Biosynthesis of Porphyrins and Related Macrocycles. Part VI.[1,2] Nature of the Rearrangement Process Leading to the Natural Type III Porphyrins

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Logical analysis of the problem posed by the constant formation in nature of type III porphyrins, *e.g.* protoporphyrin-IX (6), focuses attention on C–C bond making and bond breaking around the carbon atoms from which the interpyrrolic bridges are built (at C-5, C-10, C-15, and C-20). An approach to the study of such processes is outlined, based on double [^{13}C] labelling combined with n.m.r. spectroscopy.

[2,11-$^{13}C_2$]Porphobilinogen (17) has been prepared and applied (*a*) to determine the size of ^{13}C–^{13}C coupling for directly bonded carbon atoms in the porphyrin macrocycle and (*b*) to indicate that a biosynthetically significant ^{13}C–^{13}C coupling occurs between carbon atoms separated by three bonds. A coupled enzyme system has been developed from chicken blood cells and beef liver mitochondria which produces sufficient protoporphyrin-IX for spectroscopic and chemical work (20—30 mg); a similar system from *Euglena gracilis* is also described.

[2,11-$^{13}C_2$]Porphobilinogen diluted with unlabelled porphobilinogen has been converted enzymically into protoporphyrin-IX; the ^{13}C spectra of the dimethyl ester (i) as such, (ii) with a praseodymium shift reagent, and (iii) after chemical modification establish that the formation of type III porphyrins by both enzyme systems (avian and algal) is characterised by the same three precise features which are described.

HAEM (3), chlorophyll a (7), and cytochrome c (9) are all derived in nature from protoporphyrin-IX (6), which in turn is formed biosynthetically from uroporphyrinogen-III[3] (4). Also, uroporphyrinogen-III (4) has recently been rigorously proved [4,5] to be a precursor of the corrin macrocycle of vitamin B_{12} (8). These natural porphyrins (3) and (9), chlorins [*e.g.* (7)], and corrins [*e.g.* (8)] play a central role in living systems and knowledge of their biosynthesis, and hence that of uroporphyrinogen-III (4), is of vital importance.

Earlier work, especially by Shemin, Granick, Neuberger, Bogorad, and Rimington with others too,[3] established that uroporphyrinogen-III (4) is built from four units of porphobilinogen (1) (PBG) and that the co-operative action of two proteins (called deaminase and cosynthetase) is necessary for the natural type III isomer (4) to be formed. If PBG (1) is treated with deaminase alone, then uroporphyrinogen-I (2) is produced.† Interestingly, uroporphyrinogen-I (2) is the product one would *expect* to be formed from straightforward chemical considerations; Scheme 2 shows a plausible sequence for the interaction of PBG with deaminase. So living systems have developed over evolutionary time a highly specific way to catalyse a

† Two other isomers could in principle be produced from four PBG units; they are uroporphyrinogen-II (10) and uroporphyrinogen-IV (11). No examples of these type II and type IV isomers have been found in living systems.

[1] Part V, A. R. Battersby, E. McDonald, J. R. Redfern, J. Staunton, and R. H. Wightman, preceding paper.

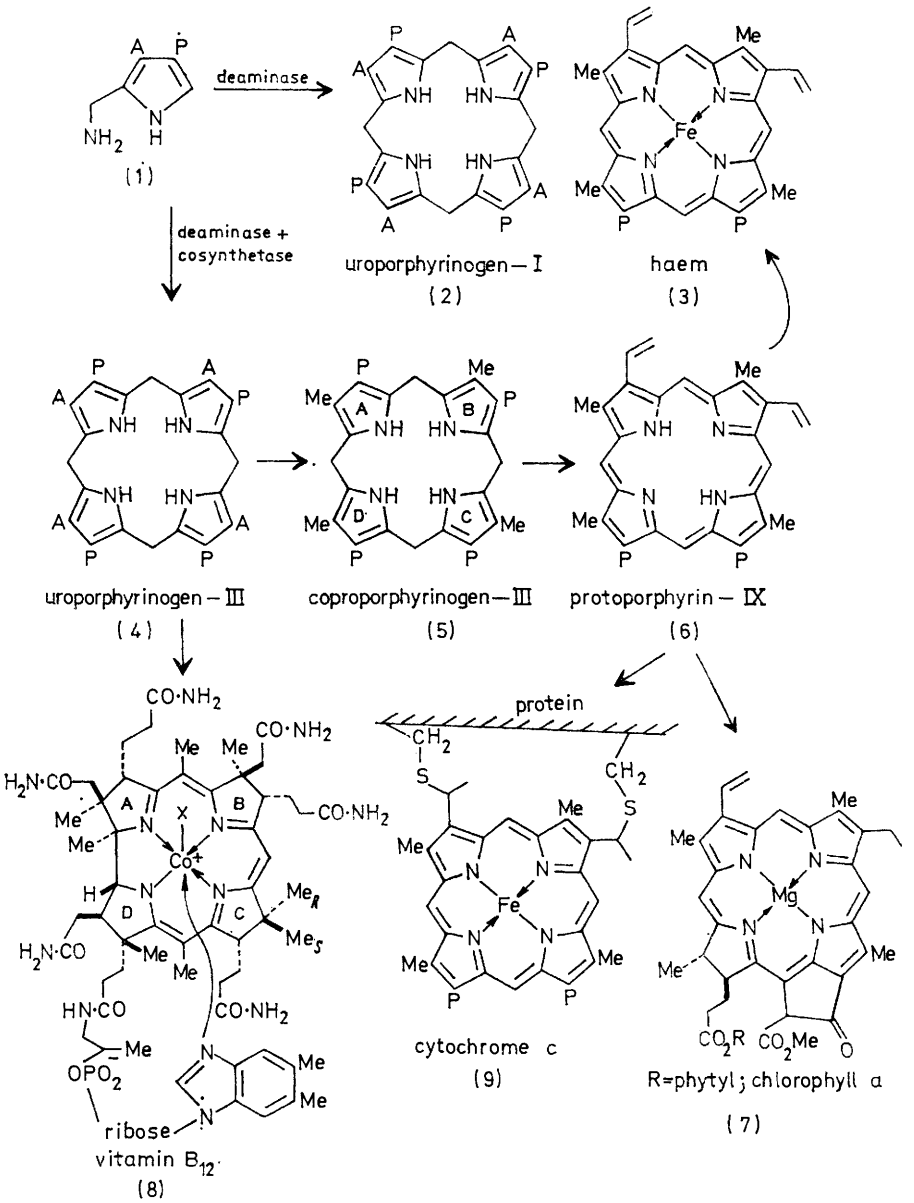
[2] Preliminary account, A. R. Battersby, E. Hunt, and E. McDonald, *J.C.S. Chem. Comm.*, 1973, 442.

[3] Reviewed to September, 1974, by A. R. Battersby and E. McDonald in 'Falk's Porphyrins and Metalloporphyrins,' 2nd edn., ed. K. M. Smith, Elsevier, Amsterdam, 1975.

[4] A. R. Battersby, M. Ihara, E. McDonald, F. Satoh, and D. C. Williams, *J.C.S. Chem. Comm.*, 1975, 436.

