Biosynthesis of Porphyrins and Related Macrocycles. Part 15.[1] Chemical and Enzymic Formation of Uroporphyrinogen Isomers from Unrearranged Aminomethylpyrromethane: Separation of Isomeric Coproporphyrin Esters

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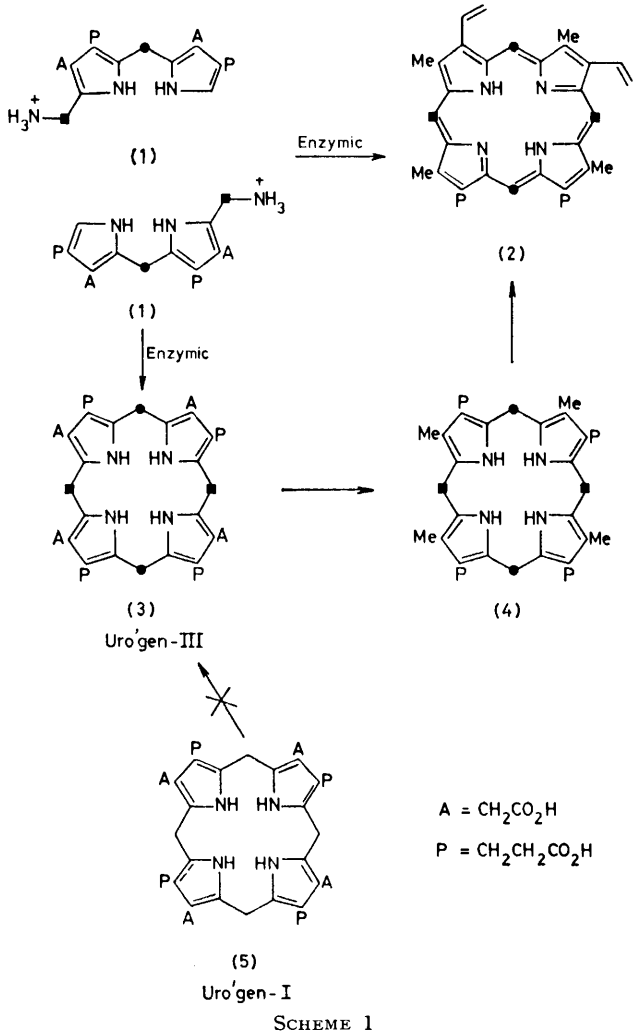
The unrearranged pyrromethane (1) is transformed chemically mainly into uro'gen-I with a smaller amount of uro'gen-IV but only traces of uro'gen-III are formed. Uro'gen-I is produced *via* a tetrapyrrolic (bilane) intermediate and when the deaminase–cosynthetase enzyme system from *Euglena gracilis* is present, this intermediate is converted into uro'gen-III. The rearrangement step for this conversion has the same characteristics found earlier for the natural biosynthetic process from porphobilinogen. Pyrromethane (1) is not a direct biosynthetic precursor of uro'gen-III and reasons are advanced why this is understandable.

Methods are developed based on high pressure liquid chromatography for the separation of all four isomeric coproporphyrin esters.

In the preceding paper,[1] it was shown that when the unrearranged pyrromethane (1) was incubated with enzymes from *Euglena gracilis*, or from duck's blood, it was transformed into protoporphyrin-IX (2) in the manner indicated by the labelling patterns illustrated in Scheme 1. In each experiment, specifically labelled uroporphyrinogen-III (uro'gen-III) (3) and copro'gen-III (4) must have acted as intermediates.[2,3] So it was essential to assess the importance of any processes by which uro'gen-III (3) might be produced *chemically* from pyrromethane (1).

