Biosynthesis of Porphyrins and Related Macrocycles. Part V.[1,2] Structural Integrity of the Type III Porphyrinogen Macrocycle in an Active Biological System; Studies on the Aromatisation of Protoporphyrinogen-IX

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Coproporphyrin-III ester (23), specifically labelled with ^{14}C or ^{3}H in the propionic acid side chains of rings A and B, has been prepared by partial synthesis (40% overall yield) from readily available protoporphyrin-IX (9). The derived samples of coproporphyrinogen-III [(4) and (5)] have been converted enzymically into two samples of protoporphyrin-IX ester [(10) and (11)], shown by degradation to be labelled at sites equivalent to those in the original samples of coproporphyrinogen-III. These results and those of other workers establish that the type III porphyrinogen macrocycle, having been biosynthesised from four porphobilinogen (PBG) units, does not subsequently undergo enzymic ' scrambling ' of the constituent pyrrole rings. This result is of crucial importance for further biosynthetic studies (see following paper).

Experiments based on isotope effects have been carried out with [11-$^{3}H_1$]PBG which provide strong evidence that the final step on the biosynthetic pathway, *viz.* the aromatisation of protoporphyrinogen-IX (6) to the corresponding porphyrin (9), is an enzyme-catalysed process. Synthetic routes are described which provide [11-^{3}H]- and [11-^{2}H]-PBG.

A CLASSICAL series of studies with mutant organisms and isolated enzymes defined[3,4] the biosynthetic sequence porphobilinogen (PBG) (1) ⟶ uroporphyrinogen-III (2) ⟶ coproporphyrinogen-III (3) ⟶ protoporphyrinogen-IX (6) ⟶ protoporphyrin-IX (9), the last substance being of vital importance, *e.g.* for the production of haem. This paper is concerned with labelling studies on the stages (3) ⟶ (6) and (6) ⟶ (9).

First, it became essential for the researches described in the following paper[5] to prove that the type III macrocycle [*e.g.* (2) and (3)], when once biosynthesised from four PBG units, does not subsequently undergo enzymic ' scrambling ' of the constituent pyrrole rings. Secondly, we aimed to find out whether or not the aromatisation step in the natural sequence (6) ⟶ (9) is enzyme-mediated.

Integrity of Type III Porphyrinogen Macrocycle.—

[1] Part IV, A. R. Battersby, J. F. Beck, and E. McDonald, *J.C.S. Perkin I*, 1974, 160.

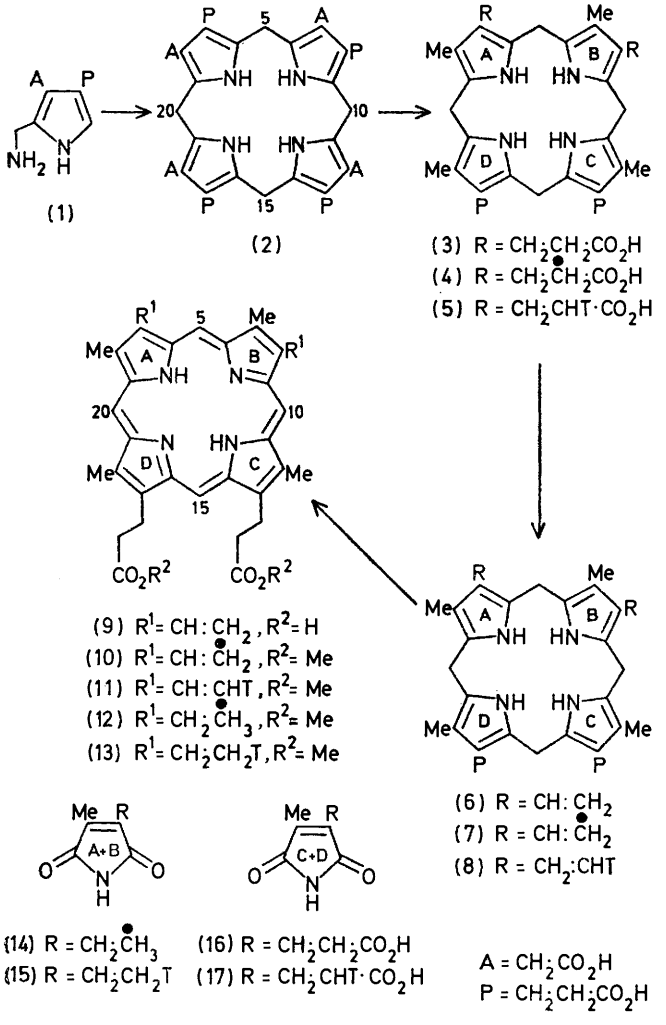
[2] Preliminary account in part, *J.C.S. Chem. Comm.*, 1972, 1118.

[3] Reviewed by B. F. Burnham, ' Metabolic Pathways,' ed. D. M. Greenberg, Academic Press, New York, 1969, vol. III, 3rd edn., p. 403.

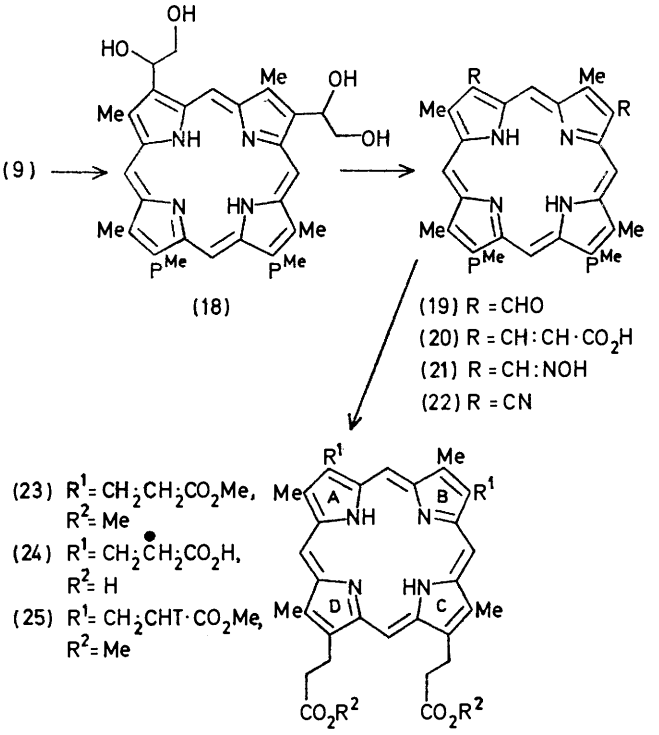
[4] Reviewed by A. R. Battersby and E. McDonald, in ' Falk's Porphyrins and Metalloporphyrins,' ed. K. M. Smith, Elsevier, Amsterdam, 1975, 2nd edn.

