Biosynthesis of Porphyrins and Related Macrocycles. Part II.[1,2] Synthesis of δ-Amino[5-^{13}C]laevulinic Acid and [11-^{13}C]Porphobilinogen: Incorporation of the Latter into Protoporphyrin-IX

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A short synthesis of δ-aminolaevulinic acid is described in which C-5 and the nitrogen atom are introduced as cyanide ion. The sequence is especially well suited to the preparation of material labelled at C-5 and it is used to afford δ-amino[5-^{13}C]laevulinic acid; ^{13}C chemical shifts are reported for the various products. [11-^{13}C]Porphobilinogen is also synthesised from [^{13}C]formaldehyde and this material is incorporated by extracts of *Euglena gracilis* into protoporphyrin-IX. The ^{13}C n.m.r. spectrum of the labelled porphyrin proves that the four signals near δ 97 arise from the *meso*-carbon atoms and that these positions are essentially equally labelled.

RESEARCH[3] during the nineteen-fifties, especially by Shemin, Neuberger, Granick, and Rimington, established that the porphyrin macrocycle [*e.g.* protoporphyrin-IX (3)] is built in nature from δ-aminolaevulinic acid (1) by way of porphobilinogen (2). One approach in the biosynthetic studies at Cambridge depends heavily upon ^{13}C n.m.r. to determine the mechanisms whereby the macrocycles of natural porphyrins and corrins are constructed from porphobilinogen. A prerequisite is the synthesis of δ-aminolaevulinic acid and porphobilinogen enriched at specific sites with carbon-13; this work and incorporation experiments with porphobilinogen are now described.

δ-Aminolaevulinic acid has been synthesised in several ways[4] and some of the routes have been used for the preparation of material labelled with nitrogen-15[4c,e]

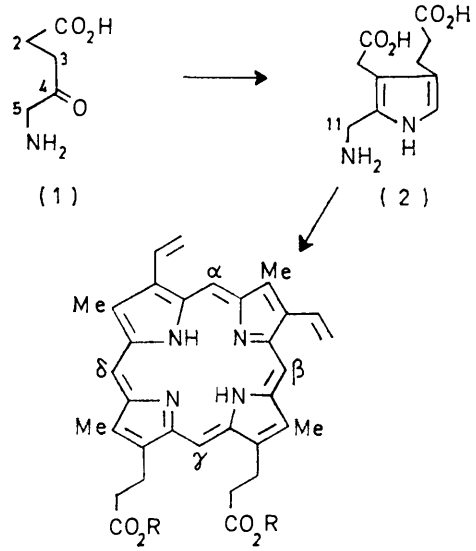
[1] Part I, A. R. Battersby, D. A. Evans, K. H. Gibson, E. McDonald, and L. Nixon, *J.C.S. Perkin I*, 1973, 1546.

[2] Preliminary communication, A. R. Battersby, E. McDonald, J. Moron, and J. Feeney, *J.C.S. Chem. Comm.*, 1972, 920.

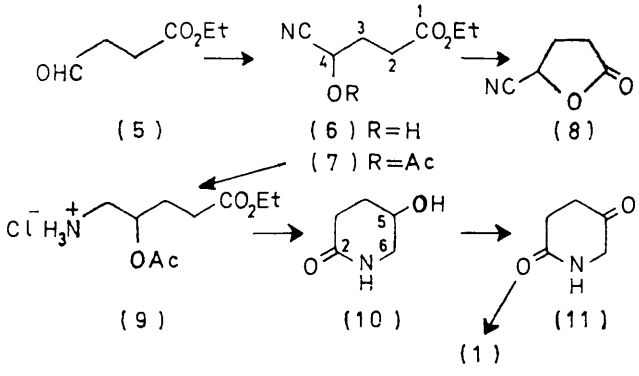
[3] Reviewed by L. Bogorad in 'The Chlorophylls,' ed. L. P. Vernon and G. R. Seeley, Academic Press, New York, 1966, p. 481; B. F. Burnham in 'Metabolic Pathways,' ed. D. M. Greenberg, Academic Press, New York, 1969, vol. III, 3rd edn., p. 403.

[4] (*a*) F. Sparatore and W. Cumming, 'Biochemical Preparations,' ed. G. B. Brown, Wiley, New York, vol. 10, 1963, p. 6; (*b*) A. A. Marei and R. A. Raphael, *J. Chem. Soc.*, 1958, 2624; (*c*) D. Shemin, C. S. Russell, and T. Abramsky, *J. Biol. Chem.*, 1955, **215**, 613; (*d*) V. M. Radimov and M. A. Gubareva, *J. Gen. Chem.* (*U.S.S.R.*), 1953, **23**, 1951 (*Chem. Abs.*, 1955, **49**, 1007); (*e*) A. Neuberger and J. J. Scott, *J. Chem. Soc.*, 1954, 1820; (*f*) A. Neuberger, J. J. Scott, and L. Shuster, *Biochem. J.*, 1956, **64**, 137; (*g*) R. W. Wynn and A. H. Corwin, *J. Org. Chem.*, 1950, **15**, 203; (*h*) A. W. Schrecker and M. M. Trail, *J. Amer. Chem. Soc.*, 1958, **80**, 6077; (*i*) L. Pichat and M. Herbert, *Bull. Soc. chim. France*, 1957, **23**, 674; (*j*) D. Shemin, *Methods Enzymol.*, 1957, **4**, 648.