[5] A. I. Scott, N. Georgopapadakou, K. S. Ho, S. Klioze, E. Lee, S. L. Lee, G. H. Temme, tert, C. A. Townsend, and I. M. Armitage, *J. Amer. Chem. Soc.*, 1975, **97**, 2548.

rearrangement process which produces the *unexpected* type III system (4) from four PBG molecules. The nature and chemical mechanism of the rearrangement process poses one of the most fascinating and important problems in the porphyrin field and it is not surprising that at least twenty-five hypothetical schemes [6] have hypothetical schemes showed that it was necessary to discover (*a*) *how many* of the four PBG units required for macrocycle formation undergo rearrangement during the biosynthesis of uroporphyrinogen-III (4); (*b*) very importantly, whether each rearrangement is *intramolecular* with respect to the PBG unit involved or



Here and elsewhere: A = $CH_2{\cdot}CO_2H$; P = $CH_2{\cdot}CH_2{\cdot}CO_2H$; A^{Me} = $CH_2{\cdot}CO_2Me$; P^{Me} = $CH_2{\cdot}CH_2{\cdot}CO_2Me$

SCHEME 1

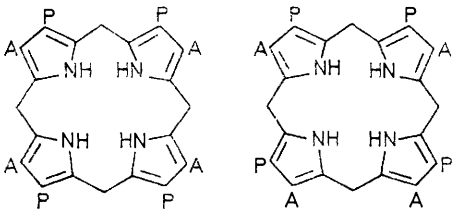
been proposed to account for the change. Some of these had been eliminated by experiments carried out before 1970 which are summarised in ref. 3 but the majority remained. This paper reports experiments which show the nature of the biosynthetic process leading to the type III porphyrins.

Analysis of the Problem.—Consideration of the various whether *intermolecular* processes are operating; (*c*) *the site(s)* at which the migrating carbon atom(s) appear(s) in the final type-III macrocycle. Our plan involved examination of all these features by appropriate applica-

[6] The majority are collected by (*a*) P. Maitland, *Quart. Rev.*, 1950, **4**, 45; (*b*) E. Margoliash, *Ann. Rev. Biochem.*, 1961, **30**, 551; (*c*) E. Bullock, *Nature*, 1965, **205**, 70.

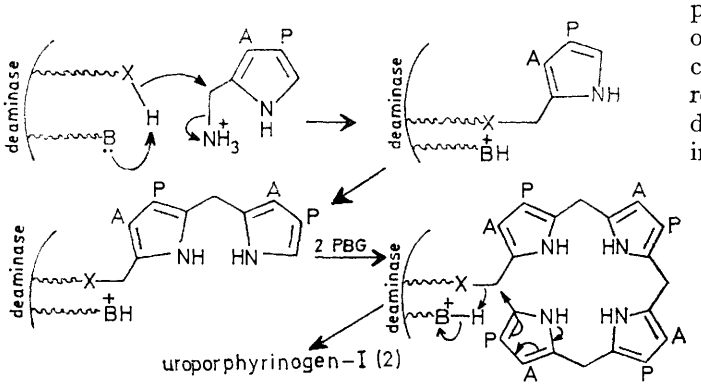
tion of carbon-13 labelling followed by a n.m.r. spectroscopic study of the biosynthetic product.

Analysis of two mechanistic proposals will amplify these points. The proposal in Scheme 3 is in a form



type II (10) type IV (11)

slightly modified from the original one;[7] the group X is either the original amino-group of one PBG unit or is a nucleophilic group on the enzyme which has displaced the amino-group of the first PBG unit to have been

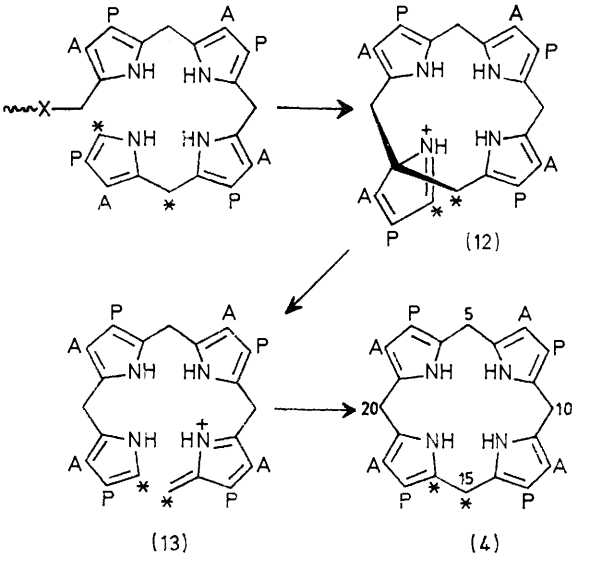


SCHEME 2

bound. Fragmentation of the spiro-intermediate (12) and then ring closure of the methylenepyrrole (13) reverses ring D. For this proposal, three PBG units are incorporated into uroporphyrinogen-III without rearrangement and the fourth undergoes intramolecular rearrangement with the migrating carbon atom being delivered to the bridge at position 15 (see asterisks on Scheme 3).

In contrast, there are three intramolecular rearrangements in Scheme 4, again drawn with minor modifications of the original form.[8] The migrating carbon atoms appear in this case at the bridges numbered 5, 10, and 15. Equally clear-cut differences distinguished most of the other hypothetical schemes and so the bridge carbon atoms (C-5, C-10, C-15, and C-20) were recognised as being of key importance for work on the nature of the rearrangement process. Accordingly, a porphyrin had to be chosen for study in which carbon-13 atoms at the four bridges gave four separate ^{13}C n.m.r. signals. Earlier work[9] had shown that the dimethyl ester of protoporphyrin-IX (14) matched this requirement and the four signals were assigned to the individual bridges by unambiguous syntheses[10] of ^{13}C-labelled forms of protoporphyrin-IX. The order of these signals, reading from low to high field, was C-5, C-10, C-20, and C-15. This same order was found to hold good for the ^{13}C signals from the bridge carbon atoms of the diacetylporphyrin[10] (15), but with the important advantage that whereas for protoporphyrin-IX ester (14) the separation between the signals from C-5 and C-15 was 1.9 p.p.m., that for the diacetylporphyrin (15) was 6.9 p.p.m. The latter porphyrin was thus chosen for the subsequent n.m.r. studies; conditions had been defined[10] for its preparation on a small scale from protoporphyrin-IX dimethyl ester (14) which was to be available from the biosynthetic experiments.

In concentrating attention on protoporphyrin-IX (6), it must not be overlooked that the rearrangement process which generates the type III macrocycle occurs somewhere on the pathway after PBG (1) but before uroporphyrinogen-III (4). Biosynthetic work on protoporphyrin-IX (6) can only give information about the nature of the biochemical rearrangement if the type III macrocycle, once formed, does not undergo further skeletal rearrangement in the biological system. The independent studies by the Münster group[11] and those described in Part V[1] established the integrity of the type III



SCHEME 3

porphyrinogen skeleton along the pathway from (4) *via* (5) to (6). Accordingly, what is discovered about the labelling patterns and ^{13}C–^{13}C couplings for protoporphyrin-IX (6) produced enzymically from suitably ^{13}C-labelled PBG also holds true for uroporphyrinogen-III

[7] J. H. Mathewson and A. H. Corwin, *J. Amer. Chem. Soc.*, 1961, **83**, 135.

[8] R. Robinson, 'The Structural Relations of Natural Products,' Clarendon Press, Oxford, 1955, p. 22.