[5] A. R. Battersby, G. L. Hodgson, E. Hunt, E. McDonald, and J. Saunders, following paper.

Valuable evidence regarding the first problem was briefly published by the Münster group [6] as our own studies were completed. They demonstrated that [5,15-^{14}C]uroporphyrinogen-III [as (2)] is converted enzymically into [5,15-^{14}C]protoporphyrin-IX [as (9)]. Our work interlocks with theirs in being based on specifically labelled coproporphyrin-III [*e.g.* (24)]. The plan was to convert the derived porphyrinogen (4) enzymically into protoporphyrin-IX (10), the labelling pattern of which could then be determined by degradation. Accordingly, the dialdehyde (19) was prepared essentially by Sparatore and Mauzerall's route,[7] (9) ⟶ (18) ⟶ (19), but with improved yield (69% overall),



the main change being in the hydroxylation procedure (see Experimental section). Condensation of the dialdehyde with malonic acid gave the known diacrylic ester [7] (20), which was esterified and hydrogenated in formic acid containing perchloric acid to yield coproporphyrin-III tetramethyl ester (23). The overall yield from the dimethyl ester of (9) to (23) from this sequence



is 40% and so it provides a convenient preparation of coproporphyrin-III from the readily available protoporphyrin-IX (9). Kenner and his co-workers have described another attractive method for the same conversion.[8]

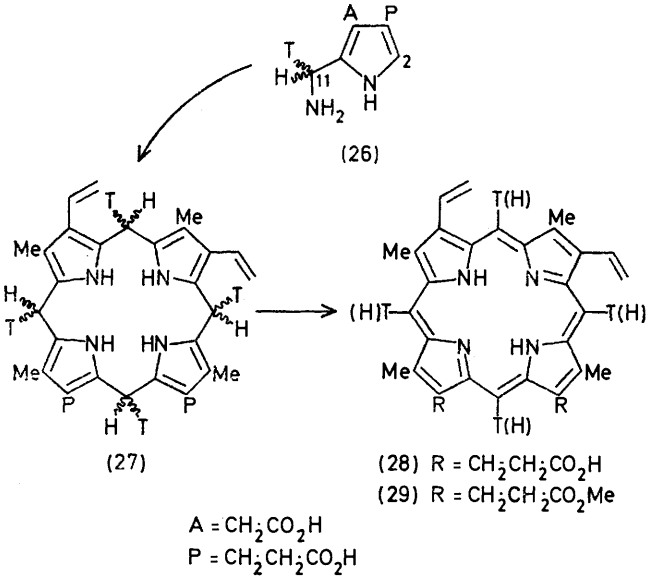
By using [2-^{14}C]malonic acid in the above sequence, coproporphyrin-III ester was obtained specifically labelled in the ring-A and ring-B side chains as indicated by ● for the derived acid (24) prepared from it by hydrolysis. This was reduced with sodium amalgam in aqueous alkali [9] to yield the labelled porphyrinogen (4) ready for immediate incubation with the preparative enzyme system from *Euglena gracilis*.[5] Even after 5 min, activity (1.5%) was incorporated into protoporphyrinogen-IX (7), which was oxidised with quinhydrone [10] to the corresponding porphyrin, isolated as its ester (10). Proof that the labelling pattern of this product was unchanged from the original material (4) was gained by hydrogenation to mesoporphyrin-IX ester (12) followed by oxidation of the derived acid to yield the imides [4] (14) and (16). The molar activity of the former imide corresponded to 49% of the molar activity of the original protoporphyrin-IX ester (10); hence 2 × 49% (98%) of the original activity of the labelled protoporphyrin-IX ester (10) was carried by rings A and B. In contrast, the activity of haematinic acid (16) was insignificant (<1%).

[6] B. Franck, D. Gantz, F.-P. Montforts, and F. Schmidtchen, *Angew. Chem. Internat. Edn.*, 1972, **11**, 421.
[7] F. Sparatore and D. Mauzerall, *J. Org. Chem.*, 1960, **25**, 1073.
[8] G. W. Kenner, S. W. McCombie, and K. M. Smith, *J.C.S. Chem. Comm.*, 1972, 1347.
[9] J. E. Falk, 'Porphyrins and Metalloporphyrins,' Elsevier, Amsterdam, 1964, p. 161.
[10] S. Sano, *J. Biol. Chem.*, 1966, **241**, 5276.

A complementary set of experiments was based on coproporphyrin-III ester, which was ^{3}H-labelled as illustrated in structure (25) in the propionate side chains of rings A and B. This was prepared from the dialdehyde (19) by condensation with malonic acid which had undergone base-catalysed exchange with tritiated water. Thorough drying of the exchanged malonic acid was essential for success in the condensation step. When a diluted sample of the ^{3}H-labelled coproporphyrin-III ester (25) was oxidised to haematinic acid (17), its molar activity accounted for only 78% of the original activity of the porphyrin. Thus, 22% of the original ^{3}H activity was located at the bridge positions of the porphyrin (25), not surprisingly, since exchange of hydrogen at these bridges is known to occur under mildly acidic conditions.[11] This extra labelling at the bridges caused no problems for studies of the enzymic conversion of the derived porphyrinogen acid (5) into protoporphyrin-IX. A longer incubation time than used above led to an incorporation of 15% [isolated as the ester (11)]. This sample was degraded (*a*) by oxidation of the derived mesoporphyrin-IX ester (13) to ethylmethylmaleimide (15) and haematinic acid (16); the former accounted for 76% of the original activity, the latter for <2%; (*b*) by the earlier route to the dialdehyde [as (19)] which carried 20% of the original activity at its bridge positions; no further loss of ^{3}H activity occurred when the dialdehyde [as (19)] was converted *via* the dioxime [as (21)] into the dinitrile [12] [as (22)]. These results define the labelling pattern of the protoporphyrin-IX (11) as being exactly the same as in the original coproporphyrinogen-III (5).

The ^{14}C and ^{3}H experiments above both show that specifically labelled coproporphyrinogen-III (3) is converted enzymically into protoporphyrin-IX (9) which is labelled at the equivalent sites. Taken together, the work at Münster [6] and that above establish that the pyrrolic units in the type-III porphyrinogen macrocycle do not undergo ' scrambling ' of their positions in the presence of a full set of active enzymes for the sequence PBG (1) ⟶ (2) ⟶ (3) ⟶ (6) ⟶ protoporphyrin-IX (9); further support came from work at Liverpool.[13] In addition, the foregoing experiments provide tracer evidence for the sequence (3) ⟶ (6) ⟶ (9) to underpin that previously available from experiments with mutants.