or at various positions [4c,i,j] with carbon-14. Our first preparation of the ^{13}C-labelled acid was from sodium [2-^{13}C]acetate which was converted into [2-^{13}C]glycine by Bloch's method.[5] This was then carried through to δ-amino[5-^{13}C]laevulinic acid (1) essentially by the sequence of Pichat and Herbert; [4i] the yield overall



from sodium acetate was 28%. A disadvantage of this synthesis is its length and also it starts from relatively expensive labelled material. We therefore developed a new synthesis wherein C-5 and the nitrogen atom are introduced as cyanide ion; the route is thus particularly



well suited to the preparation of material labelled at C-5.

The readily available ethyl 4-oxobutyrate [6] (5) reacted as its hydrogen sulphite addition compound with sodium (or potassium) cyanide to give the cyanohydrin (6); this possesses the full carbon–nitrogen skeleton of δ-aminolaevulinic acid and it carries suitable functions at the correct sites. It was recognised early in the planning that 5-hydroxy-2-piperidone (10) could act as a convenient link between the cyanohydrin (6) and δ-aminolaevulinic acid; the nitrogen atom in the piperidone is protected internally for the subsequent oxidation step to 5-oxopiperidone (11). Accordingly, the cyanohydrin (6) was converted azeotropically in hot benzene into its lactone by using acid catalysis. The structure (8) for this product follows from the high frequency carbonyl absorption at 1805 cm^{-1}, characteristic [7] of γ-lactones carrying an electron-withdrawing substituent at the γ-position, and from the n.m.r. and mass spectra (see Experimental section).

It was hoped by analogy [8] that hydrogenation of the cyano-lactone (8) would yield 5-hydroxy-2-piperidone directly, but this did not occur over palladised charcoal in trial experiments. However, the conversion was not exhaustively studied because an alternative route was successful. Acetylation of the cyanohydrin and hydrogenation of the product (7) in acidic ethanol gave a salt [presumably (9)] which was deacetylated with anhydrous ethanolic hydrogen chloride. Treatment of the dried product(s) in ethanol with sodium hydride gave 5-hydroxypiperidone (10) in 73% yield from the acetate (7), itself obtained in 92% yield from sodium cyanide. When a similar sequence of hydrogenation followed by treatment with anhydrous potassium carbonate was applied to the cyanohydrin (6), the same product (10) was obtained in lower yield. The spectroscopic properties of 5-hydroxypiperidone indicate that the hydroxy-group is axial, presumably stabilised in that conformation by hydrogen bonding to the nitrogen atom as occurs for 3-hydroxypiperidines.[9]

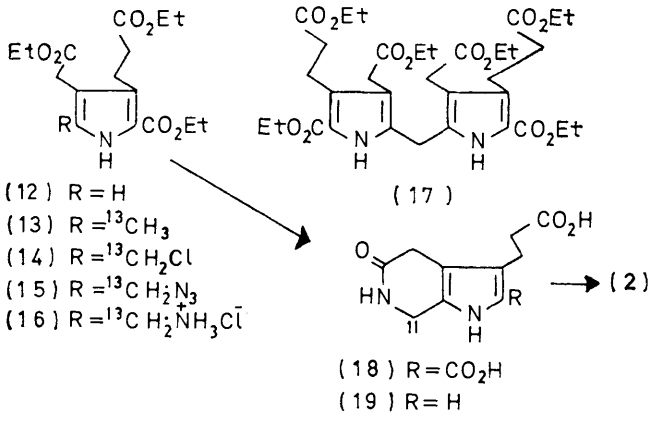
Oxidation of the hydroxypiperidone with dimethyl sulphoxide and *NN*-dicyclohexylcarbodi-imide [10] followed by acidic hydrolysis of the total product gave δ-aminolaevulinic acid hydrochloride (89%; this represents a yield of 60% overall from sodium cyanide). In contrast, the hydroxypiperidone reacted slowly with Jones reagent; only *ca.* 20% of the crystalline ketone (11) had been formed after 44 h. This inertness is probably a reflection of the internally bonded structure of the alcohol.[11]

Repetition of the foregoing synthesis starting with potassium [^{13}C]cyanide (90% enrichment) afforded δ-amino[5-^{13}C]laevulinic acid hydrochloride in 57% overall yield. ^{13}C Spectra at both natural abundance and for the enriched samples in our synthesis are reported in the Experimental section.

