Porphobilinogen

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Porphobilinogen was isolated by Westall (1952) from the urine of a patient with acute porphyria. A structure (I), to be discussed later, was assigned to it by Cookson & Rimington (1953) and Cookson (1953a) on the basis of the chemical reactions briefly recorded in these preliminary notes, and was further supported by the X-ray crystallographic investigations of Kennard (1953). In the present paper our chemical studies are described in detail, together with an improved method of isolation of porphobilinogen, and a discussion of its conversion into porphyrin.

EXPERIMENTAL

Melting points are uncorrected. Paper chromatograms of porphobilinogen and its derivatives were run using the upper layer of a mixture of *n*-butanol:water:acetic acid (4:1:4, v/v) at 17°, and developed with 1% (w/v) *p*-dimethylaminobenzaldehyde in 2.5*N*-HCl. Ultraviolet absorption spectra between ~210 and 400 mμ. were measured in ethanolic solution, except where another solvent is specified, with a Unicam SP 500 Spectrophotometer. Maximum and minimum are printed in normal type, shoulders in italics. Infrared absorption spectra were measured on a Grubb Parsons F3A spectrometer. Samples were examined as Nujol mulls. The intensity of absorption is indicated as follows: (s), strong; (m), medium; (w), weak; ?, doubtful.

Isolation of porphobilinogen

The material used initially was kindly provided by Mr R. G. Westall, and we later isolated further quantities by his method (Westall, 1952). Then, following Brockman & Gray (1953), we substituted Deacidite FF for Dowex 2. Both methods are rather slow, and the instability of porphobilinogen under these conditions often results in low yields. Neither method is suitable for urines relatively poor in porphobilinogen. The following procedure avoids the use of exchange resin and alumina chromatography, is rapid, and gives very much better yields.

Urine containing porphobilinogen was brought to pH 4–4.5 with acetic acid. Mercuric acetate (15%, w/v) solution was added until all porphobilinogen was precipitated (negative Ehrlich reaction of the supernatant); the precipitate was collected on a Büchner funnel and washed with mercuric acetate (1%, w/v). It was then suspended in water and decomposed by H₂S. After filtration and aeration, excess of lead acetate (10%, w/v) was added. The deeply pigmented precipitate was removed and washed repeatedly with 1% (w/v) lead acetate solution. AgNO₃

(20%, w/v) solution was added to the combined filtrate and washings until precipitation was complete. After filtration and washing of the precipitate with 1% (w/v) AgNO₃, the combined filtrate and washings were treated, without prior removal of Pb and Ag ions, with a slight excess of 15% (w/v) mercuric acetate solution. This mercury precipitate was almost white; it was collected by centrifuging and decomposed by passing H₂S without the addition of further fluid. HgS was easily removed by centrifuging from the pale yellow supernatant, which was freed from H₂S by aeration and then adjusted to pH 4 with aqueous NH₃. Porphobilinogen crystallized immediately in almost colourless, well formed, crystals. After chilling, the solution was filtered and the porphobilinogen washed first with water containing a little acetic acid, and then with acetone. Further quantities could usually be obtained by the same method by extracting the final HgS precipitate with a little water. Yields were about 60%.

Porphobilinogen was recrystallized for analysis by solution in 0.5*N*-NH₃ and addition of *N* acetic acid to pH 4. The crystals of *porphobilinogen monohydrate* were washed free from salt and were then air-dried. (Found: C, 49.3; H, 7.0; N, 11.4. C₁₀H₁₄O₄N₂ · H₂O requires C, 49.2; H, 6.6; N, 11.5%.) *Porphobilinogen (anhydrous)* was obtained by drying over P₂O₅ at 20°/0.1 mm. for 3 days. (Found: C, 52.8; H, 6.8; N, 12.1. C₁₀H₁₄O₄N₂ requires C, 53.1; H, 6.2; N, 12.4%.) When exposed to the atmosphere it ceased to increase in weight after 1 hr. (increase in weight, 7.9%. Theory for 1 molecule of water, 7.3%). The analysis of porphobilinogen and its salts presented considerable difficulty on account of both their instability and hydration. Porphobilinogen was optically inactive at all pH values examined.

Estimation of porphobilinogen

Vahlquist (1939) described the determination of porphobilinogen in urine by means of the Ehrlich aldehyde reagent, but the definition of his porphobilinogen unit is ambiguous. We interpret it as follows: a porphobilinogen solution, which when mixed with an equal volume of Ehrlich reagent (2%, w/v, in 5*N*-HCl) has $E_{552\text{ m}\mu}^{1\text{ cm.}} = 0.545$, contains 1 unit/ml. This is measured against a blank cell containing equal volumes of the solution to be determined and 5*N*-HCl. Under these conditions maxima are at 552 and 530 mμ. Crystalline porphobilinogen (as normally obtained) assays 130 units/mg. Here and subsequently weights refer to the monohydrate.