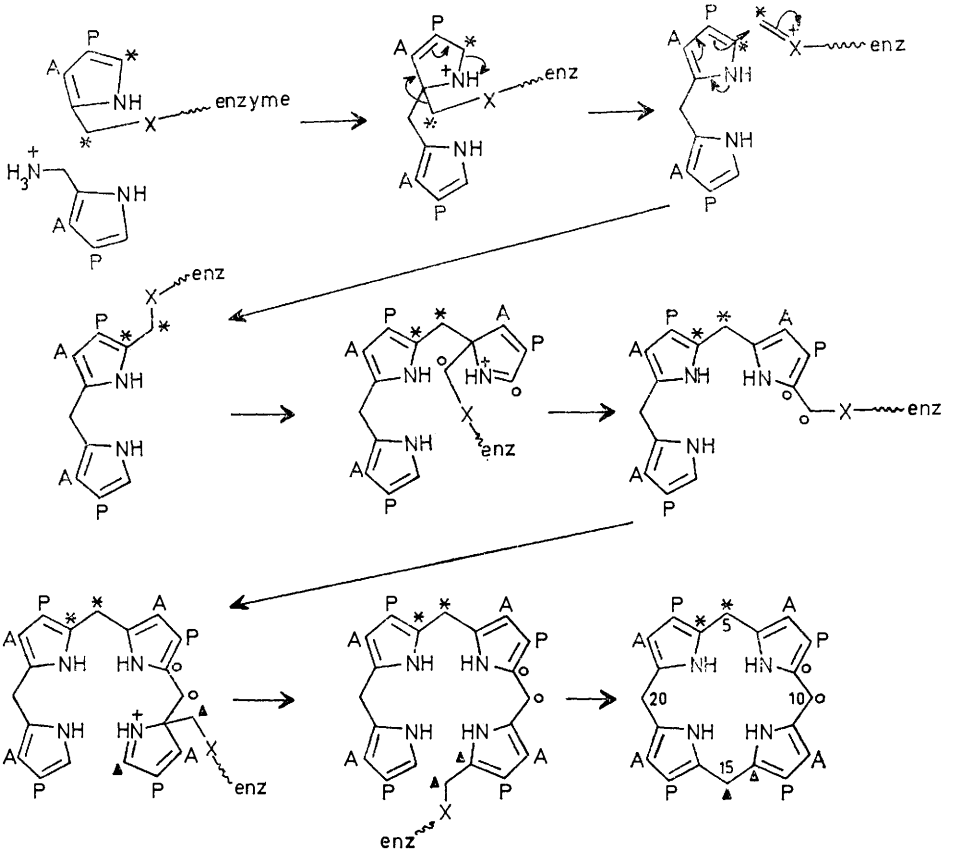
[9] A. R. Battersby, E. Hunt, E. McDonald, and J. Moron, *J.C.S. Perkin I*, 1973, 2917.

[10] A. R. Battersby, G. L. Hodgson, M. Ihara, E. McDonald, and J. Saunders, *J.C.S. Perkin I*, 1973, 2923.

[11] B. Franck, D. Gantz, F.-P. Montforts, and F. Schmidtchen, *Angew. Chem. Internat. Edn.*, 1972, **11**, 421.

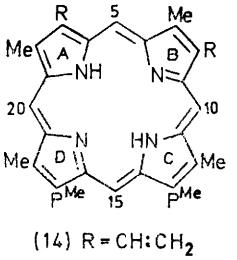
(4) from which (6) is formed. Hence the findings are directly and reliably informative about the type III rearrangement process.

The Approach by Use of ^{13}C *Labelling.*—For clarity, we will outline the full strategy for the work with carbon-13 the total chemical yield being 60—70%. It follows from arithmetic that 81% of the PBG molecules carry two ^{13}C atoms and this was confirmed by mass and n.m.r. spectrometry on the lactam ester (20) formed by treatment of the labelled PBG with diazomethane. The



SCHEME 4

later; in essence, it required the preparation of PBG such that the majority of its molecules were doubly labelled with carbon-13 at C-2 and C-11. [5-^{13}C]Aminolaevulinic acid (ALA) (16) had been synthesised [9] with 90 atom % of carbon-13; a by-product from this synthesis has recently been shown to be [6-^{13}C]piperidin-2-one (see Experimental section). The [5-^{13}C]ALA (16) was converted by partially purified aminolaevulinic acid dehydratase from ox liver [12] into [2,11-$^{13}C_2$]PBG (17), singly labelled species (18) and (19) were, of course, also present to the extent of *ca.* 9% each and yielded [$^{13}C_1$] lactams corresponding to (20).



(14) R = CH:CH_2
(15) R = COMe

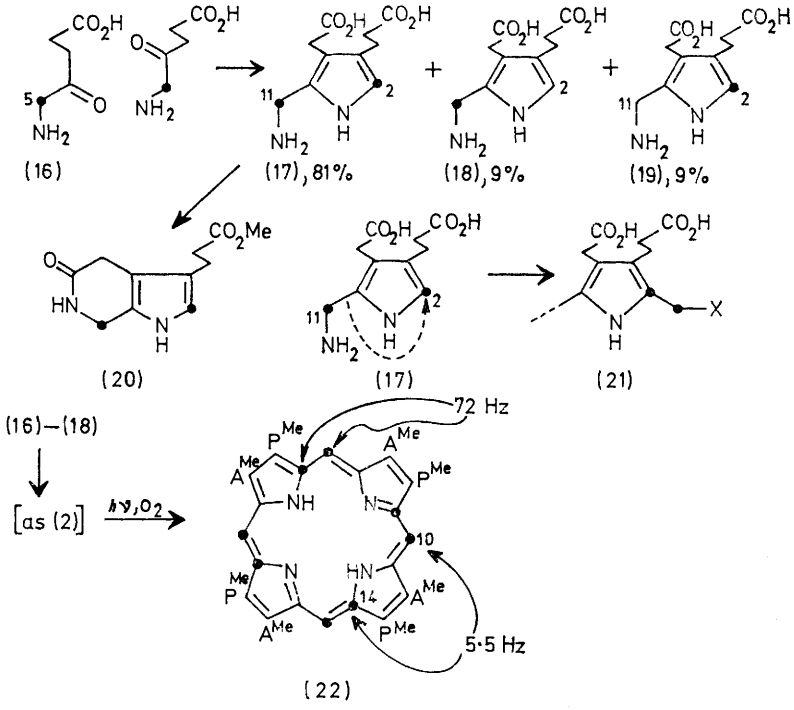
We can now consider the use of [2,11-$^{13}C_2$]PBG (17) for biosynthetic studies of the type III rearrangement. If, during the formation of uroporphyrinogen-III (4), an intramolecular change occurs of the type (17)⟶(21), this would be revealed by the signal from the affected bridge carbon atom of the isolated protoporphyrin-IX dimethyl ester (14) being split by ^{13}C–^{13}C coupling. However, no measurements had been made of the size of coupling from directly bonded ^{13}C atoms in a porphyrin macrocycle, so this basic information was gained as follows.

The mixture of labelled PBG molecules (17)—(19) containing 81% of (17) was converted into labelled uroporphyrinogen-I [as (2)] by purified deaminase isolated from spinach.[13] After aromatisation of the product (by

[12] H. A. Sanocovich, A. M. Ferramola, A. M. del C. Batlle, and M. Grinstein, *Methods Enzym.*, 1970, **17**, 216, 220.
[13] L. Bogorad, *Methods Enzym.*, 1962, **5**, 885.

light and air) the centrosymmetric uroporphyrin-I octamethyl ester (22) was isolated crystalline in 73% overall yield from PBG.