Attention turned next to the synthesis of porphobilinogen (2), which has steadily attracted considerable

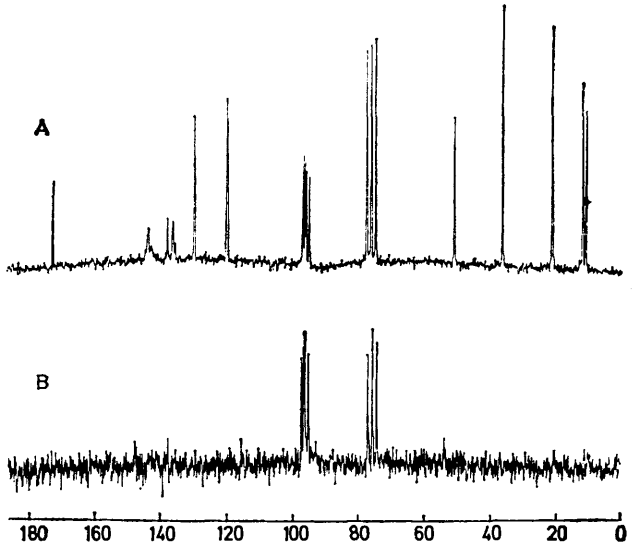
[5] K. Bloch, *J. Biol. Chem.*, 1949, **179**, 1245.
[6] D. A. Peak, R. Robinson, and J. Walker, *J. Chem. Soc.*, 1936, 753.
[7] W. Brugel, G. Stengel, F. Reichenender, and H. Suter, *Angew. Chem.*, 1956, **68**, 441; A. Schellenberger, *Chem. Ber.*, 1961, **94**, 19.
[8] C. C. Deane and T. D. Inch, *Chem. Comm.*, 1969, 813; S. Hanessian and T. H. Haskell, *J. Heterocyclic Chem.*, 1964, **1**, 55; H. Paulsen, *Angew. Chem. Internat. Edn.*, 1966, **5**, 495.
[9] G. Hite, E. E. Smissman, and R. West, *J. Amer. Chem. Soc.*, 1960, **82**, 1207.
[10] K. E. Pfitzner and J. G. Moffatt, *J. Amer. Chem. Soc.*, 1965, **87**, 5661, 5670.
[11] J. T. Nielsen, N. Elming, and N. Clauson-Kaas, *Acta Chem. Scand.*, 1955, **9**, 30.

effort [12] especially from S. F. MacDonald and his colleagues; the valuable work at Ottawa has greatly helped the present studies. It was decided to prepare



porphobilinogen from the pyrrole (13) and to introduce the ^{13}C label by reductive methylation [13] of the α-free pyrrole [14] (12) using [^{13}C]formaldehyde. Since the latter was not commercially available at the time, it was prepared by catalytic aerial oxidation of [^{13}C]methanol (60% enrichment). The reductive methylation proceeded smoothly to yield the [*methyl*-^{13}C]pyrrole (13) together with a small amount of the pyrromethane (17), as had been observed for a related case.[1,15] Sulphuryl chloride converted the pyrrole (13) into the chloromethyl derivative (14), from which the azide (15) was prepared by displacement.[16] Catalytic hydrogenation of the azide in acidic solution over palladium gave the amine hydrochloride (16), which was converted into porphobilinogen by an established route [12d] *via* the lactams (18) and (19). This sequence provided [11-^{13}C]porphobilinogen lactam (19), in which form the labelled compound was stored, in 43% overall yield from the [*methyl*-^{13}C]pyrrole (13).

The ^{13}C chemical shifts for a series of porphyrins have been reported from our group [2] and by Doddrell and Caughey,[17] and the signals have largely been assigned. In particular, the spectrum of protoporphyrin-IX dimethyl ester (4) showed a set of four sharp signals near δ 97 p.p.m. downfield from tetramethylsilane (see Figure A) which were considered [2] to arise from the *meso*-carbon atoms [marked α, β, γ, and δ on formula (4)]. The availability of [11-^{13}C]porphobilinogen allowed this important assignment to be confirmed. The labelled sample (2) was incubated with a porphyrin-producing enzyme system from *Euglena gracilis* [18,19] and the isolated [^{13}C]protoporphyrin-IX, as its dimethyl ester,* showed the four signals near δ 97 strongly enhanced (see Figure B) in addition to the three signals centred at δ 76·8 arising from the solvent. Earlier work [20] had shown that in protoporphyrin-IX derived from 5-amino[5-^{14}C]laevulinic acid, the *meso*-positions together carry half the total radioactivity (they were assayed in admixture as radioactive carbon dioxide). In the foregoing ^{13}C experiment, only four signals of essentially equal intensity and of closely similar chemical shift were observed; † these signals can thus be assigned rigorously to the *meso*-carbon atoms. Also, within the accuracy of the n.m.r. method, it is established that [11-^{13}C]porphobilinogen is biochemically transformed into protoporphyrin-IX which is equally labelled at each of the four *meso*-positions.



^{13}C N.m.r. spectra ($CDCl_3$) for protoporphyrin-IX dimethyl ester at 25·2 MHz: (A) spectrum at natural abundance using 0·06M-solution (acquisition time 0·8 s. and 182 K transients); (B) spectrum of ^{13}C-enriched sample from [11-^{13}C]porphobilinogen using 0·006M-solution (pulse delay 0·2 s, acquisition time 0·8 s and 32 K transients); δ in p.p.m. downfield from Me_4Si

The foregoing work and cognate research on assignment of the four signals to individual *meso*-carbon atoms, opens the way to studies of the rearrangement process leading to type-III porphyrins.

* An unidentified porphyrin was also isolated; this is under investigation.

† The recorded intensities varied slightly over several runs with the same sample, and small changes also occurred when different settings were used on the computer read-out.