Derivatives of porphobilinogen

Porphobilinogen hydrochloride. Westall (1952) observed that porphobilinogen forms a hydrochloride soluble in water but sparingly so in 2*N*-HCl. This salt is convenient for

the further purification of the crude base, particularly that obtained by the chromatographic methods, which is often deeply coloured and contains some uroporphyrin. The crude material was dissolved in 2*N*-HCl (1 ml./50 mg.) by warming to 45–50°. Filtration, if necessary, was carried out rapidly, and the solution immediately cooled (ice-NaCl). The hydrochloride, which crystallized in almost colourless needles, was filtered from the dark mother liquors, washed sparingly with ice-cold 2*N*-HCl and then with dry acetone. Yield about 70%. Recrystallization of the hydrochloride from 2*N*-HCl was conveniently carried out in a suitably jacketed no. 3 sintered glass funnel. (Found: C, 42.9; H, 6.3; N, 9.6; Cl, 12.8. $C_{10}H_{14}O_4N_2$, HCl, H_2O requires C, 42.8; H, 6.1; N, 10.0; Cl, 12.7%. Gain in weight on exposure to the atmosphere of a sample which had been kept over P_2O_5 at 20°/0.1 mm. for 3 days, 6.3%. Theory for one molecule of water, 6.4%.)

Porphobilinogen hydrobromide. This was prepared in the same way as the hydrochloride and is rather more soluble. Both the hydrate and anhydrous salt have been observed to crystallize together from the same solution (Kennard, 1953). The analytical sample was dried for 3 days over P_2O_5 at 20°/0.1 mm. (Found: C, 40.3; H, 5.6; N, 9.1; Br, 26.5. $C_{10}H_{14}O_4N_2$, HBr requires C, 39.1; H, 4.9; N, 9.1; Br, 26.0%. Loss in weight of hydrate on drying, 5.3%; gain in weight on exposure of dried sample to atmosphere, 5.6%. Theory for one molecule of water, 5.5%.)

Acyl derivatives of porphobilinogen. Porphobilinogen (34.9 mg.) was dissolved in *N*-NaOH (1 ml.) and acetic anhydride (0.1 ml.) added. Efforts to isolate a solid derivative after careful acidification were not successful, although the presence of material believed to be *N*-acetylporphobilinogen was recognized by paper chromatography of the much diluted reaction mixture. Ehrlich's reagent revealed only a single spot with R_F 0.79. The same result was obtained when triethylamine was used in place of NaOH. *N*-Benzoylporphobilinogen was similarly tentatively identified as the only Ehrlich-positive product in a small-scale experiment in which benzoyl chloride was used instead of acetic anhydride (R_F , 0.92).

Porphobilinogen lactam (II). Acetic anhydride (0.2 ml.) was added in portions to a cold solution of porphobilinogen (51 mg.) in water (1.9 ml.) and pyridine (0.6 ml.). The product began to separate after 15 min.; it was filtered off and washed several hours after. It was practically insoluble in the usual solvents, but could be dissolved in *N*- NH_3 and reprecipitated by 0.5*N* acetic acid. The lactam (25.7 mg., 59%) formed colourless leaflets which began to darken above 200° and melted with effervescence at *c.* 285° (R_F , 0.65). (Found: C, 57.6; H, 5.9; N, 12.9. $C_{10}H_{12}O_3N_2$ requires C, 57.7; H, 5.8; N, 13.5%.) Ultraviolet absorption in 0.01*N*-NaOH: λ_{max} at *c.* 210 m μ . (ϵ , 7500). Infrared absorption: NH stretching 3290, 3200 (s), 3100 (w) cm^{-1} ; OH stretching $\sim$ 2250 cm^{-1} ; CO stretching 1698 (—COOH), 1637 (—CONH—) cm^{-1} ; second amide band 1612 cm^{-1} . Both the solid and its solutions fluoresced a brilliant blue under a Wood's lamp. The Ehrlich colour given by the lactam appeared immediately, in contrast to that from porphobilinogen which only develops slowly. Paper chromatography of the mother liquor from the reaction showed the presence of more of the lactam and also of *N*-acetylporphobilinogen.

The lactam, identical in all respects with the above, was also formed when benzoyl chloride was used instead of acetic anhydride, but the yield of lactam was lower

(29%). Toluene-*p*-sulphonyl chloride was even less satisfactory.

The lactam was unchanged by solution in *N*-NaOH and further treatment with acetic anhydride.

Methyl ester of porphobilinogen lactam. This was prepared by treatment of the finely powdered lactam (40 mg.) with excess of diazomethane (made from 3 g. nitrosomethylurea) in ether (40 ml.) containing methanol (2 ml.). After 2 days at 4° the solvent was removed *in vacuo* and the residue recrystallized from ethanol, sublimed at 230°/0.1 mm., and the sublimate (25.9 mg.) again recrystallized from ethanol (7 ml.). The ester formed colourless platelets (24.5 mg.) which showed the same blue fluorescence as the free acid, and which melted at 248–250° (R_F , 0.83). (Found: C, 59.6; H, 6.6; N, 12.6. $C_{11}H_{14}O_3N_2$ requires C, 59.4; H, 6.3; N, 12.6%.) Ultraviolet absorption: λ_{max} at *c.* 210 m μ . (ϵ , 7500).