Aromatisation of Protoporphyrinogen-IX (6).—The ready aromatisation of porphyrinogens to porphyrins, especially in light, is well documented [14] so it was conceivable that the aromatisation step (6) ⟶ (9) in the biosynthesis might be non-enzymic. However, there were indications [15] that ' aromatase ' activity was present in certain crude enzyme preparations. Our own experiences were similar. Thus, Dr. G. L. Hodgson found when PBG (1) was incubated at pH 7.6 in the dark with the enzyme system from *Euglena gracilis*, 44% of the macrocyclic material formed was present as porphyrinogen after 0.5 h, this value falling to 12% after 1.5 h and to 1.6% after 4 h. We reasoned, as follows, that experiments with protoporphyrinogen-IX (27) ^{3}H-labelled at the bridge methylene groups should give clear



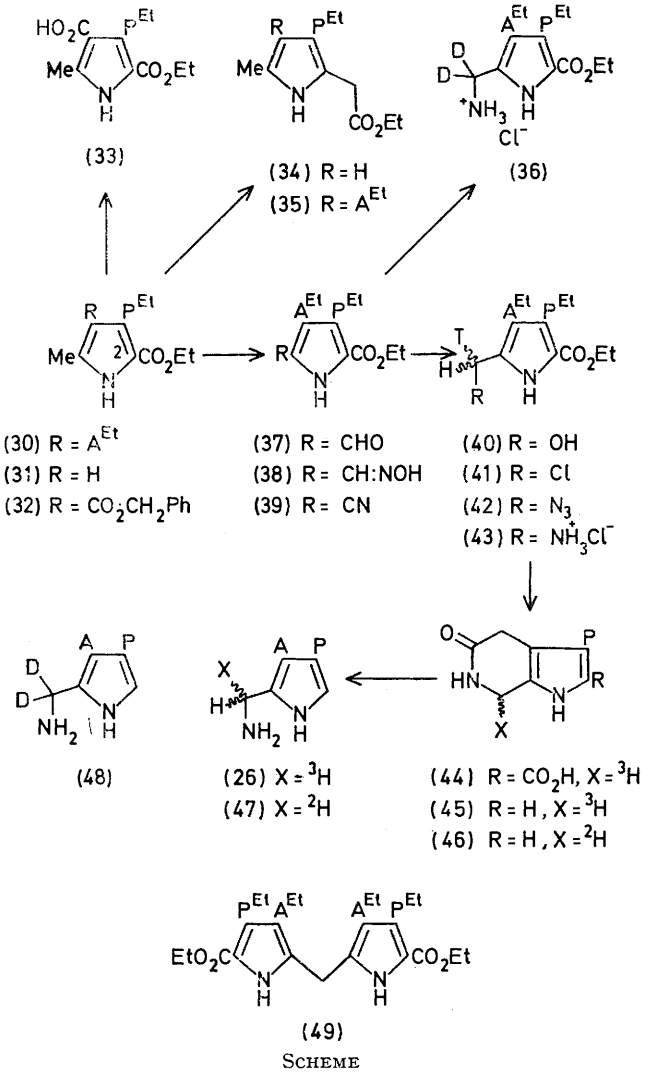
evidence for or against an enzymic process. The non-enzymic photochemical autoxidation of porphyrinogens shows an appreciable kinetic isotope effect; *e.g.* such an aromatisation of coproporphyrinogen-III [as (3)], randomly tritiated at the bridge methylenes, occurred with only 4% loss of tritium.[13] In contrast, an enzyme-catalysed aromatisation process would be expected to be *stereospecific* and hence should remove [16] 50% of the tritium from each bridge of the labelled protoporphyrinogen-IX (27). The plan was to synthesise [11-^{3}H]-PBG (26) for incubation with the enzyme systems from chicken blood and from *Euglena gracilis*; the protoporphyrinogen-IX (27) formed as the penultimate product must be ^{3}H-labelled at the four methylene bridges and the isolated protoporphyrin-IX ester (29) was to be assayed for ^{3}H retention.

The required starting material (30) for this work had been synthesised [17] by reductive alkylation [18] of the corresponding β-free material (31) prepared from the benzyl ester (32). Conditions have now been developed for debenzylation, decarboxylation, and reductive alkylation with glyoxylic acid to be carried out with pyrrole (32) as a ' one-pot ' operation, the overall yield (32) ⟶ (30) being 51%. When the conditions were

[11] J. B. Paine, tert., and D. Dolphin, *J. Amer. Chem. Soc.*, 1971, **93**, 4080.
[12] W. S. Caughey, J. O. Alben, W. Y. Fujimuto, and J. L. York, *J. Org. Chem.*, 1966, **31**, 2631.
[13] J. A. S. Cavaleiro, G. W. Kenner, and K. M. Smith, *J.C.S. Perkin I*, 1974, 1188.
[14] Ref. 9, p. 162.
[15] *E.g.* S. Sano and S. Granick, *J. Biol. Chem.*, 1961, **236**, 1173; R. J. Porra and J. E. Falk, *Biochem. J.*, 1964, **90**, 69.
[16] D. Arigoni and E. L. Eliel, *Topics Stereochem.*, 1969, **4**, 127; A. R. Battersby, *Accounts Chem. Res.*, 1972, **5**, 148.
[17] A. R. Battersby, D. A. Evans, K. H. Gibson, E. McDonald, and L. Nixon, *J.C.S. Perkin I*, 1973, 1546.
[18] M. W. Roomi and S. F. MacDonald, *Canad. J. Chem.*, 1970, **48**, 139.

too mild, the acid (33) was isolated and identified as its known ethyl ester; [19] conversely, if the debenzylation stage was too vigorous, the esters (34) and (35) were formed, undoubtedly by hydrolysis of the C-2 ester group of pyrroles (31) or (30) followed by decarboxylation and reductive alkylation.

The known aldehyde [20] (37) was prepared from (30) as usual by using sulphuryl chloride and an occasional by-product was shown spectroscopically to be the pyrromethane (49), the formation of which is unexceptional.



SCHEME

Reduction of the aldehyde (37) by borotritiide introduced the C-11 label, and the alcohol (40) with thionyl chloride gave the chloride (41). The rest of the sequence [21] (41) → (42) → (43) → (44) → (45) → (26) is shown in the Scheme. (11*RS*)-[11-$^2H_1$]PBG (47) was prepared similarly by use of borodeuteride in the reduction step; the labelled PBG (47) was shown to be completely monodeuteriated by mass spectrometry on the derived ester [methyl ester of (46)].