The content of carbon-13 at C-2 and C-11 of the PBG (17)—(19) used in the foregoing enzymic synthesis was 90 atom %, and therefore as a ^{13}C atom at C-11 of PBG is



built biochemically into a bridge carbon atom of uroporphyrinogen-I [as (2)], there is a 90% chance of its being joined *in the same molecule* to a second ^{13}C atom. Thus, in the ^{13}C n.m.r. spectrum of the isolated ester (22), *ca.* 90% of the signal from the bridge carbon atoms will be split owing to this ^{13}C–^{13}C coupling. In practice, this signal appeared as a double doublet (Figure 1) (J 72 and 5.5 Hz) centred on a broad singlet (*ca.* 10% intensity); the 72 Hz coupling was clearly due to the directly bonded ^{13}C atoms and is similar in size to that found for directly bonded sp^2-hydridised ^{13}C atoms in other systems.[14] The 5.5 Hz splitting was assigned to long-range coupling through three bonds as indicated on structure (22). It was recognised that this assignment was of crucial importance because (see later) it allows experiments to be designed to test for incorporation of PBG units intact into the type-III porphyrin macrocycle. Accordingly, the assignment was given solid experimental backing by the synthesis of two porphyrins carrying two ^{13}C atoms at positions 10 and 14;[15] the n.m.r. spectra of these products showed the 5.5 Hz coupling.

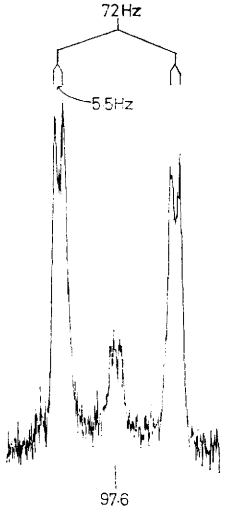


FIGURE 1 ^{13}C N.m.r. signals from the bridge carbon atoms of uroporphyrin-I octamethyl ester (22) enzymically derived from *undiluted* [2,11-$^{13}C_2$]PBG; spectral width (SW) 500 Hz, acquisition time (AT) 4 s, number of transients (NT) 10 000, run in $CDCl_3$

Enzymic Conversion of [2,11-$^{13}C_2$]*PBG into Protoporphyrin-IX* (6).—The foregoing studies set up the bridge-head from which the attack on the type III problem was possible. Enzyme systems were then required capable of producing in a practicable number of runs at least 20—30 mg in total of protoporphyrin-IX (6) from PBG to allow the necessary spectroscopic and chemical work. A cell-free extract from *Euglena gracilis* was known to yield protoporphyrin-IX from PBG on a micro-scale;[16] conditions were therefore developed for large-scale growth of this organism and for the isolation and handling

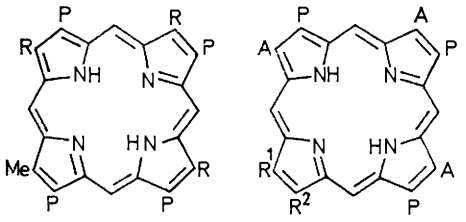
[14] J. B. Stothers, 'Carbon-13 NMR Spectroscopy,' Academic Press, New York, 1972, p. 370.

[15] A. R. Battersby, M. Ihara, E. McDonald, J. Saunders, and R. J. Wells, following paper.

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

of the soluble enzymes. Studies were also made of the effects of pH, incubation time, and various additives (see Experimental section). This work provided an enzyme system and incubation conditions which reproducibly afforded protoporphyrin-IX (6) in 35—40% isolated yield [as the dimethyl ester (14)] from 6—9 mg of PBG in a single run.

Proteins from chicken blood and beef liver were used to prepare a second enzyme system. When the total



(23) R = Me
(24) R = A

(25) R^1 = A, R^2 = P
(26) R^1 = P, R^2 = A

haemolysate from chicken blood cells [17] was centrifuged at 25 000 *g*, it gave a supernatant free from ferrochelatase [3] which is particle bound; this enzyme catalyses the insertion of iron into protoporphyrin-IX (6) to give haem (3), and its presence would have caused partial or complete loss of the desired porphyrin (6). The assembly of enzymes in the supernatant converted PBG, under oxygen-free nitrogen, into a mixture of porphyrinogens (75—80% total yield) which were aromatised, and the resultant porphyrins were separated to give (typical yields) coproporphyrin-III (23) (56%), uroporphyrin-III (25) (9%), and the heptacarboxylic acid, phyriaporphyrin-III [18] * (24) (10%). Thus, by treating PBG with supernatant for 20 h *with exclusion of oxygen* to generate largely coproporphyrinogen-III (5), followed by addition of beef liver mitochondria and incubation in air for a further 24 h, protoporphyrin-IX (6) was formed in up to 60% of the total porphyrin yield. Beef liver mitochondria contain coproporphyrinogenase,[3] which brings about oxidative decarboxylation of the propionate side chains on rings A and B of coproporphyrinogen-III (5). Typically, a single run with this coupled enzyme system yielded 12—14 mg of protoporphyrin-IX dimethyl ester (14).

A key step for the enzymic incorporation experiments was dilution of the foregoing [2,11-$^{13}C_2$]PBG (17)—(19) with four parts of unlabelled PBG; the proportion of labelled molecules in the diluted product is thereby much reduced but, importantly, the presence of 81% of *doubly labelled* molecules in the ^{13}C-labelled PBG present is left unchanged. This has the result that the majority of single molecules in the protoporphyrin-IX enzymically produced contain only one doubly labelled PBG unit and so carry only two ^{13}C atoms. If we neglect any small secondary isotope effects, then one quarter of the molecules of protoporphyrin-IX will have ring A derived from a labelled PBG unit, another quarter will have ring B so labelled, and similarly for rings C and D. Thus dilution allows each bridge and each pyrrole ring of the protoporphyrin-IX to be examined separately.

The studies previously outlined now allow the following analysis. (*a*) If ring A and the C-20 bridge of protoporphyrin-IX [see (14)] are derived *intact* from one PBG unit, by far the major signal from C-20 will appear as a 5.5 Hz doublet. (*b*) In contrast, if the PBG unit forming ring A has undergone *intramolecular rearrangement* so that its original aminomethyl residue provides C-5 [see (14)] as (17) ⟶ (21), then the major signal from C-5 will be a 72 Hz doublet centred on a small singlet. (*c*) Finally, if the C-11 methylene group of the PBG unit which forms ring A takes part in an *intermolecular process* (*i.e.* it becomes separated and attached to a different PBG unit), then the major signal from that carbon, at whichever bridge it appears, will be a singlet.

This analysis has been carried out for ring A and its attached methylene carbon atom but the same holds true for the other three rings. Thus the enzymic experiment was expected to afford precise information concerning

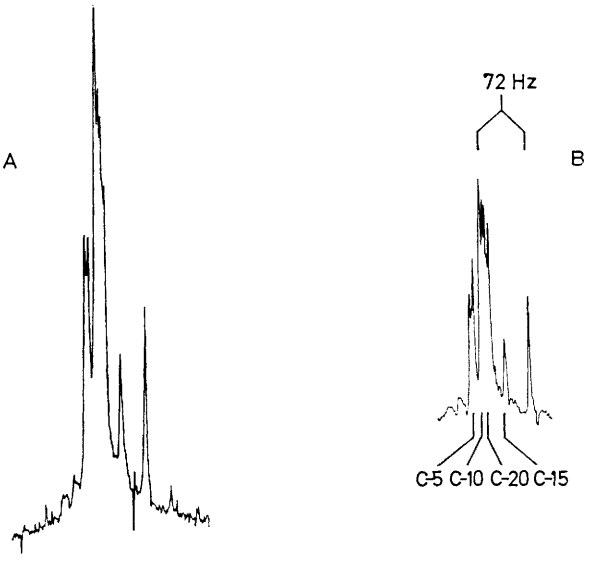


FIGURE 2 ^{13}C N.m.r. signals from the bridge carbon atoms of protoporphyrin-IX dimethyl ester (27)—(30) enzymically derived from *diluted* [2,11-$^{13}C_2$]PBG; (A) by using the chicken blood–beef mitochondria system, SW 2 000 Hz, AT 1.0 s, NT 159 000; (B) by using the cell-free extract from *Euglena gracilis*, SW 1 000 Hz, AT 4.0 s, NT 15 645; both run in $CDCl_3$

the biosynthetic process as it affects all four units which constitute the macrocycle.