[12] (*a*) G. P. Arsenault and S. F. MacDonald, *Canad. J. Chem.*, 1961, **39**, 2043; (*b*) S. F. MacDonald and R. J. Stedman, *ibid.*, 1955, **33**, 458; (*c*) A. H. Jackson, D. M. MacDonald, and S. F. MacDonald, *J. Amer. Chem. Soc.*, 1956, **78**, 505; (*d*) A. H. Jackson and S. F. MacDonald, *Canad. J. Chem.*, 1957, **35**, 715; (*e*) B. Frydman, S. Reil, M. E. Despuy, and H. Rapoport, *J. Amer. Chem. Soc.*, 1969, **91**, 2338; (*f*) H. Plieninger, P. Hess, and J. Ruppert, *Chem. Ber.*, 1968, **101**, 240; G. W. Kenner, K. M. Smith, and J. F. Unsworth, *J.C.S. Chem. Comm.*, 1973, 43.

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[14] E. J. Tarleton, S. F. MacDonald, and E. Baltazzi, *J. Amer. Chem. Soc.*, 1960, **82**, 4389.

[15] *Cf.* R. V. Gregorovich, K. S. Y. Liang, D. M. Clugston, and S. F. MacDonald, *Canad. J. Chem.*, 1968, **46**, 3291.

[16] A. Treibs and K. Jacob, *Annalen*, 1970, **737**, 176.

[17] D. Doddrell and W. S. Caughey, *J. Amer. Chem. Soc.*, 1972, **94**, 2510.

[18] E. F. Carell and J. S. Kahn, *Arch. Biochem. Biophys.*, 1964, **108**, 1.

[19] Details of the large-scale enzyme extraction are in a paper in preparation (A. R. Battersby, J. Baldas, J. Collins, D. H. Grayson, K. J. James, and E. McDonald).

[20] D. Shemin, T. Abramsky, and C. S. Russell, *J. Amer. Chem. Soc.*, 1954, **76**, 1204; see also E. I. B. Dresel and J. E. Falk, *Biochem. J.*, 1956, **63**, 80.

EXPERIMENTAL

The general directions in Part I [1] were followed with the following additions. Solutions were dried over anhydrous magnesium sulphate unless stated otherwise; ^{13}C n.m.r. spectra were determined for solutions in $CDCl_3$ unless stated otherwise at 25·2 MHz [using proton noise decoupling and Fourier transform techniques (PFT)] on a Varian XL-100 spectrometer; the chemical shifts are reported in p.p.m. downfield from tetramethylsilane.

Ethyl 4-Acetoxy-4-cyanobutyrate and Ethyl 4-Acetoxy-4-([^{13}C]cyano)butyrate (7).—Ethyl 4-oxobutyrate [6] (2 g, 15·4 mmol) was added dropwise at 0° to a stirred solution of sodium disulphite (1·46 g, 7·7 mmol) in water (10 ml) and the solution was kept at 4° for 16 h. It was cooled to 0°, stirred and treated dropwise with sodium cyanide (755 mg, 15·4 mmol) in water (5 ml) and then stirred at 0° for a further 1 h. Saturation of the solution with sodium chloride followed by extraction with benzene afforded the crude cyanohydrin (6) (2·38 g), which was acetylated in dry pyridine (20 ml) with acetic anhydride (2 g) at 20° for 20 h. After addition of a few drops of water, the pyridine was evaporated off and the residue in ether was washed with 2N-hydrochloric acid, saturated aqueous sodium hydrogen carbonate, and saturated brine. Evaporation of the dried solution gave *ethyl 4-acetoxy-4-cyanobutyrate*, b.p. 105° at 0·3 mmHg (2·87 g, 91%) (Found: C, 53·9; H, 6·6; N, 7·0. $C_9H_{13}NO_4$ requires C, 54·25; H, 6·6; N, 7·0%), $\nu_{max.}$ (film) 1765, 1743, and 1385 cm^{-1}; τ 4·55 (1H, t, J 6·5 Hz, CH·O), 5·83 (2H, q, J 7·5 Hz, O·CH_2), 7·35—7·55 (2H, m, CH_2·CH_2), 7·65—7·95 (2H, m, CH_2·CH_2), 7·88 (3H, s, OAc), and 8·74 (3H, t, J 7·5 Hz, O·CH_2·CH_3); δ_C 170·7 (C-1), 168·2 (acetyl CO), 116·0 (CN), 60·7 (ethoxy CH_2), 60·0 (C-4), 29·1 (C-2), 27·6 (C-3), 20·2 (acetyl CH_3), and 14·2 (ethoxy CH_3); *m/e* 199 (M^+, 12%), 172 (2), 157 (50), 154 (27), 129 (28), 112 (100), 111 (26), 102 (56), 101 (46), 97 (41), 88 (31), and 85 (77).

The above procedure was repeated with potassium [^{13}C]cyanide (250 mg; 90% enrichment) to give *ethyl 4-acetoxy-4-([^{13}C]cyano)butyrate* (0·7 g, 92%). The proton n.m.r. spectrum of this product was identical with that of unlabelled material save that the signal at τ 4·55 was a quartet (J 6·5 Hz); ^{13}C-PFT n.m.r. showed strong enhancement at δ 116·0 and signals at δ_C 170·7 (s, C-1), 168·2 (d, J 1·5 Hz, acetyl CO), 60·7 (s, ethoxy CH_2), 60·0 (90% of signal as d, J 66·0 Hz, C-4), 29·1 (d, J 3·0 Hz, C-2), 27·6br (s, C-3), 20·2 (s, acetyl CH_3), and 14·2 (s, ethoxy CH_3); *m/e* 200 (M^+ for ^{13}C-material, 9%), 199 (M^+ for ^{12}C-material, 0·9%), 172 (0·7), 158 (40), 155 (23), 129 (29), 113 (100), 112 (36), 102 (55), 101 (45), 98 (36), 88 (29), and 85 (71).