Clearly, deuterium- or tritium-labelled aldehydes [as (37)] can be made by re-oxidation of the alcohols (40) or (40; ^2H in place of T) and Collins' reagent [22] carried out this conversion in 65% yield.

Interesting results were obtained in experiments aimed at synthesis of doubly labelled [11-$^2H_2$]PBG (48) by deuterogenation of the known [23] nitrile (39). Treatment of the aldehyde (37) with hydroxylamine under the described conditions [23] gave a mixture of *syn*- and *anti*-oximes [as (38)], as shown by n.m.r., which was dehydrated to yield the nitrile (39). An improved approach [64% yield of (39)] used Van Es' method [24] for direct preparation of aromatic nitriles by treatment of the aldehyde with hydroxylamine hydrochloride and sodium formate in hot formic acid. Reduction of the nitrile (39) with deuterium and platinum in ethanolic hydrochloric acid gave the salt [as (36)], but mass spectrometry showed that <5% of the product was doubly deuteriated. Since the solvent was the only likely source of protium, the deuterogenation was repeated in $CH_3{\cdot}CO_2D$–D_2O–DCl; the product (36) now contained 80% of [$^2H_2$] species. We have not attempted to improve the deuterium content still further but the now obvious experiments should bring this about.

The foregoing [11-$^3H_1$]PBG (26) was mixed with [2,11-^{14}C]PBG * to act as an internal standard; neither of the labelled carbon atoms is lost in the biosynthetic sequence to protoporphyrin-IX (28). Mention was made above of the fact that after a short treatment of PBG with the enzymes from *Euglena gracilis*,[5] a considerable part of the macrocyclic product was present in the reduced porphyrinogen form. We therefore planned to compare the ^3H retentions when the labelled PBG was treated with the enzyme system for a short (45 min) or a longer (5 h) period, the aromatisation being completed in each case by photochemical autoxidation. The results for protoporphyrin-IX (28) showed a lower ^3H retention for the longer incubation (expts. 1 and 2, Table) indicating that more of the final porphyrin had been formed by enzymic aromatisation in the longer

Incorporation of [2,11-^{14}C,11-$^3H_1$]PBG into protoporphyrin-IX [a] (9)

Expt. no.	Enzyme source (length of incubation)	% Incorporation based on ^{14}C	% Retention of tritium
1	*E. gracilis* (45 min)	21	77
2	*E. gracilis* (5 h)	10	69
3	Chicken blood–liver mitochondria (24 h)	10	61

[a] Isolated and purified as its dimethyl ester.

* Prepared enzymically from [5-^{14}C]-5-aminolaevulinic acid by Dr. E. Hunt, whom we thank.

[19] M. Piatelli, *Tetrahedron*, 1960, **8**, 266.
[20] D. M. MacDonald and S. F. MacDonald, *Canad. J. Chem.*, 1955, **33**, 573.
[21] A. R. Battersby, E. Hunt, E. McDonald, and J. Moron, *J.C.S. Perkin I*, 1973, 2917.
[22] J. C. Collins, W. W. Hess, and F. J. Frank, *Tetrahedron Letters*, 1968, 3363.
[23] A. H. Jackson and S. F. MacDonald, *Canad. J. Chem.*, 1957, **35**, 715.
[24] T. Van Es, *J. Chem. Soc.*, 1965, 1564.

experiment. The figures correspond roughly * to 60% (5 h run) and 45% (45 min run) of the protoporphyrin-IX having been aromatised enzymically, the rest by autoxidation. Incidentally, the ^3H retention values eliminate a trivial explanation of ^3H loss by chemical exchange from the bridge methylene groups; only 31% of the tritium was lost after 5 h compared with 23% after 45 min.

A second experiment with the coupled enzyme system from chicken erythrocytes–beef liver mitochondria gave further support. The [2,11-^{14}C, 11-$^3H_1$]PBG (26) was incubated *anaerobically* with the chicken blood enzymes to form [5] mainly coproporphyrinogen-III [as (3)] and after 18 h beef liver mitochondria were added. Further incubation in the dark in air for 24 h gave protoporphyrin-IX (28), which as its ester (29) showed a ^3H retention of 61% (expt. 3, Table). This corresponds to *ca.* 75—80% of the isolated protoporphyrin-IX having been formed by enzymic aromatisation. The findings for both enzyme systems thus agree.

After completion of the foregoing work, parallel results were reported by Jackson, Belcher, and their co-workers [25] for an enzyme system from chicken erythrocytes. More recently still, an enzyme has been partially purified from yeast mitochondria [26] which specifically catalyses the aromatisation of protoporphyrinogen-IX (6). This enzyme does not act on either uroporphyrinogen-III (2) or coproporphyrinogen-III (3). Thus, the tracer and enzymic studies lead to the same conclusion, *viz.* the final aromatisation step on the natural biosynthetic pathway is enzyme-catalysed.

EXPERIMENTAL

For general directions see refs. 1 and 17.

Coproporphyrin-III Tetramethyl Ester [*Ester of* (24)] ^{14}C-*Labelled at the Propionate Groups on Rings* A *and* B.—Protoporphyrin-IX dimethyl ester (590 mg) prepared [12] from haemin chloride was dissolved in tetrahydrofuran (600 ml) and water (150 ml). Osmium tetraoxide (0.1 g) in tetrahydrofuran (10 ml) was added followed by sodium chlorate (0.6 g) in water (5 ml) and after 24 h at 20 °C the tetrahydrofuran was evaporated off and water (100 ml) was added. The precipitate was collected by centrifugation and dissolved in 0.5N-hydrochloric acid (125 ml), and the solution was filtered and diluted with water (250 ml). Extraction with 1 : 1 ether–ethyl acetate removed less polar porphyrins and the organic layer was back-extracted with 0.17N-hydrochloric acid. Neutralisation of the acidic solution with sodium acetate gave the tetraol (18), sufficiently pure (t.l.c.) for the next stage (476 mg), m.p. 248—250° (lit.,[7] 238—240°); $\lambda_{max.}$ ($\varepsilon \times 10^{-3}$) 621 (3.58), 567 (6.48), 535 (8.60), 500 (13.62), and 400 nm (179).