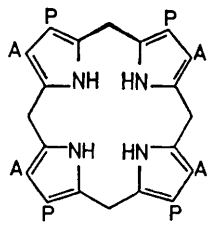


Scheme 1

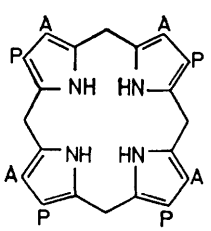
Preliminary experiments involved incubation of [^{14}C]pyrromethane[4] [(1), ^{14}C at ●] with inactivated (boiled) duck's blood, and buffer, and before work-up, a mixture[5] of uro'gens I—IV, (3) and (5)—(7), was added as carrier. The products and carrier were aromatised by iodine oxidation and the resulting mixture of uroporphyrins was converted into the corresponding octamethyl esters for purification; this mixture was found to be radioactive (*ca.* 12—13% of initial radioactivity). Clearly one or more of the isomeric uroporphyrinogens (3) and (5)—(7) had been formed chemically from pyrromethane (1) but these early experiments did not distinguish between the incubation itself and the isolation procedure.

The stability of pyrromethane (1) under various defined conditions was therefore studied (see Experimental section). These experiments served to highlight what was required before a quantitative assessment could be made of the amounts of uro'gen isomers formed chemically from pyrromethane (1). These requirements were (*a*) a technique which not only stabilised the labile uro'gens but which also destroyed any remaining pyrromethane (1) to avoid this being converted into uroporphyrins during work-up; (*b*) a means of separating quantitatively the four possible isomeric products for analysis by assay of radioactivity or by spectroscopy; and (*c*) an enzyme preparation capable of synthesising uro'gen-III (3) without transforming it further; this would allow direct comparison of the chemical and enzymic products

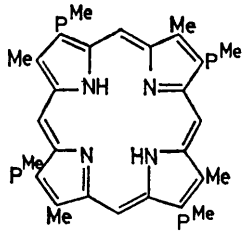
These requirements were met as follows. (*a*) It was found that 1.3% aqueous iodine solution, commonly used for this purpose, smoothly converted uro'gens and intermediate hydroporphyrins into uroporphyrins. In addition, only an insignificant amount of porphyrin was



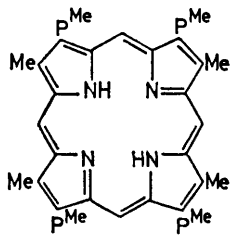
(6)
Uro'gen-II



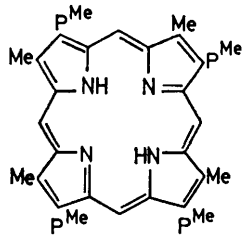
(7)
Uro'gen-IV



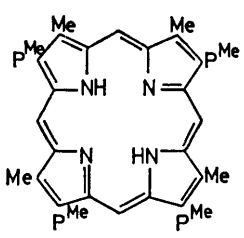
(8)
Coproporphyrin-I ester



(9)
Coproporphyrin-II ester



(10)
Coproporphyrin-III ester



(11)
Coproporphyrin-IV ester

$P^{Me} = (CH_2)_2CO_2Me$

$A^{Me} = CH_2CO_2Me$

produced under these conditions from a solution of pyrromethane (1) (freshly-prepared by neutralisation of the tetra-sodium salt [4]). The excess of iodine was reduced by sodium hydrogen sulphite and the reaction mixtures were assayed at the Soret band for total porphyrin content. (*b*) No attempt was made to analyse the isomer content of the mixture of uroporphyrins directly because it seemed likely that differences in chromatographic mobility would be greater for the corresponding coproporphyrins. The uroporphyrins were therefore converted into their octamethyl esters which were purified chromatographically before being dissolved in hydrochloric acid and heated at 180 °C for 3 h to effect clean decarboxylation of the acetate side-chains. The resultant coproporphyrins, as their tetramethyl esters (8)—(11), were analysed by high pressure liquid chromatography (h.p.l.c.). After much experimentation, two solvent systems were developed,[6] the one separating isomer I (8) from III and IV [(10) and (11)] and from II (9), the other resolving III (10) from IV (11) after several recycles (see Figure 1). (*c*) The enzyme system for producing uro'gen-III (3) was obtained by fractionation [7] of the soluble proteins released on breaking the cells of *Euglena gracilis*. Ammonium sulphate precipitation (45—70% fraction) followed by dialysis gave an effective preparation of