2-Acetyl porphobilinogen lactam methyl ester (III). A solution of the lactam ester (8.2 mg.) in boiling acetonitrile (4 ml.) was rapidly cooled; a stream of dry HCl was immediately passed into the supersaturated solution for 8 min., and the mixture was set aside overnight. The solid (0.35 g.) which remained after removal of excess of nitrile under reduced pressure, consisting largely of a salt derived from acetonitrile itself, was dissolved in water (2 ml.) and a sample suitably diluted for measurement of its spectrum. Although the product from the reaction between HCl and acetonitrile alone did not absorb light in the range studied, an aqueous solution of equivalent concentration was used in the blank cell. The acidic reaction solution had an absorption maximum at 331 m μ , which was assumed to be due to the ketimine hydrochloride, for the spectrum changed when the solution was made alkaline: The spectrum rapidly changed further as hydrolysis of the ketimine to the acetyl compound took place. Hydrolysis was complete after 3 hr. at 17° when the absorption maximum was at 306 m μ . If ϵ is 20000 at this wavelength (Cookson, 1953*b*) and the reaction had gone in quantitative yield, the optical density indicated a molecular weight of about 300 for the lactam ester (theory, 222). This upper limit to the molecular weight provided further support for the formulation of porphobilinogen as a monopyrrole.

2,4-Dinitrophenylhydrazine (13.2 mg.) was added to the main bulk of the reaction mixture (which was Ehrlich-negative). After heating the solution for a short time, blood-red needles of the 2,4-dinitrophenylhydrazone of the acetyl compound separated (2.7 mg.). The m.p. was indefinite, with decomposition above 215°. (Found: N, 19.2; $C_{19}H_{20}O_7N_6$ requires N, 18.9%.)

Ketolactam (IV). The free lactam (II) (37.2 mg.) was stirred with polyphosphoric acid (2.0 g.) at 95° for 5 min. The lactam dissolved and the mixture became red. Stirring was continued, while the temperature was raised to 110° during the next 10 min. and the colour of the mixture faded to yellow. After cooling the mixture, water was added and the precipitate (29 mg. 85%) washed with dilute aqueous NH_3 and with water. The ketolactam, which was insoluble in the usual organic solvents, was recrystallized from water (25 ml.). It formed colourless prisms (22.3 mg.) which began to sublime at 310° and then at *c.* 330° to decompose without melting (Found: C, 63.4; H, 5.4; N, 14.7. $C_{10}H_{10}O_2N_2$ requires C, 63.2; H, 5.3; N, 14.7%.) Ultraviolet and infrared absorption values are given in Table I. (λ_{max} in aqueous solution, 288.5 m μ .)

Attempts to cyclize (II) to (IV) by means of conc. H_2SO_4 , $SOCl_2$, $POCl_3$ or anhydrous HF were unsuccessful.

Table 1. Comparison of ultraviolet and infrared absorption characteristics in ethanol of the ketolactam (IV) with those of model compounds

M.p.	Ketolactam (IV)	1-Oxo-2:3- dihydropentindole (VI)	1-Oxo-1:2:3:4- tetrahydrocarbazole (V)
	> 330°	250°	169°
	Ultraviolet absorption		
$\lambda_{\max}$ (m μ .)	255 (ϵ , 8 300) 284 (ϵ , 20 600)	234 (ϵ , 19 400) 300 (ϵ , 25 500)	237 (ϵ , 16 200) 308.5 (ϵ , 23 000)
$\lambda_{\min}$ (m μ .)	223 (ϵ , 1 200)	214 (ϵ , 7 750) 258 (ϵ , 1 300)	220 (ϵ , 8 800) 264 (ϵ , 1 000)
	Infrared absorption		
In NH stretching region ν (cm. ⁻¹)	3200? 3145 (s) Broad 3060 (s) 2780 (m) 2700 (w) 2620 (w)	3205 (s) — 3060 (m) 2780 (w) — —	3300 (s) 3200 (w) — — — —
In CO region ν (cm. ⁻¹)	1654 (s) Very broad	1656 (s) 1620 (m)	1645 (s) 1620?

Synthetic experiments

1-Oxo-1:2:3:4-tetrahydrocarbazole (V) was prepared from γ -3-indolylbutyric acid by cyclization with polyphosphoric acid for 10 min. at 80°. A sample of 1-oxo-2:3-dihydropentindole (VI) was kindly provided by Dr J. Elks (see Elks, Elliott & Hems, 1944). Ethyl 2:3-dimethylpyrrole-5-carboxylate was prepared by the method of Fischer & Fink (1948). Its hydrolysis and decarboxylation provided 2:3-dimethylpyrrole.

5- β -Chloropropionyl-2:3-dimethylpyrrole. 2:3-Dimethylpyrrole (2.93 g.) dissolved in ether (10 ml.) was added to a Grignard reagent prepared from bromobenzene (4.33 g.) and Mg (0.74 g.) in ether (20 ml.). The solution was refluxed for 30 min. before being cooled to 0°, while a solution of β -chloropropionyl chloride (3.54 g.) in ether (15 ml.) was added. A deeply coloured viscous oil separated towards the end of the addition. After being refluxed for 1 hr., the mixture was again cooled to 0° before hydrolysis with NH₄Cl solution. The chloroketone, which was only slightly soluble in ether, was filtered off and recrystallized from ethanol. It formed white leaflets which melted to a carmine oil at 119°. (Found: C, 58.2; H, 6.6; N, 7.5. C₉H₁₂ONCl requires C, 58.2; H, 6.5; N, 7.6%.) Ultraviolet absorption: $\lambda_{\max}$, 256 (ϵ , 4200) and 313 m μ . (ϵ , 18 800); $\lambda_{\min}$, 225 (ϵ , 1100) and 273 m μ . (ϵ , 3100). Attempts to cyclize the chloroketone with polyphosphoric acid, SnCl₄, or AlCl₃ in tetrachloroethylene were unsuccessful.