The foregoing diluted [2,11-$^{13}C_2$]PBG was converted by the coupled chicken blood–beef mitochondria system

* The synthesis of phyriaporphyrin-III has recently been completed by Professor P. S. Clezy and his co-workers (University of New South Wales) and (shortly thereafter) by Professor A. H. Jackson and his co-workers (Cardiff), and in this laboratory.[19]

[17] *Cf.* D. Shemin, J. Abramsky, and C. S. Russell, *J. Amer. Chem. Soc.*, 1954, **76**, 1204.

[18] A. R. Battersby, E. Hunt, M. Ihara, E. McDonald, J. B. Paine, tert. F. Satoh, and J. Saunders, *J.C.S. Chem. Comm.*, 1974, 994.

[19] A. R. Battersby, E. Hunt, M. Ihara, E. McDonald, J. B. Paine, tert., and J. Saunders, in preparation.

into protoporphyrin-IX, isolated as its dimethyl ester, which gave the ^{13}C n.m.r. spectrum (from the bridge carbon atoms) shown in Figure 2A. Though the signals

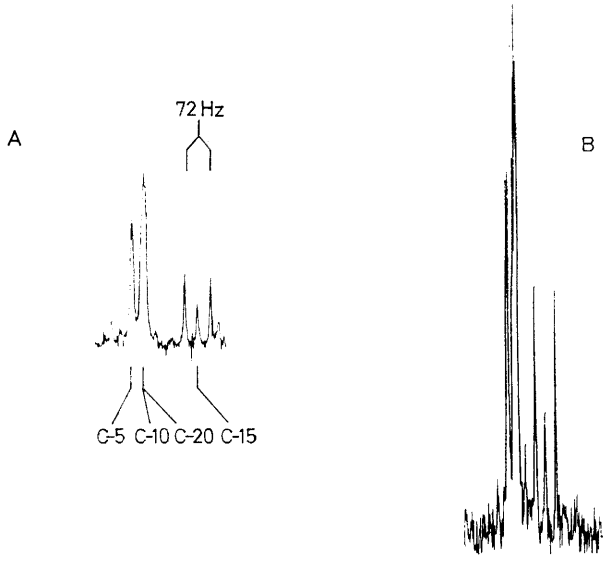


FIGURE 3 ^{13}C-labelled protoporphyrin-IX dimethyl ester as for Figure 2 run at *ca.* 15 mM in $CDCl_3$ with *ca.* 14 mM-Pr($[^2H_9]$fod)$_3$; ^{13}C signals from the bridge carbons with (A) (blood) SW 2 000 Hz, AT 1.0 s, NT 75 000; (B) (*Euglena*) SW 5 000 Hz, AT 0.8 s, NT 5 652

overlap to some extent, the observed pattern was best fitted by the signals from C-5, C-10, and C-20 being 5.5 Hz doublets and that from C-15 being a 72 Hz doublet. The signal pattern was simplified by determining the spectrum

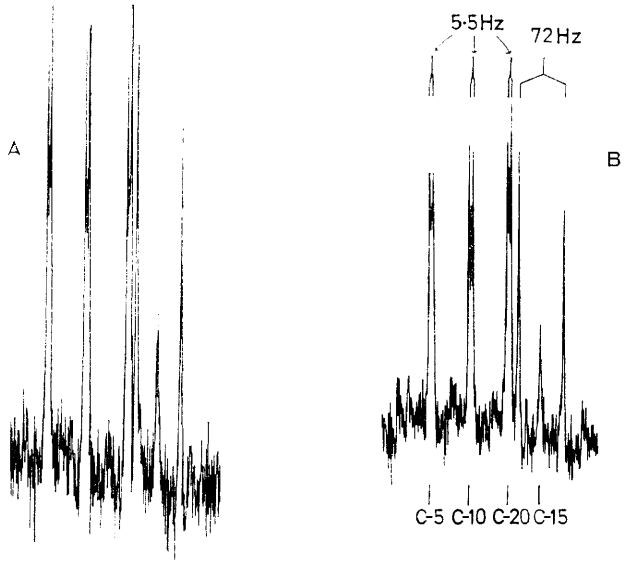
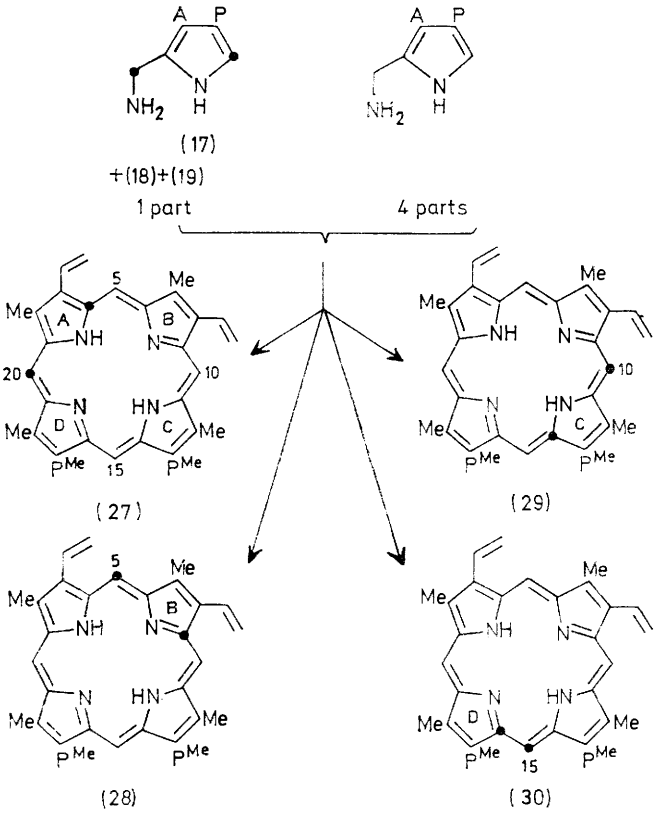


FIGURE 4 ^{13}C N.m.r. signals from the bridge carbon atoms of the diacetylporphyrin [as (15)] chemically derived from the ^{13}C-labelled protoporphyrin-IX dimethyl ester used for Figure 2; (A) (blood) SW 2 000 Hz, AT 2.0 s, NT 32 642; (B) (*Euglena*) SW 2 000 Hz, AT 2.0 s, NT 25 769

in the presence of a praseodymium shift reagent Pr($[^2H_9]$fod)$_3$, which was shown, as outlined in the Experimental section, to affect most strongly the signal from C-15 and to shift it upfield. In the resulting spectrum (Figure 3A) the signal from C-15 is clearly visible as a separate 72 Hz doublet centred on a small singlet. Finally, the biosynthetic protoporphyrin-IX was converted chemically into the diacetylporphyrin [as (15)]; the value of this derivative is clear from Figure 4A. The signals from the four bridge carbon atoms are now completely separated, with those from C-5, C-10, and C-20 as 5.5 Hz doublets and that from C-15 as a 72 Hz



doublet. When these results are combined with the rigorous assignments of signals and couplings already outlined, it follows that the labelled protoporphyrin-IX dimethyl ester is mainly composed of the doubly-labelled [$^{13}C_2$] species (27), (28), (29), and (30). The fact that the two signals of the 72 Hz doublet appear as sharp lines is further evidence in support of these deductions since species (30) is the only [$^{13}C_2$] molecule present in the mixture which lacks a ^{13}C atom three bonds removed from the ^{13}C atom at the bridge.