This product (437 mg) in stirred dioxan (250 ml) was treated with sodium periodate (1.25 g) in water (80 ml). After 24 h, water (100 ml) was added, the precipitate (largely dialdehyde with trace of monoaldehyde diol) in warm chloroform (20 ml) was treated with ether (60 ml) to yield 3,8-diformyldeuterioporphyrin-IX dimethyl ester (376 mg), m.p. 295—298° (decomp.) [lit.[7,12,27,28] in range 290—303° (decomp.)]; $\lambda_{max.}$ ($\varepsilon \times 10^{-3}$) 651 (3.57), 596 (7.55), 564 (10.0), 527 (15.0), and 437 nm (153); *m/e* 592 (M^+, 100%) and 519 ($M^+ - CH_2CO_2Me$, 20); τ ($CF_3{\cdot}CO_2H$) −1.74, −1.50 (2 H), −1.00, and −0.83 (2 H) (total 6 H, each s, bridge CH, CHO), 5.40br (4 H, t, CH_2), 5.91 and 5.94 (each 3 H, s, CO_2Me), 6.30 (12 H, s, 4 × Me), and 6.80br (4 H, t, CH_2).

Conversion of the above dialdehyde (376 mg) into the diacrylic acid (20) was performed essentially as in ref. 7; yield 430 mg; m.p. 290° (decomp.); τ ($CF_3{\cdot}CO_2H$) 0.50br (2 H, d, *J* 17 Hz, CH=C), and 2.7br (2 H, d, *J* 17 Hz, C=CH). The corresponding tetramethyl ester showed *m/e* 706 (M^+, 100%) and 633 ($M^+ - CH_2CO_2Me$, 10%).

For the ^{14}C series, sodium [2-^{14}C]malonate (6 mg; 250 μCi) in water (0.5 ml) containing unlabelled sodium malonate (24 mg) was treated with N-hydrochloric acid (0.4 ml). The residue from evaporation of this solution was dried *in vacuo* at 20 °C for 20 h, then suspended in pyridine (5 ml), and added to a solution of the above dialdehyde (60 mg) in pyridine (5 ml) and piperidine (1 drop) which had been preheated to 100 °C. After 2 h at 100 °C, unlabelled malonic acid (0.1 g) in pyridine (5 ml) was added, and after a further 2 h the solution was poured into light petroleum (100 ml; b.p. 40—60°). The precipitate was collected (centrifuge) and washed with 2 : 3 ether–chloroform to give the labelled diacrylic acid dimethyl ester [as (20)] (75 mg; 150 μCi).

A solution of the foregoing labelled product (50 mg), in methanol containing 5% concentrated sulphuric acid, was kept at 20 °C for 16 h then poured into an excess of chloroform, and the mixture was washed with aqueous sodium acetate and water. The tetraester from the chloroform in formic acid (20 ml) and 30% perchloric acid (2 drops) was hydrogenated over 10% palladised charcoal at 18 °C and 760 mmHg; uptake (1.9 mol. equiv.) was complete in 6 h. The filtered solution was added to chloroform (100 ml), then washed with aqueous sodium acetate and water. Chromatography of the residue from the chloroform on alumina (25 g) in chloroform gave labelled coproporphyrin-III tetramethyl ester (30 mg), m.p. 168—170° (from chloroform–methanol). Unlabelled material was identified by full spectroscopic and t.l.c. comparison with authentic material; *m/e* 710 (M^+, 100%) and 637 (25).

Coproporphyrin-III Tetramethyl Ester ^3H-*Labelled at the Propionate Groups on Rings* A *and* B (25).—Tritiated water (60 mg; 300 mCi) was added to pyridine (2 ml) containing malonic acid (0.1 g). After 10 min the pyridine was evaporated off on a vacuum transfer line and the residue was dried *in vacuo* (P_2O_5) for 6 h. The solid in pyridine (5 ml) was added to a solution of the dialdehyde (19), (60 mg) in pyridine (3 ml) and piperidine (1 drop) preheated to 100 °C. After 5 h the mixture was worked up as for the ^{14}C-labelled material to give the tetramethyl ester (52 mg; 245 μCi).

Enzymic Conversion of Labelled Coproporphyrinogen-III [(4) *and* (5)] *into Protoporphyrin-IX by using* Euglena gracilis.—The enzyme system from one jar of *E. gracilis* cells [5] was prepared essentially as usual.[5] Hydrolysis of the labelled coproporphyrin-III tetramethyl ester was performed with an excess of N-potassium hydroxide in 1 : 1 water–tetrahydrofuran; the tetrahydrofuran was evapor-

* For these *approximate* calculations, the ^3H retention for chemical autoxidation has been taken as 100% and for enzymic aromatisation as 50%.

[25] A. H. Jackson, D. E. Games, P. Couch, J. R. Jackson, R. B. Belcher, and S. G. Smith, *Enzyme*, 1974, **17**, 81.

[26] R. Poulson and W. J. Polglase, *J. Biol. Chem.*, 1975, **250**, 1269.

[27] H. Fischer and H. Orth, ' Die Chemie des Pyrroles,' Akademische Verlag, Leipzig, 1937.

[28] R. Lemberg and J. Parker, *Austral. J. Exp. Biol. Med. Sci.*, 1952, **30**, 163.

ated off and adjustment of the solution to pH 4 precipitated the acid, which was collected. A solution of this (1 mg ^{14}C-labelled or ^{3}H-labelled) and radioinactive dihydroxyporphyrin (8 mg) {as [24; CH(OH)·CH_2·CO_2H in place of CH_2·CH_2·CO_2H]; added for a purpose to be covered in a subsequent Paper} in aqueous 0.01N-potassium hydroxide (15 ml) was shaken in the dark with portions of freshly prepared 3% sodium amalgam (total *ca.* 10 g) until a virtually colourless solution resulted. This was filtered through a plug directly into the above enzyme solution, the mixture was gently shaken at 37 °C for 5 min (^{14}C-series) or 3 h (^{3}H-series) in air in the dark, and quinhydrone (25 mg) in ethanol (25 ml) was added. After 1 h at 20 °C, 3 : 1 ethyl acetate–acetic acid (200 ml) was added and the rest of the work-up for protoporphyrin-IX dimethyl ester was essentially as described [5] save that radioinactive carrier protoporphyrin-IX (2 mg) was added before the esterification step. After chromatography, the resultant labelled protoporphyrin-IX dimethyl ester was further diluted (20 mg inactive ester added) and purified by chromatography and crystallisation from chloroform–methanol to constant specific activity.