2:3-Dimethyl-5-pyrrolyl vinylketone. The chloropropionylpyrrole (745 mg.) was boiled for 20 min. with excess of pyridine after which the latter was removed under reduced pressure. The deliquescent quaternary salt was dissolved in a small volume of water, to which dilute NaOH was added dropwise. When no more of the vinyl ketone was precipitated, it was filtered off and washed with water. It separated from light petroleum (b.p. 60–80°) in pale lemon rhombs (396 mg.) m.p. 142–143°. (Found: C, 72.5; H, 7.6; N, 9.5. C₉H₁₁ON requires C, 72.5; H, 7.4; N, 9.4%.) Ultraviolet absorption: $\lambda_{\max}$, 210 (ϵ , 4600), 237.5 (ϵ , 5000), 346 (ϵ , 16 600), and 275 m μ . (ϵ , 2700); $\lambda_{\min}$, at 222 (ϵ , 4100), and 262 m μ . (ϵ , 2000). Attempts to cyclize the vinyl ketone were not successful.

Ethyl 2:3-dimethyl-4-morpholinomethylpyrrole-5-carboxylate. A mixture of ethyl 2:3-dimethylpyrrole-5-carboxylate (10 g.), morpholine (6.24 g.), conc. HCl (6.3 ml.), 40% (w/v) aqueous formaldehyde (6.0 ml.) and ethanol (30 ml.) was boiled under reflux. After 5 hr. the ethanol was removed under reduced pressure and water was added. The unchanged pyrrole which was precipitated, was filtered off, and boiled again with the same reagents. On making the filtrate alkaline with NaOH the morpholino compound precipitated as an oil which rapidly solidified. It recrystallized from ethanol in colourless needles, m.p. 137–138° (2.5 g.; the recovered material provided another 2.47 g. together with 5.4 g. still unchanged pyrrole). (Found: C, 63.2; H, 8.3; N, 10.4. C₁₄H₂₂O₃N₂ requires C, 63.2; H, 8.3; N, 10.5%.) Ultraviolet absorption: $\lambda_{\max}$, 243 (ϵ , 4500) and 287 m μ . (ϵ , 17 000); $\lambda_{\min}$, 220 (ϵ , 2300) and 255 m μ . (ϵ , 3900). In acid ethanol: $\lambda_{\max}$, 238 (ϵ , 3400) and 290 m μ . (ϵ , 16 500); $\lambda_{\min}$, at 215 (ϵ , 1600) and 263 m μ . (ϵ , 2900). The use of paraformaldehyde in place of formaldehyde solution in the Mannich reaction was less satisfactory.

The methiodide of the morpholino compound separated from a mixture of ethyl acetate and ethanol in slightly yellow crystals which melted at 180° with decomposition and previous darkening. (Found: C, 44.3; H, 6.3; N, 6.8. C₁₅H₂₃O₃N₂I requires C, 44.1; H, 6.1; N, 6.9%.)

Ethyl 4-2'-tris(ethoxycarbonyl)ethyl-2:3-dimethylpyrrole-5-carboxylate. An ethanolic solution (5 ml.) of the methiodide prepared from the above morpholino compound (900 mg.) was added to ethanol (10 ml.) in which Na (78 mg.) and tris(ethoxycarbonyl)methane (1.57 g.) had been dissolved. The mixture was refluxed for 6 hr. On the next day the ethanol was distilled off under reduced pressure, and water added to the residue, which was then made just acid, before extraction with ether. Evaporation of the washed and dried (Na₂SO₄) extract left a viscous oil. A small sample which was obtained crystalline by distillation at a high temperature at 0.1 mm. was used to seed the main bulk. The product (709 mg.) was washed free from traces of oil with light petroleum (b.p. 40–60°) and recrystallized from light petroleum (b.p. 60–80°) containing a trace of benzene. The highly refracting rhombs melted at 83–84° (570 mg.). (Found: C, 58.5; H, 7.0; N, 3.5. C₂₀H₂₉O₈N requires C, 58.4;

H, 7.1; N, 3.4%). Ultraviolet absorption: $\lambda_{\max}$ at 245 (ϵ , 4600) and 288 m μ . (ϵ , 17000); $\lambda_{\min}$ at 222 (ϵ , 3100) and 256 m μ . (ϵ , 4200).

Ethyl 4-2'-bis(ethoxycarbonyl)ethyl-2:3-dimethylpyrrole-5-carboxylate. A solution of the methiodide derived from the above morpholino compound (5.3 g.) in ethanol (20 ml.) was added to one of ethyl malonate (6.4 g.) in ethanol (30 ml.) in which Na (0.46 g.) had been dissolved. The mixture was refluxed for 6 hr. and then worked up as above. The excess of ethyl malonate was removed at 120°/0.2 mm., but the residue did not crystallize and chromatography on Al_2O_3 in ether-light petroleum (b.p. 40–60°) (20:80, v/v) was necessary. The pyrrole thus purified separated from light petroleum (b.p. 60–80°) in colourless crystals (3.5 g., 52%) which melted at 63–64°. (Found: C, 59.8; H, 7.7; N, 3.8. $\text{C}_{17}\text{H}_{25}\text{O}_6\text{N}$ requires C, 60.2; H, 7.4; N, 4.1%). Ultraviolet absorption: $\lambda_{\max}$ at 247 (ϵ , 4500) and 287 m μ . (ϵ , 17 700); $\lambda_{\min}$ at 220 (ϵ , 2400) and 255 m μ . (ϵ , 4200).