γ-Cyano-γ-butyrolactone (8).—A solution of the foregoing cyanohydrin (212 mg) in dry benzene (10 ml) was stirred and heated under reflux with Amberlite IR-120 resin (H-phase; 0·4 g) using a reflux variable take-off still head. Portions of the benzene were collected periodically (*ca.* 10 ml h^{-1}) with addition of an equivalent volume of fresh benzene to the still. After 10 h, the mixture was filtered, the resin was washed, and the filtrate was evaporated. Chromatography of the residue on silica gel (10 g) with benzene (20 ml) then 3 : 7 ether–benzene gave *γ-cyano-γ-butyrolactone* (156 mg) as a liquid (Found: M^+, 111·0322. $C_5H_5NO_2$ requires M, 111·0320), $\nu_{max.}$ 1805, 1140, and 995 cm^{-1}; τ 4·87 (1H, m, CH·CN) and 7·40 (4H, m, CH_2·CH_2); *m/e* 111 (M^+, 26%), 85 (15), 67 (35), 66 (10), 56 (100), and 41 (100), m^* 25·1 (67 ⟶ 41; calc. 25·09).

5-Hydroxy-2-piperidone and 5-Hydroxy[6-^{13}C]-2-piperidone (10).—Ethyl 4-acetoxy-4-cyanobutyrate, before distillation (0·5 g), in ethanol (15 ml) and concentrated hydrochloric acid (1 ml) was shaken under hydrogen at 20° and 1 atm with 5% palladised charcoal (0·5 g) for 1·5 h (uptake 126 ml). The filtered solution was evaporated and the residue in anhydrous ethanol (40 ml) was treated with hydrogen chloride until 2 g had been absorbed. After 62 h at 20° the solution was evaporated; evaporation was repeated after addition of fresh ethanol. The crystalline residue was dried at 20° (P_2O_5) for 24 h, then was dissolved in anhydrous ethanol (50 ml), and to the stirred solution was added sodium hydride (130 mg; 50% dispersion) in one portion. The mixture was stirred at 20° for 20 min and after neutralisation with glacial acetic acid (phenolphthalein) was filtered, and the solid was washed with ethanol. Evaporation of the combined solutions and chromatography of the residue in 1 : 9 methanol–chloroform on alumina gave the hydroxypiperidone (250 mg) which, recrystallised from ethanol–ether, had m.p. 145—146° (lit.,[11] 144—146°); yield 211 mg (73%), $\nu_{max.}$ (Nujol) 3210, 3120sh, and 1635 cm^{-1}; τ (D_2O) 5·78 (1H, quintet, J 4·5 Hz, CH·OD), 6·49 and 6·75 (each 1H, dd, J 13 and 4·5 Hz, ND·CH_2), and 7·28—7·76 (2H, m), and 7·82—8·25 (2H, m) (both CH_2·CH_2); δ_C (D_2O) 63·2 (C-5), 48·4 (C-6), and 27·5 and 27·3 (C-3 and C-4, not necessarily respectively); the carbonyl group was not observed when a pulse recycle time of 2·4 s was used; *m/e* 115 (M^+, 100%), 98 (8), 59 (7), 58 (13), 57 (7), 55 (8), 44 (13), and 43 (12).

In the ^{13}C series, ethyl 4-acetoxy-4-([^{13}C]cyano)butyrate (90% enrichment; 0·65 g) gave *5-hydroxy[6-^{13}C]-2-piperidone* (265 mg, 70%), m.p. 144·5—145·5°. The proton n.m.r. spectrum (D_2O) was as above save that at τ 6·49 and 6·75 90% of the signals appeared as double dd, J = 13, 4·5, and 143 Hz, and 10% as dd, J 13 and 4·5 Hz; δ_C (D_2O) 63·2 (90% of signal as d, J 39·6 Hz, C-5), 48·4 (C-6, strongly enhanced), and 27·5 and 27·3 (C-3 and C-4 as above); *m/e* 116 (M^+ for ^{13}C-material, 100%), 115 (M^+ for ^{12}C-material, 16), 99 (11), 59 (8), 58 (14), 57 (10), 55 (10), 44 (16), and 43 (14).

4-Cyano-4-hydroxybutyrate (305 mg) was hydrogenated and worked up in a similar way and the product was treated in anhydrous ethanol (30 ml) for 48 h with anhydrous potassium carbonate (0·3 g). Isolation as earlier and crystallisation from ethanol–ether gave 5-hydroxy-2-piperidone (110 mg, 49%), m.p. 143·5—145°.