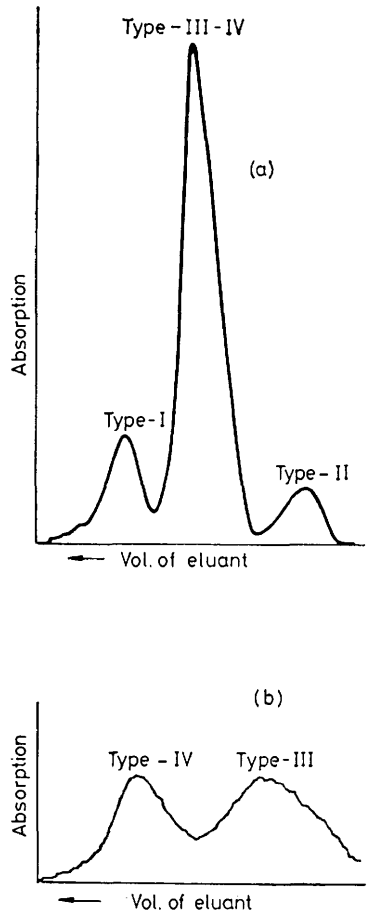


FIGURE 1 H.p.l.c. traces for analyses of coproporphyrin esters run (a) on 10μ CN normal phase column and (b) on 10μ Porasil column; u.v. analyser set at *ca.* 390 nm. See Experimental section for solvents used

deaminase–cosynthetase which converted porphobilinogen into pure uro'gen-III (3).

Using these techniques it was found [8] that in a 16 h incubation at 37 °C and pH 7.2, a 1.7mM solution of [^{14}C]pyrromethane (1a) (Scheme 2) was converted *chemically* in 32% yield into a mixture of uro'gens, the composition of which is shown as the ' Chemical run ' in the Table. The major product, not unexpectedly, was uro'gen-I (5a) presumably formed by head-to-tail condensation to give the aminomethylbilane (12a) which

Proportions of uro'gen isomers formed from pyrromethane (1a)

	Uro'gens formed (% of total)			
	Type-I	Type-II	Type-III	Type-IV
Chemical run	68 ± 2	1 ± 0.5	3 ± 2	28 ± 2
Enzymic run	15 ± 1	2 ± 1	54 ± 2	29 ± 2

cyclises to uro'gen-I (5a); related head-to-tail condensations were known.[9] The smaller, but significant, yield of uro'gen-IV (7a) is probably produced by head-to-head reaction of two pyrromethane molecules (1a) to form the pyrrolenine (13a) which can lose $CH_2{=}\dot{N}H_2$ in a retro-Mannich reaction. The resultant symmetrical bilane (14a) can recombine with the eliminated C_1-unit at C-1 or C-19 to form bilane (15a), or its equivalent, ready for ring-closure to uro'gen-IV (7a). Importantly, only a trace of uro'gen-III (3a) was formed chemically [*ca.* 3% of the mixture ≡ 0.05% overall yield from (1a)]. Independent work [10] has also demonstrated the chemical formation of uro'gen-I (5a) and uro'gen-IV (7a) from pyrromethane (1a).

The contrast between the foregoing chemical run and the parallel one in the presence of the deaminase-cosynthetase enzyme system is striking (see 'Enzymic run', Table). Uro'gen-III (3a) is now the major product formed enzymically at the expense of uro'gen-I (5a) only; the amount of uro'gen-IV (7a) formed is not affected by the enzymes. Since in addition, it was firmly established [2] that uro'gen-I (5a) is not converted into uro'gen-III (3a) by deaminase-cosynthetase, the following clear conclusion can be drawn: that the enzymes are not acting directly on the pyrromethane (1a) but on a chemically formed intermediate *en route* to uro'gen-I (5a). We felt this intermediate must be the aminomethylbilane * (12a) produced in specifically labelled form from the labelled pyrromethane (1a).