Neither the hydrolysis of this compound nor that of the foregoing tris(ethoxycarbonyl)ethyl derivative was investigated in detail. Both gave free acids which were extremely soluble in water and could not be isolated. Hydrolysis of the malonic ester derivative (1.28 g.) by boiling water (4 ml.) containing ethanol (1 ml.) and NaOH (0.73 g.) for 4 hr., followed by the addition of HCl to pH 3.5, gave a solution of the polycarboxylic acid, which was decarboxylated to haemopyrrole carboxylic acid (0.10 g.) by heating to 160° in a Carius tube for 6 hr. Much tar was formed, and doubtless better conditions for the decarboxylation could be found.

Conversion of porphobilinogen into uroporphyrin

Conversion in acid at 100°. A solution of porphobilinogen (25 mg.) in 0.5N-HCl (50 ml.) was heated at 100° for 20 min. The amount of porphyrin, which was estimated spectrophotometrically in a suitably diluted sample, was found to increase on exposure to air, as the initially formed porphyrinogen became oxidized. Air was drawn through the main solution to complete this oxidation; at the end of the reaction the solution contained 16.5 mg. uroporphyrin (77.5% yield) estimated spectrophotometrically. The porphyrin was precipitated at pH 3.1 (sodium acetate) and removed by centrifuging. The brown mother liquor was re-acidified and the trace of porphyrin (15.6 μg .) estimated; this solution had

an absorption band at 480 m μ . and gave an intense green fluorescence with zinc acetate (cf. Brockman & Gray, 1953). A paper chromatogram of a sample of the unpurified porphyrin precipitate run in lutidine (Nicholas & Rimington, 1949, 1951) showed only an octacarboxylic porphyrin spot. Esterification of the bulk of the porphyrin with methanolic HCl gave a quantitative yield of the octamethyl ester (18.8 mg.) which melted at 258–260°. The Falk & Benson (1953) method of chromatography showed only a single spot in the uroporphyrin III position with very slight tailing.

A sample (8 mg.) of this uroporphyrin was decarboxylated in the usual way (Fischer & Zerweck, 1924); the yield was 70%, as determined photometrically. The porphyrin was isolated and esterified with methanolic HCl, and the absence of substances other than coproporphyrins was demonstrated by chromatography on Al_2O_3 (Brockmann grade V) in benzene- CHCl_3 . Paper chromatograms run by the method of Chu, Green & Chu (1951) on samples, both before and after the Al_2O_3 chromatography, showed only single spots with very slight tailing in the coproporphyrin III position. The chromatographed coproporphyrin ester separated from methanol- CHCl_3 in needles with the appearance of coproporphyrin III ester; the mother liquor was only faintly coloured. The melting point of this material was taken on a micro hot stage, the sample being squeezed between coverslips so that it was translucent. Individual needles melted between 132 and 145°, but much solid remained up to ca. 160° and a few crystals remained until 190°. When the temperature was lowered the oil readily recrystallized in long pleochroic laths, which began to melt again at c. 160°, but did not melt completely until 197°. This behaviour was visible both in ordinary light and between crossed Nicols. A sample of coproporphyrin III tetramethyl ester prepared from *Corynebacterium diphtheriae* melted at 148–150° and resolidified while the temperature was raised; it melted again at 170–177°.

Conversion at various pH values at 20°. Four solutions, made up with the compositions shown in Table 2, were set aside in the dark for 17 days. The time after which a definite pink fluorescence became visible when illuminated with a Wood's lamp gave a rough indication of the rate of porphyrin formation. At the end of 17 days the unchanged porphobilinogen still present was estimated as described above and the uroporphyrin was estimated by the method of Rimington & Sveinsson (1950). The bulk of each solution

Table 2. Conversion of porphobilinogen into uroporphyrins in various solutions at 20°

Pbg = porphobilinogen					
Composition	pH	Time of appearance of fluorescence (days)	Uroporphyrin yield in ($\mu\text{g.}/\text{mg. Pbg}$)	Unchanged Pbg (mg.)	Apparent composition of uroporphyrin mixture (%)
A. 2.4 mg. Pbg, 50 mg. Na_2CO_3 , 0.5 ml. water, 4.5 ml. normal urine	>10	7	65	0	'I' 60 'III' 40
B. 2.3 mg. Pbg, approx. 8.1 ml. HOAc, 4.75 ml. normal urine, approx. 1.2 ml. 2N-NaOH	6.5	2	160	0	'I' 0 'III' 100
C. 2.0 mg. Pbg, 5.0 ml. 0.2N- NH_3	>10	4	80	0	'I' 50 'III' 50
D. 2.2 mg. Pbg, 5.0 ml. 0.5N-HCl	<1	No fluorescence purple pigment formed	0.35	1.0	Not enough material

was then adjusted to pH 4.2, and the porphyrin in each adsorbed on talc. The porphyrins were esterified and purified by chromatography on MgO as described by Nicholas (1951), and then run on paper chromatograms in kerosene-dioxan (Falk & Benson, 1953). For reasons outlined below (see Discussion) the results have been expressed in terms of uroporphyrin I and III mixtures which give similar patterns (Table 2), although in the case of *B* and *C* the strongest fluorescence of the 'III' spot was on the low R_F side of the spot, whereas pure uroporphyrin III gives symmetrical fluorescence.