Piperidine-2,5-dione (11).—A solution of 5-hydroxy-2-piperidone (115 mg) in water (1 ml) was mixed with acetone (7 ml) and stirred at 20° while Jones reagent (0·3 ml) was added. After the mixture had been stirred for 1 day, more reagent (0·1 ml) was added, and after a further 20 h, 1 : 1 benzene–ethanol (10 ml) and potassium carbonate were added. The filtered solution was evaporated (to 2 ml) and then passed over a short column of alumina (4 g) in 1 : 1 benzene–ethanol. Evaporation of the eluate gave a gum which crystallised from ethanol–ether to give the *dione* (20 mg, 18%), m.p. 136—138° (Found: C, 53·2; H, 6·0; N, 12·4. $C_5H_7NO_2$ requires C, 53·1; H, 6·2; N, 12·4%), $\nu_{max.}$ 1730 and 1680 cm^{-1}; *m/e* 113 (M^+, 100%), 87 (31), 86 (19), 85 (15), 58 (50), 57 (23), 45 (12), and 44 (35).

δ-Aminolaevulinic Acid Hydrochloride and [5-^{13}C] Material (1).—Dry pyridine (0·08 ml) and trifluoroacetic acid (0·04 ml) were added at 20° to a solution of 5-hydroxy-2-piperidone (115 mg, 1·0 mmol) in dry dimethyl sulphoxide (1·5 ml) containing *NN*-dicyclohexylcarbod i-imide (620

3 mmol). After the mixture had been kept at 20° for 1 day, water (a few drops) and ethyl acetate (20 ml) were added and the solution was filtered to remove urea, which was well washed with ethyl acetate. The ethyl acetate was evaporated from the combined solutions to give piperidine-2,5-dione in dimethyl sulphoxide. This was mixed with water (6 ml) and concentrated hydrochloric acid (12 ml) and the solution was heated under reflux for 4 h. It was evaporated (to *ca.* 3 ml), diluted with water (30 ml), and decolourised with the minimum of charcoal. Evaporation of the filtered solution (finally at 60° and 0·1 mmHg) gave a residue which in 0·05N-hydrochloric acid was applied to a column of Amberlite IR-120 resin (H-phase; 25 ml bed volume) which had been equilibrated with 0·05N-hydrochloric acid. Elution first with 0·05N-hydrochloric acid (150 ml) removed neutral impurities, and elution with *N*-hydrochloric acid then gave δ-aminolaevulinic acid hydrochloride, which crystallised from ethanol–ether (yield 149 mg, 89%), m.p. 144—146°, homogeneous on silica in 12 : 5 : 3 n-butanol–water–acetic acid and with i.r. (Nujol) and n.m.r. (D_2O) spectra identical with those of authentic material; τ (D_2O) 5·77 (2H, s, $CO{\cdot}CH_2{\cdot}N$) and 6·9—7·3 (4H, m, $CH_2{\cdot}CH_2$); δ_C (D_2O) 204·1 (C-4), 177·5 (C-1), 48·2 (C-5), and 35·45 and 28·55 (C-3 and C-2, not necessarily respectively).

The procedure was repeated on 5-hydroxy[6-^{13}C]-2-piperidone (90% enrichment; 230 mg) to yield δ-amino-[5-^{13}C]laevulinic acid hydrochloride (297 mg, 89%), τ (D_2O) 5·77 (2H, 90% of signal as d, *J* 143 Hz, $CH_2{\cdot}N$) and 6·9—7·3 (4H, m, $CH_2{\cdot}CH_2$); δ_C (D_2O) 48·2 (C-5), strongly enhanced.

Ethyl 3-(2-*Ethoxycarbonylethyl*)-4-*ethoxycarbonylmethyl*-[5-^{13}C-methyl]*pyrrole*-2-*carboxylate* (13).—[^{13}C]Methanol was oxidised to [^{13}C]formaldehyde in a much simplified version of the apparatus described under Method IV in ref. 21. The molybdenum oxide–ferric oxide catalyst was oxidised by being heated in a straight quartz tube (electric furnace) at 540° in a stream of air for 2 h and then at 380—390° for 0·5 h in air at 140 ml min^{-1}. A reservoir holding [^{13}C]methanol (0·5 g) was then attached to the quartz tube and the alcohol was carried in the air stream over the catalyst at 380—385° during 20—30 min. The issuing gases were passed through glacial acetic acid (50 ml) and the water which collected at the cold end of the quartz tube was washed into the main solution with acetic acid. An aliquot portion of this solution was mixed with ice and water and neutralised with 2N-sodium hydroxide before being mixed with an ethanolic solution of dimedone (10%; 15% in excess of the theoretical amount). The yield of derivative showed that formaldehyde was produced in 50—80% yield; in the ^{13}C experiment, the yield was 63% (60% enrichment).