On the basis of this interpretation, it is possible to rationalise the earlier results from incubations of the pyrromethane (1) with enzyme systems which were reported from this laboratory [1] and elsewhere.[13,14] The incubations in Cambridge using [^{14}C]pyrromethane (1a) with enzymes from duck's blood were generally run at a higher concentration of pyrromethane (36—69 μM) than in related experiments with the enzymes from *Euglena* (1—26 μM in this case). The former set of experiments resulted in a higher 'incorporation' into the final porphyrin (3.5—9%) than was found for the latter set (0.2—0.5%). These observations are consistent with the intermediacy of bilane (12a) since its rate of formation chemically is proportional to [pyrromethane]^2. This conviction that chemical formation of bilane (12a) from the pyrromethane (1) is an essential step prior to the enzymic reactions led to the choice for the present work of a still higher concentration of pyrromethane (1.7mM). The yield of uro'gen-III (3a) in the enzymic experiment was then 17%, corresponding to an isolated quantity of 2.5 mg of enzymically formed uroporphyrin-III.

Frydman and Frydman *et al.*[13,14] did not observe enzymic incorporation of pyrromethane [as (1)] at 80—300 μM into uro'gen-III (as 3) and the key difference was the time of incubation. Their incubations were for 1 h whereas ours lasted for 17 h. In our hands, the conversion of ^{13}C-pyrromethane [as (1)] into urogen-III was dramatically lower at 35 min than at 17 h [1] as would be expected when the enzymic step depends on the evidently slow chemical formation of bilane (12).

Following this lead that the bilane (12) is an intermediate, it was possible to check whether the enzymic rearrangement step implicit in the second stage of the transformation (1) ⟶ (12) ⟶ (3) has the characteristics established [15] for PBG [as (16)] the natural substrate: namely, intact incorporation into C-20/ring A; C-5/ring B; C-10/ring C; and *intramolecular* rearrangement to generate C-15 and ring D.

For this study, the [$^{13}C_2$]pyrromethane (1b) was required and two synthetic approaches were investigated. Related [$^{13}C_2$]pyrromethanes had earlier been prepared [15] from [2,11-$^{13}C_2$]PBG (16) *via* the pyrrole (17). Unlabelled (17) was therefore reacted with PBG lactam ester (19) to afford the formylpyrromethane (20) but deformylation could not be achieved using $(Ph_3P)_3$-RhCl. However, direct coupling of PBG [as (16)] with PBG lactam (18) by modification of Müller's method [9] gave, after esterification with diazomethane, the required pyrromethane (21a) in 55% yield, which was isomerically pure (h.p.l.c.). In a control experiment, PBG alone under the same reaction conditions gave neither PBG lactam ester (19) nor the pyrromethane (21a), and so the method appeared suitable for preparing *specifically labelled* pyrromethane (1b) from diluted [2,11-$^{13}C_2$]-PBG; [15] this was confirmed by ^{13}C n.m.r. analysis of the labelled product (21b). This doubly labelled pyrromethane (21b) (*with* 19% *of the molecules carrying two* ^{13}C-*atoms*) was hydrolysed to (1b), and then incubated with the deaminase-cosynthetase enzyme system from *E. gracilis*. The mixture of isomeric coproporphyrin esters (8)—(11) was isolated as earlier giving type-I (8), (15%), type-III (7) (57%), and type-IV (11) (28%) (h.p.l.c.). The ^{13}C n.m.r. spectrum was recorded from the mixture, with and without the shift reagent Pr([$^2H_9$]-fod)$_3$ (see Figure 2).