DISCUSSION

Structure of porphobilinogen

A close structural connexion between porphobilinogen and uroporphyrin has been evident since Waldenström & Vahlquist's (1939) demonstration of the formation of uroporphyrin when solutions of porphobilinogen are heated under suitable conditions. This transformation suggested that the substituents of any pyrrole ring present in porphobilinogen must be acetic acid and propionic acid residues, respectively, for oxidation of uroporphyrin yields carboxylated haematinic acid (MacDonald, 1952*a*). There remained many possibilities for the structure of porphobilinogen. The reactions by which we have definitely established its structure as 2-aminomethyl-4-2'-carboxyethyl-3-carboxymethylpyrrole (I) are summarized in the chart and described in the experimental part. The lactam (II) is a key compound, and its formation by treatment of porphobilinogen dissolved in aqueous pyridine with an acylating agent is of particular interest. It is evident that a mixed anhydride between the acetic acid side chain of porphobilinogen and the

acylating agent is an intermediate in this reaction. For cyclization to take place this mixed anhydride must cleave in the appropriate way, and in addition the amino group must exist as the free base. The dual effects of pH and nature of the acylating agent were not studied, but the production of (II) in 59% yield under our conditions shows that the reversible attack on the carboxyl group by acetic anhydride is very much more rapid than acetylation of the amino group. This is not the case when the reaction is carried out in sodium hydroxide solution, for then the *N*-acetyl derivative is apparently the exclusive product.

There was a possibility that porphobilinogen had a structure (VII) similar to (I), but with the positions of the two β -substituents reversed; this would have led to a seven-membered lactam ring with an acetic acid side chain on the other side of the pyrrole nucleus. Such a structure, which is compatible with all the other reactions, was excluded by cyclization of (II) to the ketone (IV) by means of polyphosphoric acid. The only neutral crystalline product, which could have been derived from the alternative seven-membered ring structure for the lactam (VII), would have been an eight-membered ring diketone formed by condensation of pairs of such molecules. This unlikely formulation is inconsistent with the properties of our product, for, being unstrained, it would have been expected to have had the ultra-violet absorption of a normal 2-acyl pyrrole (e.g. 2-acetyl-4-ethyl-3:5-dimethylpyrrole, $\lambda_{\max}$. 308 m μ . Cookson, 1953*b*). In fact, our product has maximum absorption at 284 m μ , i.e. at a wavelength about 20 m μ . shorter than this. A hypsochromic shift relative to the acyclic compounds is well

known in the case of the *cyclopentenones* (Gillam & West, 1942), and is due to the strain in a five-membered ring (French & Wiley, 1949). 1-Oxo-1:2:3:4-tetrahydrocarbazole (V) and 1-oxo-2:3-pentindole (VI), the ultraviolet absorption data for which are shown in Table 1, further illustrate the effect, but being indoles, naturally neither has absorption identical to that of the pyrrole (IV). Individual groups in the same environment have characteristic absorption in the infrared, irrespective of the structure of distant parts of the molecule, and therefore the direct comparison of the infrared spectra of the indoles with that of (IV) is permissible. The resemblance of the infrared spectrum of (IV) to the five-membered rather than to the unstrained six-membered indole derivative is significant, but this evidence is less impressive than that from the ultraviolet owing to the necessity of working with nujol mulls, and to the overlap in (IV) of the amide band at 1640 cm^{-1} with that due to the keto group. Originally it had been intended to prepare 2:3-dimethylpyrrolo-(4:5-3':2')-cyclopentenone as a model compound. The attempts to synthesize this ketone, which are described in the experimental section, were not encouraging, and so the indoles mentioned above were used instead.

It may be noted in connexion with these experiments that although an electron-attracting group in the α -position of a pyrrole prevents substitution in the adjacent free β -position by the Gattermann-Hoesch reaction (MacDonald, 1952*b*), such a pyrrole can still take part in the Mannich reaction (cf. Treibs & Ott, 1953).

The pK values of porphobilinogen measured by Granick & Bogorad (1953) are those to be expected for structure (I) (cf. Branch & Calvin, 1941).

δ -Aminolaevulinic acid ($\text{H}_2\text{N} \cdot \text{CH}_2 \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CO}_2\text{H}$), a specific precursor of haem (Shemin & Russell, 1953) and of porphyrins (Neuberger & Scott, 1953) was shown by Dresel & Falk (1953) to be enzymically converted into porphobilinogen. The structure I for the latter is readily intelligible when seen as the result of the condensation of two molecules of δ -aminolaevulinic acid; the alternative structure VII would not be readily produced.