Degradation of Protoporphyrin-IX Dimethyl Ester.—(a) *To maleimides.* A typical carbon-14 run was as follows. A solution of labelled protoporphyrin-IX dimethyl ester (160 mg; 3.88 × 10^5 disint. per 100 s per mmol) in formic acid (40 ml) containing 30% perchloric acid (4 drops) was shaken with hydrogen and 10% palladised charcoal (40 mg) at 20 °C and 760 mmHg; uptake 2.15 mol. equiv. The filtered solution in chloroform (100 ml) was washed with aqueous sodium acetate, then evaporated, and the residue crystallised from chloroform–methanol to yield mesoporphyrin-IX dimethyl ester (3.46 × 10^5 disint. per 100 s per mmol), m.p. 208—210° (140 mg). This, in 1 : 1 concentrated sulphuric acid–water (9 ml) was stirred at 20 °C for 1.5 h, then cooled to 0 °C, and a solution of chromium trioxide (430 mg) in water (9 ml) was added dropwise with stirring. The mixture warmed to 20 °C; it was then stirred for 20 h and extracted with ethyl acetate (5 × 30 ml). A solution in water (5 ml) of the residue from evaporation of the extract was adjusted to pH 7.5 with sodium hydrogen carbonate and was then extracted with chloroform (3 × 20 ml) to yield ethylmethylmaleimide (20 mg), m.p. 65—66° [from toluene–light petroleum (b.p. 40—60°)]. Acidification of the alkaline layer and extraction with ethyl acetate gave haematinic acid (40 mg), m.p. 112—113° (from ethyl acetate–pentane). Both products were identified by comparison with authentic samples and were recrystallised to constant specific activity. The former showed 1.89 × 10^5 disint. per 100 s per mmol (*i.e.* 3.78 × 10^5 disint. per 100 s per 2 mmol) and the latter 44 disint. per 100 s per mg.

(b) *To the dinitrile* [*as* (22)]. The steps as far as the dialdehyde [as (19)], specific activity 6.88 × 10^7 disint. per 100 s per mmol derived from protoporphyrin-IX dimethyl ester showing 3.29 × 10^8 disint. per 100 s per mmol, followed the directions given above. The product (30 mg), hydroxylamine hydrochloride (15 mg), and sodium carbonate (8 ml) in pyridine (3 ml) were heated under reflux for 30 min. The mixture was dissolved in chloroform (30 ml) and was washed with 2N-hydrochloric acid, then water, and the residual dioxime, from evaporation, was heated under reflux with acetic anhydride (10 ml) for 1 h. Chloroform (50 ml) was added, the solution was washed with water, and the residue from evaporation was fractionated on alumina (25 g) in chloroform to yield the dinitrile (20 mg), specific activity 6.80 × 10^7 disint. per 100 s per mmol, m.p. 270—272° (from chloroform–methanol); $\nu_{max.}$ 2 200 cm^{-1}; *m/e* 588 (M^+, 100%); τ −0.20, 0.40, 0.98, and 1.50 (each 1 H, s, bridge CH), 5.75 (4 H, m, CH_2), 6.30 (6 H, s, 2 × OMe), and *ca.* 6.7 (16 H, m, porphyrin Me and CH_2).

Ethyl 3-(2-*Ethoxycarbonylethyl*)-4-*ethoxycarbonylmethyl*-5-*methylpyrrole*-2-*carboxylate* (30).—Acetic anhydride (reagent grade; 40 ml) was added slowly to hydriodic acid (25 ml) at 0 °C, followed by phosphinous acid (7 ml), and the solution was allowed to warm to 20 °C. The pyrrole benzyl ester [29] (32) (3.88 g) was added and the mixture was stirred at 50—60 °C for 1 h; the ester (32) had dissolved after *ca.* 35 min, and shortly afterwards a precipitate of the carboxylic acid (33) appeared. Acetic acid (10 ml) and acetic anhydride (20 ml) were then added dropwise over 5 min and the stirring was continued at 65—75 °C for 75 min while the precipitate dissolved. The solution was cooled to 40 °C and glyoxylic acid monohydrate (2.8 g) was added in 4 portions during 15 min; the mixture was then stirred at 40—45 °C for a further 20 min before evaporation. Water (10 ml) was added to the residue which at 4 °C gave the acidic product [2.02 g; acid corresponding to (30)]. After drying at 50 °C *in vacuo*, the product was esterified in ethanolic 7% hydrogen chloride (15 ml) at 20 °C for 6 h. Evaporation of the solution and crystallisation of the product from hexane gave the triethyl ester (30) (1.72 g, 51%), m.p. and mixed m.p. 62—64° (lit.,[17] 62—65°).

During trial runs, (*a*) if heating at 65—75° was insufficient for complete decarboxylation of (33) then the corresponding ethyl ester was isolated; m.p. 101—104° (lit.,[19] 94—95°); *m/e* 325 (M^+ for $C_{16}H_{23}NO_6$); $\nu_{max.}$ 3 340, 1 725, and 1 695 cm^{-1}; $\lambda_{max.}$ 222 and 274 nm; τ 8.7 (9 H, m) and 5.80 (6 H, m) (3 × OEt), 7.50 (3 H, s, pyrr-Me), 7.46 and 6.61 (each 2 H, t, pyrr-CH_2·CH_2·CO), and −0.06br (1 H, s, NH); (*b*) if the period of heating at 65—75 °C was too long (16 h), two products were isolated after chromatography of the ethyl esters on alumina (dichloromethane elution). 3-(2-*Ethoxycarbonylethyl*)-2-*ethoxycarbonylmethyl*-5-*methylpyrrole* (34) showed *m/e* 267 (M^+ for $C_{14}H_{21}NO_4$); $\nu_{max.}$ 3 340, 1 725, and 1 695 cm^{-1}; $\lambda_{max.}$ 223 nm; τ 8.8 (6 H, m) and 5.9 (4 H, m) (2 × OEt), 7.85 (3 H, s, pyrr-Me), 7.53 and 7.34 (each 2 H, t, pyrr-CH_2·CH_2·CO), 3.52 (2 H, s, CH_2·CO), and 4.34 (1 H, d, *J* 2 Hz, pyrr β-H). 3-(2-*Ethoxycarbonylethyl*)-2,4-*bis*-(*ethoxycarbonylmethyl*)-5-*methylpyrrole* (35) showed *m/e* 353 (M^+ for $C_{18}H_{27}NO_6$); $\nu_{max.}$ 3 470 and 1 730 cm^{-1}; $\lambda_{max.}$ 222 nm; τ 8.8 (9 H, m) and 5.9 (6 H, m) (3 × OEt), 7.86 (3 H, s, pyrr-Me), 7.55 and 7.34 (each 2 H, t, pyrr-CH_2·CO), and 6.67 and 6.46 (each 2 H, s, pyrr-CH_2·CO).