In the presence of shift reagent, a large upfield shift occurs *only* for the signals from C-15 of coproporphyrin-III ester (10b) and coproporphyrin-IV ester (11b) since these carbon atoms are the only ones in the three isomers present to be flanked † by two ester groups [16] (Scheme 2). The rigorous assignment of the shifted 70 Hz doublet to C-15 of coproporphyrin-III ester (10b) and the other assignments marked on Figure 2, are based on the following: (*a*) the content of type-I (8b) was halved (h.p.l.c.) by a combination of chromatography on cellulose and preparative h.p.l.c. and the spectrum was re-run with shift reagent so allowing assignment of the signal for type-I (8b); (*b*) sufficient unlabelled coproporphyrin-III ester (10) was added to enhance (with precise fit of chemical shift) the small signal centred in the 70 Hz doublet; (*c*) the signal intensity of the shifted 70 Hz doublet is correct for correspondence with the major component (type-III) but not for the lesser type-IV; (*d*) ^{13}C-enrichment at C-15 for the type-IV isomer can

* Subsequent studies [11,12] with synthetic aminomethylbilane (12) have confirmed this interpretation.

† Coproporphyrin-II ester [labelled (9)] can be neglected since it was present in barely detectable quantity by h.p.l.c.

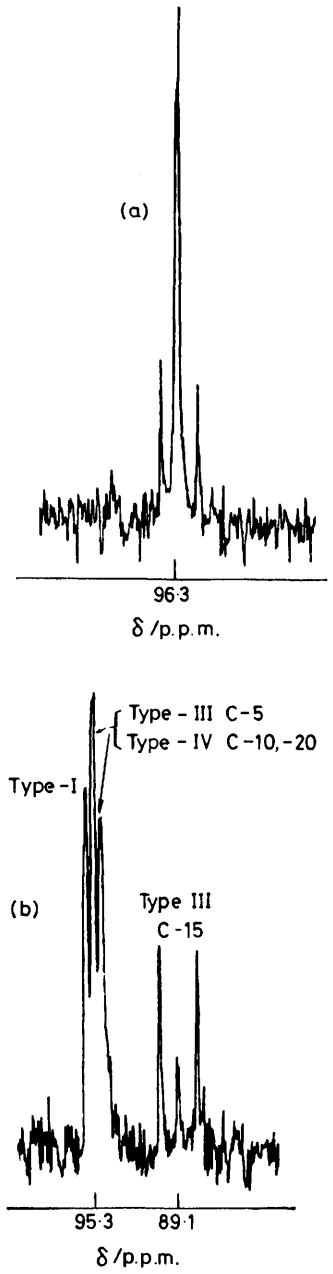
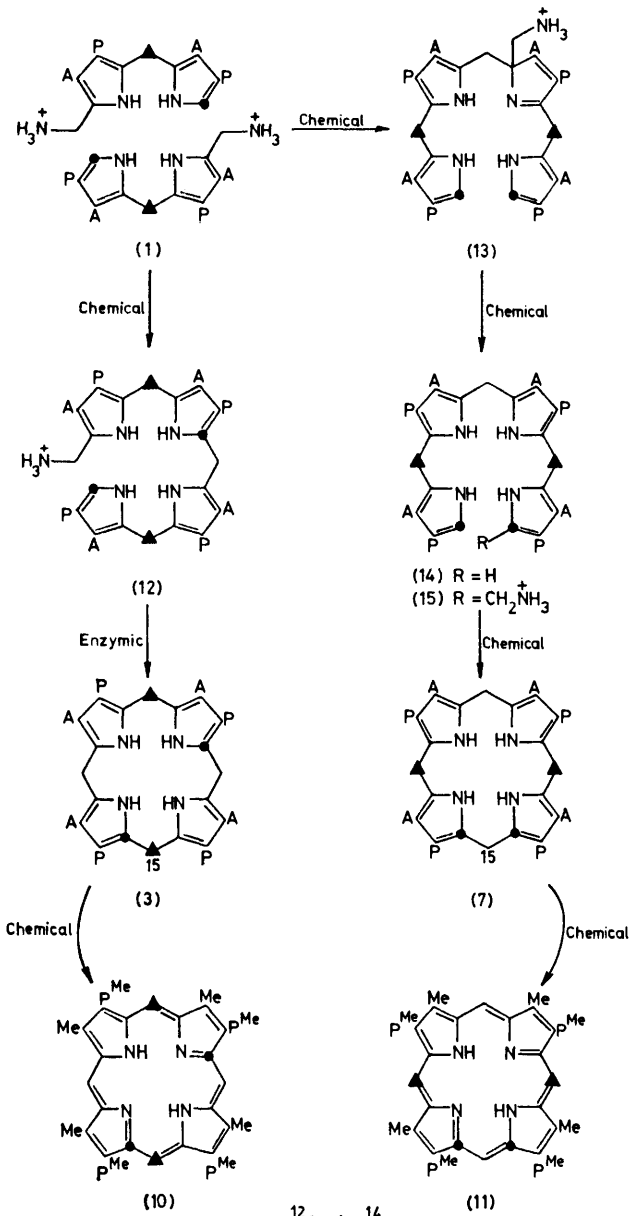