Freshly distilled hydriodic acid (*d* 1·94; 9 ml) was mixed with cooled acetic anhydride (4·5 ml) and then hypophosphorous acid (3·6 ml) was added. After the mixture had been stirred for 15 min, the α-free pyrrole (12) (1·04 g, 3·06 mmol) was added, followed dropwise by the foregoing solution of [^{13}C]formaldehyde (18 ml, 2·97 mmol). The solution was stirred at 20° for 1 h, then evaporated to dryness, and the residue was treated with water (3 ml). The solid which had separated after 16 h at 0° was collected, dried, and esterified for 20 h at 20° in ethanolic 7% hydrogen chloride (8 ml). The precipitate (61 mg) was identified as the pyrromethane (17) by its n.m.r. spectrum and by comparison with an authentic sample from Dr. K. H. Gibson; τ 5·6—6·0 (12H, overlapping q, $O{\cdot}CH_2$), 6·14 (2H, 60% of signal as d, *J* 128 Hz, and 40% as s, bridge CH_2), 6·90 and 7·45 (4H, each, t, *J* 7 Hz, $CH_2{\cdot}CH_2{\cdot}CO$), and 8·55—8·9 (18H, overlapping t, $CH_2{\cdot}CH_3$). The main solution was evaporated and the residue in 1 : 9 ether–benzene was fractionated on silica gel (40 g) to give the [^{13}C-*methyl*]-pyrrole (575 mg), m.p. 61—63° (from benzene–pentane), identified by comparison with authentic ^{12}C-material; it was sufficiently pure as such for the next stage; later fractions contained less pure material (159 mg). Rechromatography of the material in mother liquors and later fractions gave more crystalline pyrrole (108 mg; total yield 66%), τ 0·8br (s, NH), 5·6—6·05 (overlapping q, $O{\cdot}CH_2$), 6·6 (s, $CH_2{\cdot}CO$), 7·0 and 7·48 (each t, *J* 8 Hz, $CH_2{\cdot}CH_2{\cdot}CO$), 7·8 (60% of signal d, *J* 128 Hz, 40% of signal s, Me), and 8·6—8·9 (overlapping t, $O{\cdot}CH_2{\cdot}CH_3$); δ_C (natural abundance in C_6D_6) 172·3 and 171·1 ($CH_2{\cdot}CO_2Et$), 160·6 (CO_2Et), 129·2, 119·5, and 117·3 (weak signals, C-2, C-3, and C-4 of pyrrole), 121·5 (strong signal, C-5 of pyrrole), 60·5 and 59·9 ($O{\cdot}CH_2Me$), 35·6 ($CH_2{\cdot}CH_2{\cdot}CO$), 31·1 (CH_2CO), 21·1 ($CH_2{\cdot}CH_2{\cdot}CO$), and 14·9 ($CH_2{\cdot}CH_3$).

Ethyl 3-(2-*Ethoxycarbonylethyl*)-4-*ethoxycarbonylmethyl*-5-*azido*[^{13}C]*methylpyrrole*-2-*carboxylate* (15).—The foregoing [^{13}C-*methyl*]pyrrole (749 mg) in ether (15 ml) was treated at 0° with ethereal 0·1M-sulphuryl chloride (25·4 ml; 15% excess) and after the addition, stirring was continued for 20 min as the temperature rose to 20°. The ether was evaporated off below 20°; the residue was washed with pentane (6 ml) and dried *in vacuo*. This chloromethylpyrrole (14) in acetone (16 ml) and water (8 ml) was stirred at 20° with sodium azide (287 mg) for 20 min and the acetone was then evaporated off. Recrystallisation of the precipitate from hexane gave the [^{13}C]*azide* (754 mg), m.p. 89·5—90·5° [Found for ^{12}C material: C, 54·2; H, 6·5%; *m/e* 352·1630 ($M^+ - N_2$). $C_{17}H_{24}N_4O_6$ requires C, 53·7; H, 6·4%; $M - N_2$ requires 352·1633], δ_C (C_6D_6) 171·8 and 170·5 ($CH_2{\cdot}CO_2Et$), 160·7 (CO_2Et), 129·9, 128·2, 119·3, and 116·6 (pyrrole ring), 60·7, 60·2, and 60·0 (3 × $O{\cdot}CH_2Me$), 45·6 (CH_2N_3), 35·5 ($CH_2{\cdot}CH_2{\cdot}CO$), 30·0 ($CH_2{\cdot}CO$), 21·2 ($CH_2{\cdot}CH_2{\cdot}CO$), and 14·3 ($CH_2{\cdot}CH_3$); *m/e* 380 (M^+), 352, 340, 339 (100%), 293, 279, 256, and 255.

Early preparations of ^{13}C-labelled azide were from the bromomethylpyrrole [1] (14; Br in place of Cl); with ^{13}C-methylene-labelled material; this showed τ 0·45br (s, NH), 5·48 (60% of signal as d, *J* 155 Hz, 40% as s, CH_2Br), 5·5—6·2 (overlapping q, $O{\cdot}CH_2$), 6·51 (s, $CH_2{\cdot}CO$), 7·0 and 7·48 (both t, *J* 8 Hz, $CH_2{\cdot}CH_2{\cdot}CO$), and 8·5—9·0 (overlapping t, $O{\cdot}CH_2{\cdot}CH_3$).

5-*Carboxy*[11-^{13}C]*porphobilinogen Triethyl Ester Hydrochloride* (16) *and* [11-^{13}C]*Porphobilinogen* (2).—This sequence for unlabelled material involved known compounds and authentic samples of all the substances, save the first, were available from cognate work; [1] products were identified here by full spectroscopic and t.l.c. comparison in all cases.