Ethyl 3-(2-*Ethoxycarbonylethyl*)-4-*ethoxycarbonylmethyl*-5-*formylpyrrole*-2-*carboxylate* (37).—(a) *From the* 5-*hydroxymethylpyrrole* (40). The pyrrole (40) (20 mg) in dry dichloromethane (1 ml) was added to a suspension of chromium trioxide–pyridine complex (61 mg) in dry pyridine (2 ml) and dichloromethane (4 ml). The mixture was stirred at 20 °C for 1.5 h, then filtered and evaporated. Saturated brine (10 ml) was added to the residue and extraction with ethyl acetate (5 × 10 ml) gave the aldehyde (37), m.p. 80—81° (from aqueous ethanol) (13 mg, 65%); *m/e* 353 (M^+ for $C_{17}H_{23}NO_7$); $\nu_{max.}$ 3 440, 1 733, 1 715, and 1 667 cm^{-1}; $\lambda_{max.}$ 233 and 301 nm; τ 8.73 (9 H, m) and 5.85 (4 H, q) and 5.60 (2 H, q) (3 × OEt), 7.40 and 6.92 (each 2 H, t, pyrr-CH_2·CH_2·CO), 6.16 (2 H, s, pyrr-CH_2·CO), and 0.14 (2 H, s, NH and CHO).

[29] S. F. MacDonald, *J. Chem. Soc.*, 1952, 4176, 4184.

(b) *From the pyrrole* (30). The method of ref. 20 was followed. A by-product occasionally formed was the pyrromethane (49), m.p. 144—145° (from aqueous ethanol) (lit.,[30] 145—146°); *m/e* 662 (M^+ for $C_{33}H_{46}N_2O_{12}$); $\lambda_{max.}$ 285 nm; $\nu_{max.}$ 3 340, 1 730, and 1 705 cm^{-1}; τ 8.72 (18 H, m) and *ca.* 4.8 (12 H, m) (6 × OEt), 7.47 and 6.95 (each 4 H, t, 2 × pyrr-CH_2·CH_2·CO), 6.42 (4 H, s, 2 × pyrr-CH_2CO), and 5.14 (2 H, s, pyrr$_2CH_2$).

Ethyl 3-(2-*Ethoxycarbonylethyl*)-4-*ethoxycarbonylmethyl*-5-*hydroxymethylpyrrole*-2-*carboxylate* (40).—The foregoing 5-formylpyrrole (118 mg) in stirred dry ethanol (2.5 ml) was treated with sodium borohydride (20 mg) in portions and after 30 min the solution was adjusted to pH 7 with 3N-hydrochloric acid. The residue from evaporation was partitioned between water and chloroform and the product from the chloroform crystallised from ether–hexane to give the *alcohol* (40) (113 mg, 95%), m.p. 82—83° (lit.,[31] 99°) (Found: C, 57.6; H, 7.2; N, 3.7%; M^+, 355. $C_{17}H_{25}NO_7$ requires C, 57.5; H, 7.1; N, 3.9%; M^+, 355); $\lambda_{max.}$ 276 nm; $\nu_{max.}$ 3 460 and 1 735 cm^{-1}; τ (after D_2O shake) 8.75 (9 H, m) and 5.85 (6 H, m) (3 × OEt), 7.40 and 7.05 (each 2 H, t, pyrr-CH_2·CH_2·CO), 6.50 (2 H, s, pyrr-CH_2·CO), and 5.45 (2 H, s, pyrr-CH_2·O).

When sodium [$^2H_4$]borohydride was used, the product had M^+ 356 and the ^1H n.m.r. signal at τ 5.45 integrated for 1 H.

For the preparation of [11-$^3H_1$] material (40), a solution of the 5-formylpyrrole (37) (300 mg) in propan-2-ol (10 ml) was stirred for 45 min with potassium borohydride (0.56 mg). Potassium borotritiide (10.05 mg; 54.3 mCi) was then added, the mixture was stirred in the dark at 20 °C, and after 13 h more propan-2-ol (2 ml) was added followed at 47 h by potassium borodydride (10 mg). At 51 h 2N-hydrochloric acid was added (to pH 7), the solution was evaporated, and the residue was dissolved in chloroform and water (pH 7). The aqueous layer at pH 8 was extracted with chloroform (3 × 4 ml); all chloroform extracts were combined, washed with water, and evaporated to leave the crude alcohol (320 mg). This was chromatographed on silica (p.l.c. in the dark in 1 : 1 ether–chloroform) and the product was eluted with methanol to give the tritiated alcohol (40) (172 mg; 22 mCi; radiochemical yield 40%).

[11-$^3H_1$]*Porphobilinogen Lactam* (45) *and* [11-$^2H_1$]*Porphobilinogen Lactam* (46).—The foregoing 5-hydroxymethylpyrrole (100 mg) was dissolved in stirred thionyl chloride (1 ml; freshly distilled from iron filings) and after 1 h at 20 °C the mixture was evaporated to leave the chloromethylpyrrole [as (41)], which was sufficiently pure to be used in the next stage. The reactions from this point through to the PBG lactams were as in ref. 21, so only the quantities and certain key spectroscopic results are given below.

The 5-hydroxy[11-$^3H_1$]pyrrole (40) (172 mg; 22 mCi) yielded the azide (42) (160 mg; 16.8 mCi) and from it, the salt (43) (135 mg; 13.5 mCi), m.p. 191—193°. This was diluted with unlabelled material (to give 246 mg; 13.5 mCi) before conversion into 5-carboxy[11-$^3H_1$]porphobilinogen (163 mg; 8.9 mCi) and, after dilution to 200 mg, on to the corresponding lactam (76 mg; 4.4 mCi; diluted with unlabelled material to 159 mg). This afforded [11-$^3H_1$]porphobilinogen lactam (93 mg; 3.21 mCi).

For the $^2H_1$-series, the azide (95 mg) was prepared, *m/e* 381 (M^+), τ 5.59 (1 H), and this yielded 5-carboxy[11-$^2H_1$]porphobilinogen (78 mg), τ 5.50 (1 H, s). The derived lactam showed, τ 5.74br (1 H, s), and finally [11-$^2H_1$]porphobilinogen (18 mg) was obtained, *m/e* for methyl ester 223 (M^+), τ [$(CD_3)_2SO$] 5.76 (1 H, s).