Waldenström porphyrin

Urines from cases of acute porphyria suffering attacks invariably contain porphobilinogen. Not infrequently uroporphyrin is absent from the freshly voided urine but forms from porphobilinogen more or less rapidly on standing. Such uroporphyrins have all been termed 'Waldenström porphyrins', notwithstanding variations of techniques of preparation and isolation. They are in fact chemical artifacts. Failure to appreciate this point and, possibly, the influence of the concept of the dualism of the porphyrins have led to some of the

present confusion. Much work has been devoted to the study of Waldenström porphyrins employing such methods of separation and identification of porphyrins and their isomers as are available. In the absence of pure specimens of all four uroporphyrin isomers it has not been possible to test these methods directly on a known complex mixture.

Waldenström (1935) and Mertens (1936) identified the porphyrin from acute porphyria urines as uroporphyrin III, Grinstein, Schwartz & Watson (1945) consider it to be uroporphyrin I together with a heptacarboxylic porphyrin, while Nicholas & Rimington (1953) suggested on the basis of results obtained with several techniques that it was a mixture of about 75% uroporphyrin III and 25% uroporphyrin I.

We have as yet developed no new method of porphyrin isomer analysis, but certain predictions can be made on purely chemical grounds, which are relevant to this problem. Before the isolation of porphobilinogen, the porphyrin derived from it was the only tangible clue to its structure; now the position is reversed.

Formation in vitro of porphyrins from pyrroles of the type of porphobilinogen

Electrophilic attack on a pyrrole will tend to take place at the point of highest negative charge, but the reaction rate, being controlled by the energy of activation, will be favoured by a transition complex of low energy. The reaction, therefore, normally takes place by direct displacement so that the pyrrole ring system may be retained (i.e. pyrrolines are not formed). The group replaced must be able to give up an electron pair and separate as a cation or a combination of neutral and positive fragments. Such groups include $-\text{H}$, $-\text{CH}_2\text{OH}$, $-\text{CH}_2\text{NH}_2$ or pyrrolylmethylene, but not alkyl or $-\text{CH}_2\text{NH}_3^+$. The term 'free position' is customarily given only to one bearing a hydrogen atom, but it must be remembered that positions are not 'blocked' if substituted by other groups which are readily replaceable. Although there is no fundamental difference between condensations involving displacement of hydrogen or of a larger group, it will be convenient for the following discussion to call these two reactions of the nucleophilic component A 1 and A 2 respectively.

A compound such as porphobilinogen which contains two replaceable groups can react in several ways so that the product of each individual step, let alone of the condensation of four molecules into a porphyrin, cannot be predicted in the absence of data on reaction rates, etc. Moreover, these and the reaction mechanism, itself are doubtless dependent on pH and many other factors (e.g. the $-\text{CH}_2\text{NH}_2$ group can react according to A 2 only as free base).

Scheme C

It is, nevertheless, of value to discuss the composition of the isomeric porphyrin mixtures which would be obtained under various circumstances. We have experimentally observed the formation of uroporphyrinogen as an intermediate. Only the condensation in acid solution is discussed, although obviously a parallel scheme would apply for base catalysis, where the initial step would be removal of a proton from the pyrrole nitrogen. The acid-catalysed reaction is illustrated by the S_E1 reaction in which porphobilinogen first loses ammonia and the resulting carbonium ion either attacks another pyrrole acting as an electrophilic component or reacts with water (scheme B).

If the α -hydrogen atom is more easily displaced than aminomethyl or hydroxymethyl, reactions according to B and A1 will lead to uroporphyrinogen I and after oxidation to uroporphyrin I.

If the aminomethyl or hydroxymethyl side chain is displaced in preference to the α -hydrogen atom (A2), but the latter in preference to α -pyrrylmethylene (A1) the product will be a mixture of uroporphyrins III and IV with a small amount of II (assuming equal speeds of reaction). Inspection of scheme C in which the porphobilinogen unit is abbreviated PA_ (P indicating the propionic and A the acetic acid residues in the $\beta\beta'$ positions and

the horizontal dash the link between units) will show that even if reaction A2 did occur to some extent throughout the synthesis, it would have little effect on the final products, since such reactions would in most cases lead to compounds which are already intermediates in this scheme. (R in A1 or A2 is an α -pyrrylmethylene or the cation formed by the addition of a proton to formaldehyde).

Actual examples of a similar order of ease of substitution of groups by electrophilic reagents have been observed in phenols (which owing to the operation of similar electronic displacements are analogous to pyrroles). For example, Ziegler & Zigeuner (1949) and Ziegler (1951) investigating the reaction between diazonium salts and hydroxybenzyl alcohols found cases in which the ease of replacement of groups was in the order $-\text{CH}_2\text{OH} > -\text{H}$ *ortho* to phenolic $-\text{OH} > \textit{ortho}$ hydroxybenzyl.

A proton could also act as the substituting reagent, and although reaction A1 ($\text{R}=\text{H}$) would cause no change, reaction A2 would yield formaldehyde and pyrrole- β -acetic- β' -propionic acid. The reactivities of the α -positions of this compound are probably very similar, so that the equilibrium mixture obtained by this mechanism would contain comparable amounts of the isomeric α -hydroxymethylpyrroles. If such isomerization is much more

rapid than the condensation between pyrroles, the pairs of side chains in the final porphyrin will be arranged at random, and a mixture of isomeric uroporphyrins in the proportions $\frac{1}{2}$ I, $\frac{1}{2}$ II, $\frac{1}{2}$ III, and $\frac{1}{2}$ IV will be obtained, irrespective of the details of condensation. This distribution of isomers arising from the random condensation of opso-type pyrroles has been independently noted by S. F. MacDonald (personal communication).