FIGURE 2 ^{13}C N.m.r. spectra from *meso*-bridges of [^{13}C]coproporphyrin esters determined in $CDCl_3$ (a) in absence of shift reagent (b) in presence of $Pr([^2H_9]fod)_3$; chemical shifts downfield from Me_4Si

only occur by serious scrambling of the labels and all the evidence (see preceding paper also) is against this. Scheme 2 shows the probable major route to uro'gen-IV (7b) which does not enrich C-15.

With the shifted 70 Hz doublet securely assigned to C-15 of coproporphyrin-III ester (10b) it follows that the labelling pattern * of the enzymically formed uro'gen-III is as (3b) [17] (Scheme 2). Thus, uro'gen-III production involves rearrangement which is intramolecular with respect to ring D, *i.e.* ^{13}C at C-15 has become bonded to the ^{13}C-atom of the pyrrole molecule to which it was originally attached exactly as found [15] for the labelled natural substrate [2,11-$^{13}C_2$]PBG (16).



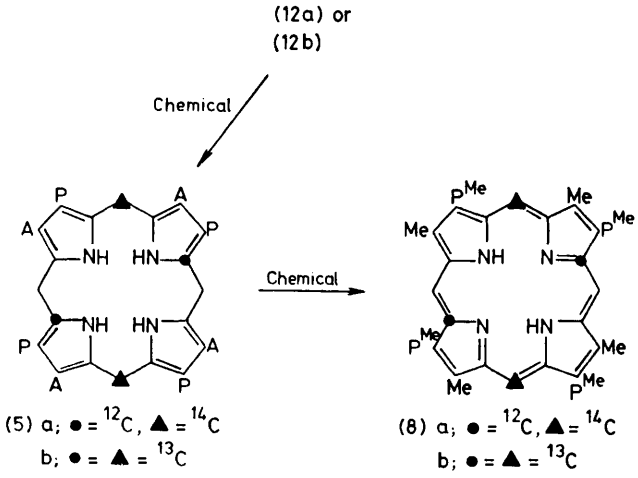
SCHEME 2

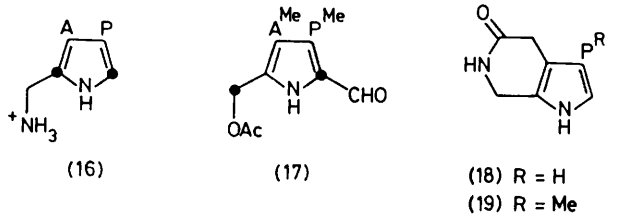
The results from this paper and the preceding one [1] now combine to give a clear picture. (*a*) Pyrromethane (1) is transformed chemically under mild conditions mainly into uro'gen-I (5) with a smaller amount of uro'gen-IV (7); only traces of uro'gen-III (3) are formed chemically. Uro'gen-I is formed *via* an intermediate 'X'. (*b*) Pyrromethane (1) is not a direct biosynthetic precursor of uro'gen-III (3) but in the presence of deaminase–cosynthetase, the intermediate 'X' is