A solution of the foregoing [^{13}C]azide (668 mg) in ethanol (50 ml) containing concentrated hydrochloric acid (0·3 ml) was shaken with palladium black (150 mg) and hydrogen at 21° and 1 atm for 5·5 h. The filtered solution was evaporated to 30 ml, then diluted with ether, and the precipitate (total 638 mg after work-up of initial mother liquor) was washed with ether. This 5-carboxy[11-^{13}C]porphobilinogen triethyl ester hydrochloride was homogeneous on silica in methanolic ammonia and was pure enough for the next stage. Unlabelled material was recrystallised from ethanol–

[21] A. Murray, tert., and D. L. Williams, ' Organic Syntheses with Isotopes,' Interscience, New York, 1958, Part I, p. 608.

ether; m.p. 192—193° (lit.,[12d] 193—195°); τ [$(CD_3)_2SO$] 1·55br (s, NH), 5·6—6·1 (6H, overlapping q, $O{\cdot}CH_2$), 6·0 (2H, s, $N{\cdot}CH_2$), 6·41 (2H, s, $CH_2{\cdot}CO$), 7·11 and 7·59 (2H each, t, *J* 8 Hz, $CH_2{\cdot}CH_2{\cdot}CO$), and 8·6—8·9 (9H, overlapping t, $OCH_2{\cdot}CH_3$); the salt dissociated in the inlet of the mass spectrometer [*m/e* 354 (M^+), 324, 308, 281 (100%), 267, and 235].

All the foregoing ^{13}C-labelled ester hydrochloride was heated on a steam-bath with 2N-sodium hydroxide for 1 h, then the solution was cooled and adjusted to pH 4·5 with acetic acid to yield 5-carboxy[11-^{13}C]porphobilinogen (440 mg), m.p. 233—234° (decomp.) (lit.,[12d] m.p. 230—233°). Unlabelled material showed τ ($CF_3{\cdot}CO_2D$) 5·52 (2H, s, $CH_2\overset{+}{N}$), 6·20 (2H, s, $CH_2{\cdot}CO$), and 6·82 and 7·19 (2H each, distorted t, $CH_2{\cdot}CH_2{\cdot}CO$).

The published steps [12d] were followed to afford carboxy-[11-^{13}C]porphobilinogen lactam (18) (249 mg) plus less pure material (69 mg) from mother liquors. These two fractions were separately decarboxylated and the two batches of [11-^{13}C]porphobilinogen lactam were purified before combination [total yield of (19), 177 mg], m.p. 285° (decomp.) [lit.,[12d] 282—284° (decomp.)]; *m/e* 209 (M^+ of ^{13}C-material), 208 (M^+ of ^{12}C-material), 180, 179, 166, 165, 150, 149, 136, and 135. This product was hydrolysed with aqueous 2N-potassium hydroxide to yield [11-^{13}C]porphobilinogen which was used directly.

Incorporation of [11-^{13}C]*Porphobilinogen into Protoporphyrin-IX* (3).—2N-Potassium hydroxide (2 ml) was added to the foregoing [11-^{13}C]porphobilinogen lactam (18·7 mg). The hydrolysis was shown to be complete in 6 h by t.l.c. on silica with 1 : 24 glacial acetic acid–methanol as solvent. After the solution had been adjusted to pH 7 with 0·1N-acetic acid, it was added to the enzyme system prepared from three growth-batches of *Euglena gracilis* [19] in pH 7·4 phosphate buffer (*ca.* 250 ml). Dithiothreitol (*ca.* 10 mg) was added, the solution was divided among three 250 ml Erlenmeyer flasks wrapped in aluminium foil, and these were gently shaken in air at 30° for 16 h. The combined solution was stirred in bright daylight for 0·5 h and was then mixed with 1 : 3 acetic acid–ethyl acetate (*ca.* 400 ml), and the precipitated protein was removed by centrifugation. Saturated aqueous sodium acetate (*ca.* 50 ml) was added and after equilibration the aqueous layer was separated and washed twice with ethyl acetate. The combined organic layers were washed repeatedly with saturated sodium acetate (*ca.* 50 ml portions) until the aqueous layer was colourless; each aqueous layer was back-washed with ethyl acetate (which was added to the main solution). Finally the combined solution in ethyl acetate was washed with one-tenth its volume of aqueous 3% sodium acetate and then with aqueous 15% hydrochloric acid until the acidic extracts were colourless. After the combined acidic solution had been overlayered with ether, it was adjusted to pH 4 with sodium carbonate. Exhaustive extraction with ether (washing with water) yielded protoporphyrin-IX and other porphyrins; this residue was dissolved in a few drops of glacial acetic acid and treated with an excess of distilled ethereal diazomethane. After the excess of reagent had been removed by adding acetic acid, the solution was evaporated and the residue was fractionated by p.l.c. on silica (1 mm thickness) with chloroform. Protoporphyrin-IX dimethyl ester and an unidentified porphyrin (*ca.* 25% of the total porphyrins and currently under investigation) were the main products. The former (0·87 mg) was eluted from the silica with chloroform and to it was added a further portion (0·44 mg) of ^{13}C-labelled material from an earlier experiment on a smaller scale. This material (1·31 mg, 60% enrichment) was diluted with [^{12}C]protoporphyrin-IX dimethyl ester (5·49 mg) and after chromatography on alumina (Grade IV) in chloroform was recrystallised from chloroform–methanol to yield the product (6·25 mg) used for the ^{13}C-PFT n.m.r. spectrum shown in the Figure (B).

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