Accordingly, the nature of the rearrangement process by which type III porphyrins are biosynthesised is defined, and is characterised by the following features: (*a*) the three PBG units which form ring A and its attached C-20 bridge, ring B and the C-5 bridge, and ring C with its C-10 bridge are all incorporated intact without rearrangement; (*b*) the PBG unit which forms ring D is built in with rearrangement which is intramolecular with respect to that PBG unit; and (*c*) the rearranged carbon atom forms the bridge at C-15.

It is of considerable interest with regard to the evolutionary process that exactly the same features characterise the biosynthetic process which generates type III porphyrins in an alga (*Euglena gracilis*), despite its different stage of development in comparison with a chicken. This will be clear from Figures 2B, 3B, and 4B. Current work is extending the study to bacteria.

The foregoing findings on the nature of the rearrangement process leading to the type III porphyrins eliminate most of the speculative mechanistic schemes mentioned at the outset. Studies by incorporation and trapping of intermediates [20,21] between PBG and uroporphyrinogen-III (4) and by complementary methods will tighten the net still further.

EXPERIMENTAL

Most general directions are given in ref. 22. ^{13}C N.m.r. spectra were determined for solutions in $CDCl_3$ which had been stored in brown bottles over alumina; the n.m.r. tubes were flushed with nitrogen before sealing with the usual plastic cap. These precautions were necessary to prevent decomposition of sensitive porphyrins, especially protoporphyrin-IX. Spectra were recorded using a pulse width of 75 μs and with a sensitivity enhancement equal to the acquisition time. Spectra were run initially with a spectral width of 5 000 Hz, and for some samples subsequent spectra were run with a spectral width of 2 000 Hz, having an improved resolution. The latter spectra required a correspondingly longer acquisition time, and therefore more accumulation time; with small samples of labelled compounds, periods up to 30 h were needed. The labelled porphyrins produced biosynthetically exhibit signals of significance only in the region for sp^2 carbon atoms and so ^1H noise decoupling (1 000 Hz bandwidth) was centred 10 p.p.m. downfield from tetramethylsilane.

[5-^{13}C]*Aminolaevulinic Acid* (16) *and* [6-^{13}C]*Piperidin-2-one*.—The synthesis was carried out as previously [9] on the same scale to the point where 5-hydroxy[6-^{13}C]piperidone (*ca.* 210 mg) was isolated by chromatography on alumina. A less polar product was further purified by chromatography on silica in benzene, then with chloroform to elute an oil which was distilled at 140 °C and 13 mmHg. It crystallised from ether-pentane at −20° to give [6-^{13}C]piperidin-2-one as hygroscopic needles (45 mg), τ (CCl_4) 1.76br (1 H, NH), 6.72 (2 H, 90% d of br signals, *J* 140 Hz and 10% br, CH_2N), 7.79 (2 H, m, CH_2·CO), and 8.23 (4 H, m, CH_2·CH_2); *m/e* 101 ($M^+ + 1$, 6%), 100 (M^+ for ^{13}C, 100%), 99 (70), 98 (11), 72 (10), 71 (12), and 70 (34). Unlabelled material showed $\nu_{max.}$ 1 650 cm^{-1}; τ 1.68br (1 H, NH), 6.68br (2 H, CH_2N), 7.72 (2 H, m, CH_2·CO), and 8.16 (4 H, m, CH_2·CH_2); *m/e* 100 ($M^+ + 1$, 6%), 99 (M^+, 100%), 98 (18), 97 (5), 71 (11), and 70 (25), as reported.[23]

Preparation of [2,11-$^{13}C_2$]*Porphobilinogen* (17).—Aminolaevulinic acid dehydratase was purified to stage 4 of the published method [12] and the protein (*ca.* 25 g) in 0.067M-phosphate buffer at pH 6.8 (25 ml) was dialysed against the same phosphate buffer (3 × 2 l, each for 2 h) at 4 °C. Assay of the resultant enzymic solution (74 ml) for dehydratase activity was performed as follows. The enzyme solution (1 ml), pH 6.8 phosphate buffer (1.3 ml) and 25mM-L-cysteine in pH 6.8 phosphate buffer (0.1 ml) were incubated in oxygen-free nitrogen at 37 °C for 1 h. Aqueous 5-aminolaevulinic acid hydrochloride (17 mM; 0.3 ml in pH 6.8 phosphate buffer) was then added and after 10 min at 37 °C saturated aqueous copper sulphate was added (0.1 ml). After centrifugation, the supernatant was assayed for PBG by Ehrlich's reagent.[24] This enzyme preparation (1 ml) typically formed 0.08 mg of PBG in 10 min and was suitable for preparative runs. Occasionally, enzyme of 2—3 times this activity was used.

For the labelled preparation, aminolaevulinic acid dehydratase (*ca.* 1.5 g; total activity 22.5 μmol PBG h^{-1}) in pH 6.8 0.067M-phosphate buffer (50 ml) containing L-cysteine (6 mg) was degassed, flushed with oxygen-free nitrogen, and incubated for 1 h at 37 °C with gentle agitation (oxygen excluded). [5-^{13}C]Aminolaevulinic acid hydrochloride [9] (90 atom % ^{13}C; 50 mg, 0.296 mmol) in water (2 ml) was adjusted to pH 6.8 with 1M-Tris buffer and was added to the foregoing enzyme solution again with exclusion of air. The mixture was shaken gently at 37 °C in the dark for 12 h.

The mixture at 0 °C was then treated with saturated aqueous copper sulphate (2 ml), and the precipitated protein was collected by centrifugation and washed with 0.5M-ammonium hydroxide (2 × 5 ml). The main solution and washings together contained 91 μmol PBG (62%). For preparation of the *diluted* [2,11-$^{13}C_2$]PBG, a solution of unlabelled PBG (370 μmol) in 0.5M-ammonium hydroxide was added at this stage; when undiluted [2,11-$^{13}C_2$]PBG was required, this step was omitted. The solution of labelled PBG was adjusted to pH 4 with acetic acid and aqueous 15% mercury(II) acetate solution (6 ml) was added. A suspension of the precipitated solid in water (30 ml) was treated with hydrogen sulphide for 15—30 min, and the sulphides were collected and treated again in water with hydrogen sulphide; this retreatment was carried out four times. The combined aqueous solutions contained 431 μmol of PBG, isolated by freeze drying as a faintly pink solid which was stored in the dark at 4 °C over potassium hydroxide.

A sample of undiluted [2,11-$^{13}C_2$]PBG was treated with an excess of ethereal diazomethane to yield the corresponding lactam ester, identified with authentic unlabelled material.[25] Its mass spectrum showed M^+ 224 and its ^{13}C n.m.r. spectrum strong peaks at δ_C 36.4 (C-11) and 115.0 (C-2).

Enzymic Preparation of Uroporphyrin-I Octamethyl Ester [*Ester of* (26)] *from* [2,11-$^{13}C_2$]*PBG*.—The enzyme deaminase was isolated from frozen spinach leaves and was purified 32-fold by Bogorad's method; [13] the resultant solution catalysed the consumption of 8.3 × 10^{-3} μmol of PBG per ml per min and it was stored at −20 °C.

