyrin I (m.p. 293°), an 8 carboxyl porphyrin (m.p. 234°), a 7 carboxyl porphyrin (m.p. 242°) and a 5 carboxyl porphyrin.

such zones of pigment which cannot be moved from the top of adsorbent columns are usually either unesterified material or are composed of porphyrin esters held there by impurities.

The diffuse bands (*A* ii and *A* iii) which were eluted together were rechromatographed on MgO. Development with 100 vol. $CHCl_3$/0·2 vol. methanol caused slow separation of a band from which an ester was crystallized in small hair-like needles (m.p. 244°, α-band 624·4 mμ.). Paper chromatography showed an 8-carboxyl spot only.

Isolation of porphyrins with 7 and 5 carboxyl groups respectively

Increasing the proportion of methanol to $CHCl_3$, after this band had been collected, removed the remainder of the pigment as a broad diffuse zone (*C* ii) passing slowly down the column. Paper chromatography showed this to be a mixture of porphyrins with 8, 7 and 6 or 5 carboxyl groups respectively. Further separation was effected by putting the material, dissolved in $CHCl_3$, on to a column of Al_2O_3, grade IV, packed in benzene and developing with $CHCl_3$. Four bands appeared, the lowest (*D* i, α, 622·3 mμ.) was faint, and although there was insufficient pigment for crystallization, paper chromatography showed it to be a 5 carboxyl porphyrin accompanied by a trace of coproporphyrin. The next band (*D* ii, α, 623·2 mμ.) was fairly strong, but was not sharply separated from the one above it (*D* iii, α, 624 mμ.). They were therefore combined and the material crystallized, first from $CHCl_3$/methanol (m.p. 222°) then from benzene/light petroleum, being obtained in straight needles, m.p. 242°. It was still heterogeneous, paper chromatography showing a 7 carboxyl porphyrin together with some 6 carboxyl and possibly also some 5 carboxyl porphyrin. Rechromatography upon MgO grade III gave a sharply defined lower band (*E* i) containing the 7 carboxyl porphyrin alone, as shown by the paper chromatogram; long straight needles, m.p. 242°.

The uppermost band (*D* iv) in the Al_2O_3 column from which these porphyrins were obtained was compact and intense (α, 625·9 mμ.). It was eluted by acid methanol, the pigment transferred to $CHCl_3$ and chromatographed upon MgO, grade III, which removed some brown material and afforded a porphyrin ester (*F* i) crystallizing in aggregates of hair-like needles and giving an 8 carboxyl spot on paper chromatography. The melting point was raised by recrystallization from benzene/light petroleum to 234°. This material