Ethyl 3-(2-*Ethoxycarbonylethyl*)-4-*ethoxycarbonylmethyl*-5-*hydroxyiminomethylpyrrole*-2-*carboxylate* (syn- *and* anti-*Forms*) [*as* (38)].—Hydroxylamine hydrochloride (278 mg) in water (2 ml) was adjusted to pH 5 with anhydrous sodium acetate and added to a solution of the aldehyde (37) (706 mg) in ethanol (8 ml). The mixture was heated under reflux for 45 min, poured into ice–water (10 ml) and kept at 4 °C. The oximes separated as crystals and an oil which later solidified (563 mg, 77%); *m/e* 368 (M^+ for $C_{17}H_{24}N_2O_7$); $\lambda_{max.}$ 235 and 298 nm; $\nu_{max.}$ 3 450, 3 300, and 1 730 cm^{-1}; τ 8.72 (9 H, m), 5.88 (4 H, q), and 5.65 (2 H, q) (3 × OEt), 7.50 and 6.99 (each 2 H, t, pyrr-CH_2·CH_2·CO), 6.46 and 6.39 (together 2 H, pyrr-CH_2CO), and 2.1 and 1.92 (together 1 H, pyrr-CH=N).

Ethyl 3-(2-*Ethoxycarbonylethyl*)-4-*ethoxycarbonylmethyl*-5-*cyanopyrrole*-2-*carboxylate* (30).—A solution of the aldehyde (37) (3.18 g) in formic acid (13.5 ml) was warmed with hydroxylamine hydrochloride (720 mg) and sodium formate (1.13 g) until all had dissolved. After the mixture had been heated under reflux for 1 h, it was cooled and diluted with water (27 ml), and the nitrile (2.38 g) was collected and recrystallised from ether–hexane (yield 2.03 g); m.p. 101—103° (lit.,[23] 96—98°); *m/e* 350 (M^+ for $C_{17}H_{22}N_2O_6$); $\lambda_{max.}$ 271 and 280 nm; $\nu_{max.}$ 3 340, 3 260, 2 230, and 1 735 cm^{-1}; τ 8.8 (9 H, m), 5.93 (4 H, q), and 5.64 (2 H, q) (3 × OEt), 7.50 and 7.07 (each 2 H, t, pyrr-CH_2·CH_2·CO), and 6.41 (2 H, s, pyrr-CH_2·CO).

5-*Carboxy*[11-$^2H_2$]*porphobilinogen Triethyl Ester Hydrochloride*.—A solution of the 5-cyanopyrrole (39) (140 mg) in CH_3·CO_2D (15 ml), D_2O (9.4 ml), and 20% DCl in D_2O (0.13 ml) was shaken with deuterium and platinum oxide (48 mg) for 5 h at 19 °C and 765 mmHg (uptake 1.12 mol. equiv.); the CH_3·CO_2D was made by heating acetic anhydride (42.6 ml) and D_2O (9.4 ml) for 1.5 h under reflux at 110 °C. The catalyst was filtered off, the filtrate was evaporated, and the residue was crystallised from ethanol–ether to give the ester hydrochloride (106 mg), m.p. 192—196°; the spectra for this product were as published [21] save *m/e* 356 (M^+ for [$^2H_2$] material).

[2,11-^{14}C, 11-$^3H_1$]*Porphobilinogen*.—[11-$^3H_1$]Porphobilinogen lactam (15.15 mg; 3.25×10^6 disint. mm^{-1} mg^{-1}) was suspended in 2N-sodium hydroxide (0.4 ml) under nitrogen and kept at 20° in the dark for 3 days. [2,11-^{14}C]-Porphobilinogen (5.46 mg; 7.38×10^5 disint. mm^{-1} mg^{-1}) was then added, glacial acetic acid was added dropwise at 0 °C, and at about pH 5 porphobilinogen crystallised. The solid was collected and the supernatant was adjusted to pH 4 to give a second crop. The total product was washed with cold water, acetone, and ether successively, and dried; yield 20 mg (91%), ^3H 2.25×10^6 disint. min^{-1} mg^{-1}; ^{14}C 1.84×10^5 disint. min^{-1} mg^{-1}; $^3H/^{14}C$ = *ca.* 12.2; m.p. 170—173° (decomp.) [lit.,[23] 170—174 and 172—175° (decomp.)]

For accurate determination of the ^3H : ^{14}C ratio in this product, a sample (0.4 mg) suspended in methanol (0.2 ml) was treated with an excess of diazomethane. After 2 h, radioinactive PBG lactam methyl ester (5 mg) was added and the solution was evaporated. Recrystallisation of the diluted sample from methanol gave a (constant) ^3H : ^{14}C ratio of 11.9 : 1; m.p. 249—251° (lit.,[17] 248—251°).

[30] S. F. MacDonald and K. H. Michl, *Canad. J. Chem.*, 1956, **34**, 1768.

[31] K. S. N. Prasad and R. Raper, *Nature*, 1955, **175**, 629.

Incubation of Doubly Labelled PBG with the Enzyme System from Euglena gracilis.—The foregoing porphobilinogen (3.9 mg; $^{3}H : ^{14}C$ 11.9 : 1; ^{14}C activity 1.8×10^{5} disint. min^{-1} mg^{-1}) was incubated aerobically in the dark with a cell free extract of *E. gracilis* in the usual way.[5]

Half the reaction mixture was quenched after 45 min with 3 : 1 ethyl acetate–acetic acid and subjected to photochemical autoxidation before isolating protoporphyrin-IX. A spectroscopic assay of the Soret band at 408 nm gave at the time of quenching an optical density (O.D.) of 1.07, and this rose to 2.4 after autoxidation was complete. The other half of the reaction mixture was incubated in the dark for a total of 5 h, and protoporphyrin-IX was then isolated in the same way. This sample at the time of quenching gave O.D. 2.34, unchanged after autoxidation.

Each sample of protoporphyrin-IX was converted into the dimethyl ester, which was diluted with radioinactive ester before chromatography on alumina and then recrystallisation from chloroform–methanol to constant activity.

Incubation of Doubly Labelled PBG with the Coupled Enzyme System from Chicken Blood and Beef Mitochondria.—Radioactive PBG (3.1 mg; $^{3}H : ^{14}C$ 11.9 : 1; ^{14}C activity 1.8×10^{5} disint. min^{-1} mg^{-1}) was incubated with the enzyme system from chicken blood [5] under nitrogen for 18 h in the usual way [5] and then for a further 24 h after adding beef liver mitochondria.[5] Protoporphyrin-IX was isolated as normally [5] and was converted into the dimethyl ester. At constant specific acitivity, this showed $^{3}H : ^{14}C$ 7.3 : 1.

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