[2,11-$^{13}C_2$]PBG (90 atom % at each labelled centre; 26.3 μmol) was added to a mixture of deaminase solution (15 ml), 0.1M-Tris buffer at pH 8.2 (20 ml), and aqueous 0.1M-ethylenediaminetetra-acetic acid (0.8 ml at pH 8). After the solution had been degassed and flushed with oxygen-free nitrogen, it was shaken gently at 37 °C in the dark for 16 h with strict exclusion of air. Air was then passed through the solution in sunlight for 1 h, the pH was adjusted to 4.0 with acetic acid, and the precipitated

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uroporphyrin-I was collected by centrifugation and washed with water (2 × 1 ml). The dried porphyrin was treated with 5% sulphuric acid in anhydrous methanol (20 ml) at 20 °C for 17 h and the resultant ester was isolated by dilution with water (adjust pH to 4.0 with sodium carbonate) and extraction with chloroform. Purification by chromatography on alumina in chloroform gave the octamethyl ester (4.5 mg, 73%). The ^{13}C n.m.r. spectrum is shown in Figure 1.

Coupled Enzyme System from Chicken Blood Cells and Beef Liver Mitochondria (with Dr. B. MIDDLETON).—Chicken blood (2 l) was freshly collected over 30 min in a flask containing heparin (2 ml; 1 000 units ml^{-1}). It was cooled to 5 °C, centrifuged at 0—5° for 30 min at 2 000 *g*, and after removal of the supernatant the compacted cells were gently resuspended in aqueous 0.9% sodium chloride (2 l) at 5 °C. The cells (*ca.* 500 ml) were again centrifuged down and made up with water to 1.5 l; after 1 h, heparin (2 ml) was added and the mixture was centrifuged at 25 000 *g* for 1.5 h at 0—5 °C. The supernatant enzyme solution (630 ml) was used immediately or stored at −20 °C (solution A).

Freshly collected beef liver (500 g) was homogenised (at 0—4 °C here and subsequently) in a Waring blender with aqueous 0.3M-sucrose and the product was centrifuged at 1 000 *g* for 10 min. The supernatant was then centrifuged at 10 000 *g* for 10 min and the pellet of mitochondria was resuspended in aqueous 0.3M-sucrose (300 ml) and if necessary, stored at −20 °C (solution B).

These two solutions were used co-operatively as in the following experiment.

Enzymic Formation of Protoporphyrin-IX from Diluted [2,11-$^{13}C_2$]*PBG by Coupled Enzyme System.*—[2,11-$^{13}C_2$]PBG which had been diluted with four parts of unlabelled PBG (total 80 mg) dissolved in 1M-Tris–hydrochloric acid buffer (40 ml; pH 7.8) was added to the chicken blood enzyme (solution A) (400 ml; 242 mg protein per ml converts 0.12 μmol PBG per h per ml). After degassing and flushing with oxygen-free nitrogen, the solution was gently shaken at 37 °C in the dark under nitrogen for 20 h. The beef liver preparation (solution B) (90 ml) was then added and gentle shaking, in air, was continued in the dark for a further 24 h.

The total solution was added to 3 : 1 ethyl acetate–acetic acid (2 l), the solution was kept in sunlight for 30 min and centrifugation removed the precipitated protein which was washed with 3 : 1 ethyl acetate–acetic acid (3 × 300 ml). The combined organic solutions were washed with saturated aqueous sodium acetate (1.5 l) and the aqueous layer was extracted with ethyl acetate (2 × 200 ml). After the combined organic solutions had been washed with aqueous 3% sodium acetate (200 ml), they were extracted with aqueous 0.5% hydrochloric acid (100 ml portions) until no further coproporphyrin was extracted (u.v. spectroscopic analysis) (organic layer called solution C). The combined acidic extracts were overlayered with an equal volume of ether and solid sodium carbonate was added in portions with shaking until the porphyrin passed into the ether. Two further extractions with ether were carried out and the three ether layers were dried over sodium chloride and evaporated to give coproporphyrin-III, which was treated further as below.

Solution C was extracted to completion with aqueous 15% hydrochloric acid (100 ml portions) and the extracted porphyrin was recovered with ether as for coproporphyrin-III. The product was dissolved in a few drops of glacial acetic acid; this solution was diluted with ether and treated with an excess of ethereal diazomethane. After 1 min, the remaining diazomethane was destroyed with acetic acid and the residue from evaporation was chromatographed on alumina (20 g) in methylene chloride. Crystallisation of the main fraction from chloroform–methanol gave ^{13}C-labelled protoporphyrin-IX dimethyl ester (12 mg), identified by full comparison with authentic material.

A solution of the coproporphyrin-III fraction above in 0.067M-phosphate buffer (100 ml; pH 7.2) was flushed with nitrogen, and freshly prepared 3% sodium amalgam was added in small portions as the mixture was shaken in the dark under nitrogen until it was colourless. The solution was decanted, adjusted to pH 7.2 with acetic acid, and then shaken gently at 37 °C in the dark in air for 20 h with beef liver mitochondria (45 ml of solution B). Protoporphyrin-IX was isolated from the final solution, esterified, and purified as above.

The above sequence was repeated on more *diluted* [2,11-$^{13}C_2$]PBG (same batch as above; 60 mg); the total yield of pure protoporphyrin-IX dimethyl ester from both runs was 26 mg. This material and the diacetylporphyrin ester (15) derived from it yielded the spectra in Figures 2A, 3A, and 4A.

Addition of dithiothreitol (1 mg for each 20 ml of enzyme solution A) was found not to affect the yield of protoporphyrin-IX in trial experiments with unlabelled PBG.

Studies on the Enzyme Preparation from Chicken Blood Cells.—(*a*) The enzyme solution A (10 ml) was mixed with pH 7.8 1.0M-Tris–hydrochloric acid buffer (1 ml) containing PBG (10 μmol). After the mixture had been degassed and flushed with oxygen-free nitrogen, it was gently shaken at 37 °C for 18 h in the dark with exclusion of air. (*b*) The same mixture was made as in (*a*) but sodium chloride (97 mg) was added to make the solution 150mM in NaCl. (*c*) As (*b*) but with sodium chloride (194 mg) equivalent to 300 mM in NaCl. Mixtures (*b*) and (*c*) were then treated as for (*a*). Each solution was worked up for porphyrins as in the foregoing experiment with the following results: (*a*) 0.455 μmol 'uroporphyrin fraction' (24%) and 1.45 μmol of coproporphyrin-III (76%) in a total porphyrin yield of 77%; (*b*) 0.73 μmol 'uroporphyrin fraction' (40%) and 1.1 μmol coproporphyrin-III (60%) in a total porphyrin yield of 74%; (*c*) 0.96 μmol 'uroporphyrin fraction' (58%) and 0.71 μmol coproporphyrin-III (42%) in a total porphyrin yield of 67%.

The 'uroporphyrin fraction' was fractionated by p.l.c. on silica with 7 : 10 aqueous 1.5M-ammonia–lutidine as solvent and was found to contain coproporphyrin-III (20%; R_F 0.7), uroporphyrin-III (38%; R_F 0.11), and phyriaporphyrin-III (42%; R_F 0.13).

All porphyrin samples were shown to be type III rather than type I isomers by decarboxylation to coproporphyrin-III followed by chromatography.[24]

The foregoing results show that decarboxylation of uroporphyrinogen-III is inhibited by high concentrations of sodium chloride.

Effect of PBG Concentration on the Enzymic Decarboxylation of Uroporphyrinogen-III.—Three identical enzyme solutions (11 ml each) were prepared from solution A as for the foregoing experiments. To one (*d*) was added 13.5 μmol PBG (*i.e.* 1.23mM), to another (*e*) was added 6.75 μmol PBG and to the last (*f*) 3.37 μmol PBG. They were incubated and worked up as above. The yields of 'uroporphyrin fraction' were (*d*) 0.37 μmol (25%); (*e*) 0.11 μmol (11.5%); (*f*) 0.034 μmol (6.5%), and those of coproporphyrin-III were (*d*) 1.45

μmol (75%); (*e*) 0.85 μmol (88.5%); (*f*) 0.47 μmol (93.5%) in total porphyrin yields of (*d*) 57%, (*e*) 57%, (*f*) 60%.