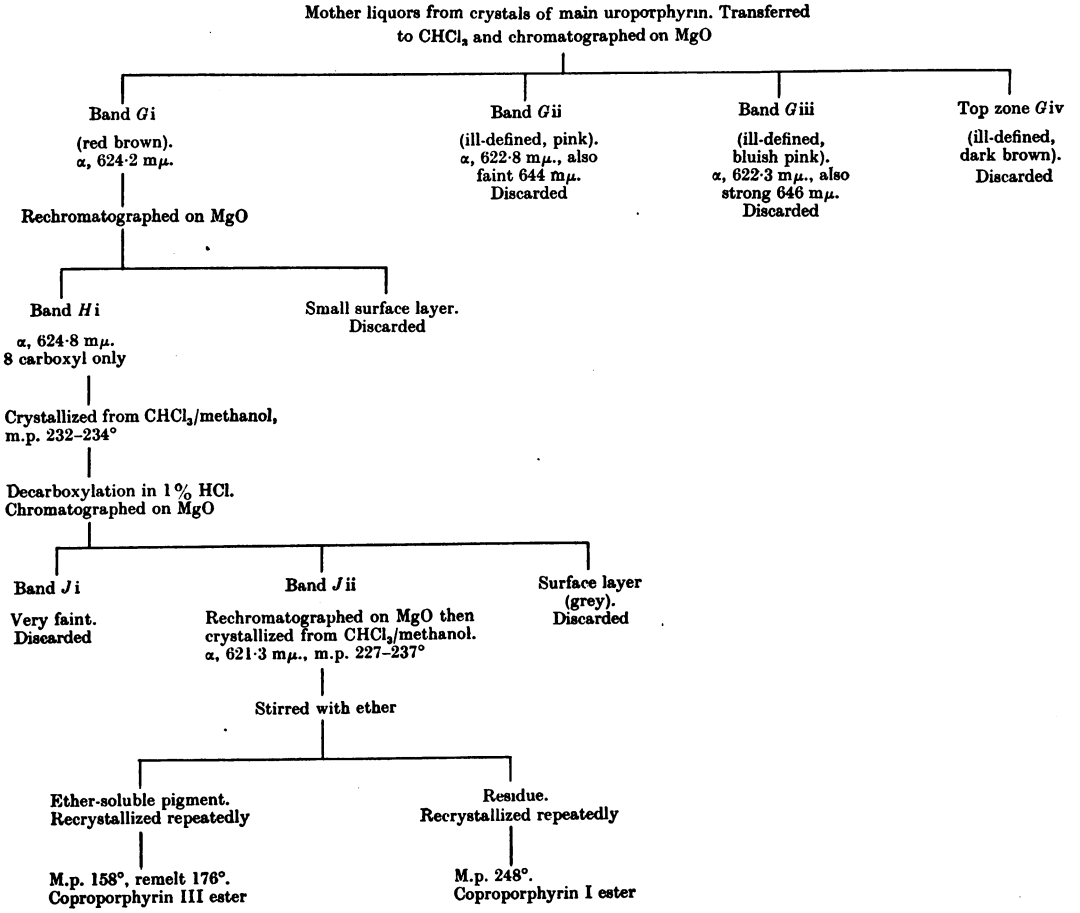

Fig. 2. Investigation of mother liquors of crystallization of main uroporphyrin I ester. Isolation of an association (m.p. 232–4°) of uroporphyrins I and III and its decarboxylation to coproporphyrins I and III.

was probably similar to that described in the preceding section with m.p. 244° and the material (m.p. 234°), the isolation of which is now to be described from the mother liquor of the first crystallization of the main uroporphyrin fraction.

Investigation of mother liquor from crystallization of main uroporphyrin fraction (see Fig. 2)

Isolation of an association of uroporphyrins I and III. The mother liquor from which the main crop of uroporphyrin (712 mg., m.p. 286°, see above) had separated, was deep red in colour. The pigment was transferred to $CHCl_3$ and placed on a column of MgO, grade III (15 × 2·5 cm.), which was developed with 100 vol. $CHCl_3$/0·6 vol. methanol. A complex chromatogram resulted with a rapidly moving brownish-red band (*G*i) surmounted by three less well defined zones (*G*ii, *G*iii and *G*iv) varying in colour from pink to bluish red. The α-bands of these fractions varied progressively from 622·8 to 622·3 mμ., but all exhibited absorption also at 644–646 mμ. *G*i was the only band investigated in detail. Rechromatography on MgO gave a compact band (*H*i) which separated from a small surface layer. It was collected (α, 624·8 mμ.) and found to contain only 8 carboxyl porphyrin by paper chromatography. Crystallization from $CHCl_3$/methanol afforded small aggregates of hair-like needles, m.p. 232–234°. As the quantity was small, it was all used for decarboxylation in 1% HCl at 180–190°. The resulting ether-soluble porphyrin was esterified and crystallized in aggregates of rather broad needles. For purification, it was chromatographed upon MgO, grade III, which removed some grey material and a minute, rapidly descending band (*J*i). The main band (*J*ii) was chromatographed again and the $CHCl_3$ solution (α, 621·3 mμ.) mixed with hot methanol. The crystals (m.p. 227–237°) left a deeply coloured mother liquor. Repeated recrystallizations from $CHCl_3$/methanol yielded typical coproporphyrin I ester (m.p. 248°). From the combined first and second mother liquors, coproporphyrin III ester (m.p. 158°, remelt 176°) was eventually obtained by recrystallizing the ether-soluble fraction of the dry residue from $CHCl_3$/methanol and then from benzene/light petroleum. The isolation of both coproporphyrins I and III indicates that the original 8 carboxyl material with m.p. 234° was an association of uroporphyrins I and III.

Investigation of the acetic acid extract of the crude porphyrin precipitate

Isolation of coproporphyrin I. The crude porphyrin precipitated from the urine by acidification had been boiled with acetic acid and then washed with hot acetic acid until this remained colourless. The combined extract and washings was brownish black in colour, and a test showed that all the porphyrin it contained was extractable by ether. Transference was effected into this solvent by addition of sodium acetate and much water. The ether layer was thoroughly washed and the porphyrin shaken out into 0·1 N-HCl. From this solution, it was precipitated at the isoelectric point, dried, esterified and chromatographed upon a column of MgO, grade III, packed in $CHCl_3$. Development with 100 vol. $CHCl_3$/1 vol. methanol eluted a main pink band (α, 621 mμ.) preceded by a narrow brown zone, and left a dark residue on the surface of the adsorbent. From the main eluate, the porphyrin was crystallized in curved needles (m.p. 246–248°; 4 carboxyl spot by paper chromatography). It was thus coproporphyrin I, possibly mixed with a small quantity of the series III isomer.