The discovery by Corwin & Andrews (1937) of the formation of tripyrrylmethanes during the synthesis of dipyrromethenes from aldehydes and pyrroles with a free α -position, which can then split into any of several different dipyrromethenes, is often quoted as a cause of isomerization. Redistribution of the charge on the salt of the dipyrromethene enables the bridge carbon atom to acquire a positive charge; and reaction according to A I, where this cation is represented by R^+ , sets up the equilibrium with the tripyrrylmethane. It is obvious that a dipyrromethane could not take part in such a reaction as the substituting reagent, and that therefore this mechanism does not concern the lower state of oxidation considered throughout the foregoing discussion.

Limitations of some methods of porphyrin isomer analysis

It can be seen from these simplified examples how mixtures of isomeric porphyrins can be obtained from a single pyrrole precursor, and how series III and IV porphyrins can predominate. It can also be confidently predicted that the exclusive formation of uroporphyrin III from porphobilinogen is not possible by chemical means. In the light of this, we have re-examined the uroporphyrin obtained by heating porphobilinogen in acid solution, which in most respects resembles uroporphyrin III. The results which are described in the experimental part show that no single method of isomer analysis is infallible, although a definite decision is usually possible when a combination of methods is used.

It has been established by Falk & Benson (private communication) that chromatography according to Chu *et al.* (1951) can only separate coproporphyrins I and II from coproporphyrins III and IV, but is unable to distinguish between the individual constituents of each pair. It appears from the present work that the same limitation applies to the Falk & Benson (1953) method for uroporphyrins. A more direct test of this point is not possible until pure uroporphyrins II and IV become available. Previous experimental data should be reconsidered with this reservation. The melting points of porphyrin esters are not reliable tests of purity; there are a number of examples in the literature of mixtures of different porphyrins (not even isomers) which melt within a degree of the melting point of

one constituent alone. Uroporphyrin esters which melt at about 260° are a further example.

Consideration of the mechanism of the conversion of porphobilinogen into uroporphyrins suggested that the composition of the isomeric mixture might vary with the pH of the solution. Table 2 summarizes the results of experiments which confirmed this idea. The fact that (according to chromatograms) about half the uroporphyrin formed in alkaline solution consisted of uroporphyrin I (and/or II), whereas that formed at pH 6.5 consisted entirely of uroporphyrin III (and/or IV) emphasizes the artificial nature of Waldenström porphyrin. It may be significant that at the Minnesota School, 1% of Na_2CO_3 is sometimes used as a preservative for porphyrin urine (cf. Schwartz, Zieve & Watson, 1951), for these were the conditions of Expt. A in Table 2. Scarcely any conversion into porphyrin took place when a solution of porphobilinogen in dilute hydrochloric acid was kept at room temperature (D, Table 2), although a high yield was obtained from a similar solution at 100° . Evidently the higher temperature is necessary for the decomposition of the salt of porphobilinogen, and at room temperature it was protected in this form. The violet pigment which slowly appeared may have been the result of oxidation.

Porphobilinogen in the biosynthesis of porphyrins

Numerous schemes for the biosynthesis of porphyrins have been put forward in the past. None was satisfactory as in the main they endeavoured to account for the production of I and III series porphyrins on purely chemical grounds. They usually involved as a final stage the condensation between two unlike dipyrromethenes which were arbitrarily forbidden to react with themselves. It was always assumed that the only position in a pyrrole at which substitution could take place was a 'free' position (i.e. one bearing a hydrogen atom), and thus any attempt to fit porphobilinogen into such a scheme would also require the presence of 'isoporphobilinogen' (VII). No evidence has been found for the existence of an isomer of porphobilinogen in biological material; moreover, the foregoing discussion shows this to be unnecessary.

It has been shown that porphyrin formation from porphobilinogen in a chicken red cell haemolysate is an enzymic process, since porphobilinogen was recovered unchanged from a control experiment in which the haemolysate had been boiled (Falk, Dresel & Rimington, 1953). The isomeric composition of the porphyrins elaborated *in vivo* is the result of enzymically directed catalysis and need not be the same as that resulting from *in vitro* transformations, although the fundamental processes involved are presumably similar.

SUMMARY

1. Porphobilinogen has been shown to be 2-aminomethyl-4-2'-carboxyethyl-3-carboxymethyl-pyrrole (I)

2. 'Waldenström porphyrin' is an artifact derived from porphobilinogen by chemical means. It is a mixture of uroporphyrins, the composition of which varies according to the conditions under which it is formed.

3. The mechanism of porphyrin formation *in vitro* from such pyrroles as porphobilinogen is discussed. It is concluded that uroporphyrin III cannot be the sole product.

4. A mixture of isomeric uroporphyrins has been found, which behaved exactly as uroporphyrin III on kerosene-dioxan chromatography. It appears likely that this method can only separate uroporphyrins I and II from uroporphyrins III and IV.

5. No evidence has been found for the existence of an isomer of porphobilinogen.

6. The participation of porphobilinogen in porphyrin biosynthesis is briefly considered.

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