A plot of the ratio of uroporphyrin fraction to coproporphyrin-III against PBG concentration yields a straight line passing through the origin.

Large-scale Growth of Euglena gracilis.—The growth medium contained (per litre): KH_2PO_4 0.4 g; $MgSO_4,7H_2O$ 0.5 g; L-glutamic acid 5 g; DL-malic acid 2.0 g; $CaCO_3$ 0.1 g; $(NH_4)_2HPO_4$ 0.2 g; $FeSO_4,(NH_4)_2SO_4,6H_2O$ 14 mg; $ZnSO_4,7H_2O$ 4.4 mg; $MnSO_4,4H_2O$ 2.04 mg; $CuSO_4,5H_2O$ 3.1 mg; $CoSO_4,7H_2O$ 4.8 mg; H_3BO_3 0.57 mg; $(NH_4)_6Mo_7O_{24},4H_2O$ 0.25 mg; vitamin B_1 1 mg; vitamin B_{12} 5μg. *Euglena gracilis* (strain number 1224/5Z) from the Culture Centre of Algae and Protozoa, Cambridge, was grown in the above medium at 30 ± 2° illuminated by two parallel banks of 3 × 5 ft fluorescent light tubes (65/80 W, Cryselco ' warm white '), with 13 in between the banks. An alternating cycle of light to dark (16 h to 8 h) was maintained. Normal techniques were used for sterilisation, for protection of growth vessels, and for sterile transfer of solutions. Cells plus medium (3.4 ml) from the 500 ml conical flasks (see later) were added to medium (23 ml) in a protected sterile boiling tube, and after illumination for 3 days the contents of the tube were added to medium (230 ml) in a 500 ml conical flask. This was illuminated for 4 days and the contents (less 3.4 ml) were then transferred to gently stirred medium in a 5 l conical flask. After illumination for 3 days the contents of 14 such flasks were centrifuged at 6 000 rev. min^{-1} at 4 °C on an M.S.E. ' High Speed 18 ' centrifuge fitted with a continuous action rotor. The cells were removed from the head with deionised water, and centrifuged at 1 000 *g* for 5 min, and the solid was resuspended in 0.05M-sodium phosphate buffer at pH 7.6 and centrifuged at 1 000 *g* for 5 min. Finally, the solid was resuspended in the same buffer (200 ml) and dithiothreitol (10 mg) was added; the suspension was stored as *ca.* 2 × 250 ml portions in closed polyethylene jars at −20 °C. This material gave enzyme preparations of undiminished activity after several months of storage.

Enzymic Formation of Protoporphyrin-IX from Diluted [2,11-$^{13}C_2$]*PBG by Enzymes from* Euglena gracilis.—The following description relates to one of the above frozen jars of *E. gracilis* cells and buffer. The jar was transferred to a refrigerator at 5 °C for 15—20 h before use and the resultant broken cell mass was centrifuged at 0 °C and 17 000 *g* for 30 min. After the supernatant had been filtered through a cotton plug, it was made 0.02M with respect to ethylenediaminetetra-acetic acid (EDTA) with a solution of EDTA (10 g) dissolved in 1M-Tris buffer (50 ml) at pH 7—8. The final enzyme solution was adjusted to pH 7.6 with 1M-Tris buffer or 1N-hydrochloric acid as necessary; the pH of the enzyme solution should be kept within 6.5—8.0 at all times.

PBG (6—9 mg) was then added in a small volume of pH 7.6 Tris buffer and the total solution was divided (50—75 ml per flask) among 250 ml conical flasks completely covered by aluminium foil. The flasks were then shaken at 30 °C in air in a shaker–water bath (120 rev. min^{-1} on the motor) for 30—40 min. An equal volume of 3 : 1 ethyl acetate–acetic acid was added to the combined solutions and, after thorough swirling, the mixture was exposed to sunlight and air for 15 min. Centrifugation at 1 500 rev. min^{-1} gave a clear organic layer which was collected by suction. The extraction of the aqueous sludge was repeated thrice with an equal volume of 3 : 1 ethyl acetate–acetic acid. Extraction of the combined organic layers with one-fifth of its volume of saturated aqueous sodium acetate removed small quantities of uroporphyrin-III and similar highly polar porphyrins. The coproporphyrin-III and protoporphyrin-IX were then extracted together into aqueous 15% hydrochloric acid (extraction with *ca.* 5% of the total organic volume until porphyrin removal was complete). Further work-up, esterification, and chromatographic isolation of the protoporphyrin-IX dimethyl ester were performed as for the blood–liver coupled enzyme system described earlier.

In the ^{13}C-labelled series, [2,11-$^{13}C_2$]PBG diluted as before with four parts of unlabelled PBG (total 140 mg) was divided among the enzyme preparations from 16 jars of *E. gracilis*. The incubations *etc.*, were run as above to give ^{13}C-labelled protoporphyrin-IX dimethyl ester (30 mg, 35%) which yielded the spectra in Figures 2B, 3B, and 4B; the diacetylporphyrin ester [as (15)] derived from it gave the spectrum in Figure 4B.

The conditions used above for the incubation of PBG with the enzyme system were developed from an extensive study of the various factors. Incubation time and weight of PBG used were studied by 50 experiments in which the time was varied from 1 min to 17 h and the weight of PBG used was 1.5—12 mg. Other experiments demonstrated the beneficial effect of EDTA [26] and, by determining PBG removal and porphyrin formation, allowed selection of the optimum pH for the overall multiple enzymic steps.

Proof that Pr([$^2H_9$]fod)$_3$ *most affects the* C-15 *Bridge of Protoporphyrin-IX Dimethyl Ester.*—The following experiment was carried out before the foregoing optimisation of conditions; the contrast in yield is striking. To the enzyme from one jar of frozen *E. gracilis* in pH 7.0 0.05M-phosphate buffer was added a solution of [5-^{13}C]aminolaevulinic acid hydrochlorine (90 atom % ^{13}C; 40 mg) in water adjusted to pH 6.5. The mixture was incubated as above for 18 h and then worked up as previously to give protoporphyrin-IX dimethyl ester (0.97 mg). This was diluted with unlabelled ester (5 mg) and recrystallised to give the sample for ^{13}C n.m.r. spectroscopy. A 3mM-solution of this product in $CDCl_3$ which was 1.7mM with respect to Pr([$^2H_9$]fod)$_3$ showed the triplet signal (J 72 Hz) which necessarily arises from C-15, at δ_C 93.4 p.p.m., the doublet (J 5.5 Hz) from the C-20 bridge at 96.2 p.p.m., and the double doublets (J 72 Hz and 5.5 Hz) from the C-5 and C-10 bridges at 97.6 and 96.8 p.p.m. (not necessarily respectively). These observations prove that the shift reagent causes the largest shift of the signal from the C-15 bridge.

Conversion of ^{13}C-*Labelled Protoporphyrin-IX Dimethyl Ester into the Corresponding Diacetylporphyrin* [*as* (15)].—The protoporphyrin-IX ester (26.6 mg) from the avian blood–liver enzyme system was converted as earlier [10] into the diacetylporphyrin ester (13.2 mg, 47%). Similarly the product from the algal enzyme system (30 mg) yielded the diacetylporphyrin ester (13.4 mg, 42%).

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[26] See also S. Sano and S. Granick, *J. Biol. Chem.*, 1961, **236**, 1173.