DISCUSSION

The application of standardized conditions of chromatography (Nicholas, 1951) and of paper chromatography (Nicholas & Rimington, 1949, 1951*a*) resulted in the detection and isolation from the urine of this congenital porphyrinuric of the following porphyrins: (1) uroporphyrin I, ester m.p. 293°; (2) a well crystallized 8 carboxyl porphyrin, ester m.p. 244°; (3) a crystalline 8 carboxyl porphyrin, ester m.p. 232–234°, possibly identical with the above but less pure. Decarboxylation of this material, which after hydrolysis gave only an 8 carboxyl spot by paper chromatography, afforded both coproporphyrin I (ester m.p. 248°) and coproporphyrin III (ester m.p. 158°, remelt 176°) proving that it was an association of the two uroporphyrin isomers I and III; (4) a 7 carboxyl porphyrin, the methyl ester of which crystallized in fine straight needles, m.p. 242°; (5) coproporphyrin I, ester m.p. 246–248° (possibly mixed with a little coproporphyrin III); (6) a 5 carboxyl porphyrin was also detected, but was not completely freed from accompanying coproporphyrin.

The yield of crude uroporphyrin I ester represented 91·7% of the total porphyrin present in the urine and 84% of the crude material was recovered as pure ester, after chromatographic removal of the 7 carboxyl and 5 carboxyl porphyrin esters (minimal in amount) and also the associations of 8 carboxyl porphyrin esters, presumably those of uroporphyrins I and III, which behaved chromatographically as entities and had melting points 234 and 244°. The quantities of these fractions did not exceed a few mg. The amount of coproporphyrin I ester ultimately isolated from the acetic acid washing of the crude porphyrin precipitate was 11·5 mg.

It is clear from the study of this case that the pattern of urinary porphyrin excretion in congenital porphyria may be more complex than has been previously realized. The association of uroporphyrins I and III in a complex, which crystallizes well and behaves as a chemical entity, is reminiscent of the claim by Grinstein, Schwartz & Watson (1945) and Watson, Schwartz & Hawkinson (1945) that the Waldenström esters (m.p. 255–260°) of acute porphyria urines are non-homogeneous. The above-named authors considered them to be associations of uroporphyrin I with a heptacarboxylic porphyrin (ester m.p. 208°) belonging to the III series. The evidence relative to the number of carboxyl groups in the latter is confined to an elementary analysis and methoxyl determination. The difficulty of obtaining small amounts of porphyrin esters in pure condition is great (cf. Fischer's

(1915*a*) original conclusion that uroporphyrin was a 7 carboxyl porphyrin). In this laboratory we incline to the view that uroporphyrins I and III may form an association, which is possibly molecular in character, and that this will separate chromatographically from the remaining isomer, whichever happens to be in excess. Authentic uroporphyrin III has been prepared from another source (Nicholas & Rimington, 1951*b*) and has an ester m.p. of 264°. Pure uroporphyrin I octamethyl ester has been found consistently to have m.p. 293°.

Although the possibility cannot be denied that the porphyrin ester of m.p. 255° separated chromatographically by Fischer & Hofmann (1937) from Petry's urinary uroporphyrin was in fact the ester of uroporphyrin III, it would seem more likely, in view of the findings recorded in this paper, that the material in question was an association of the two uroporphyrin isomers. No decarboxylation experiments were described by Fischer & Hofmann (1937) to support their view that it was uroporphyrin III. Similarly, it seems probable that the material with m.p. 255° described by Rimington (1936) as crystallizing out of the mother liquors of his preparation of uroporphyrin I octamethyl ester from bovine congenital porphyria urine and also the ester (m.p. 260–261°) separated chromatographically during the same study were associations of the kind now reported. Indeed coproporphyrin I was identified at this time among the products of decarboxylation of one of these bovine pigments.

SUMMARY

1. An investigation has been made, using chromatographic methods, of the porphyrins present in the urine of a case of congenital porphyria.

2. The pure uroporphyrin I octamethyl ester (m.p. 293°) isolated represented 77% of the total porphyrin of the urine.

3. Fractions were also isolated, with melting points 234 and 244°, behaving as entities on the chromatogram and consisting of 8 carboxyl porphyrins only. Decarboxylation yielded coproporphyrins I and III, hence these fractions are regarded as associations (possibly molecular) of uroporphyrins I and III. The view is held that in mixtures of esters of the two isomers these associations will form and separate chromatographically from the remaining isomer, whichever happens to be in excess. Reports from previous investigations are examined in the light of this suggestion.

4. Small quantities were also isolated of coproporphyrin I (ester m.p. 246–248°) and of a 7 carboxyl porphyrin with ester m.p. 242°.

5. A 5 carboxyl porphyrin was detected, but was not completely separated from accompanying coproporphyrin.

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