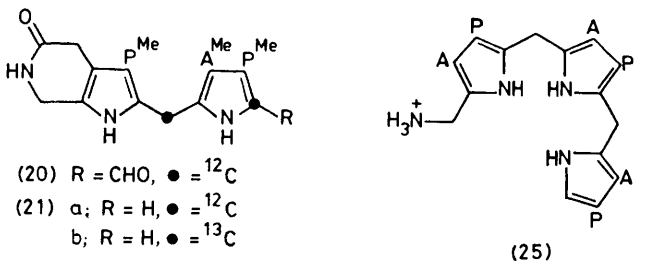
* The fact that the three separated signals around δ 95.3 in the shifted spectrum are narrow doublets (3—3.5 Hz from three-bond coupling [18]) is also in full accord with the labelling patterns shown in Scheme 2.

accepted by the enzymes, is diverted away from chemical ring-closure to uro'gen-I (5), and is enzymically rearranged to form uro'gen-III (3). (*c*) The enzyme-catalysed rearrangement of 'X' has exactly the characteristics found for the natural substrate PBG (9).

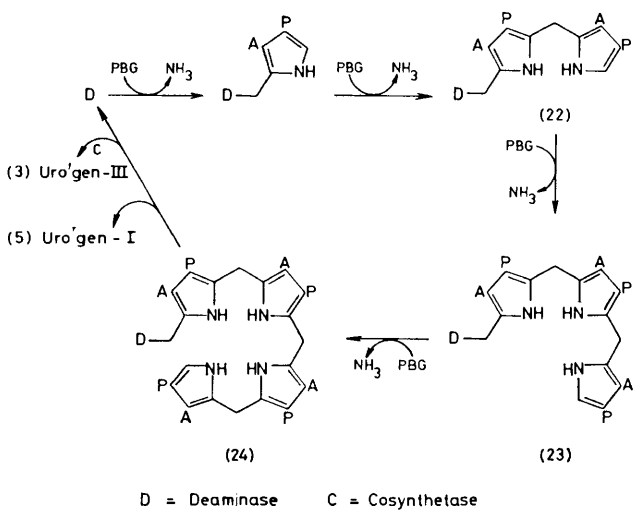
The deduction was made that 'X' is the aminomethylbilane (12) and this leads to the important conclusion that *the rearrangement step in type-III porphyrin biosynthesis occurs at the tetrapyrrole level.* Studies with such tetrapyrroles will be described in subsequent papers.







In view of the foregoing interpretation and conclusions, it is necessary to comment on the inability of the unrearranged pyrromethane (1) to act directly as a biosynthetic precursor of uro'gen-III (3) since there certainly must be a head-to-tail PBG dimer of closely related structure as a normal biosynthetic intermediate. Our view [19] is that assembly of the unrearranged linear tetrapyrrole intermediate (24) takes place entirely on the deaminase enzyme (Scheme 3), and that intermediates (22) and (23) cannot be generated from free enzyme and pyrromethane (1) or tripyrrole (25).[20]



SCHEME 3

Further discussion of the precise details of the conversion of the tetrapyrrole system into uro'gen-I (deaminase alone) or uro'gen-III (with cosynthetase present) will appear in later papers, but we note here that the important isolation [21,22] of aminomethylbilane (12) from incubations of PBG [as (16)] with deaminase in the presence of ammonium ions may involve the displacement of enzyme from the intermediate (24) or the trapping of a reactive product from it.

EXPERIMENTAL

Except as noted below, general directions are given in refs. 4 and 15.

Studies on the Stability of the Pyrromethane (1).—(a) *At* pH 9. A solution of the pyrromethane (1) (1.4 μmol) in buffer (3 ml) at pH 9 was kept at room temperature and assayed at $\lambda_{max.}$ 400 nm (Soret) for formation of porphyrin. Approximate yields of porphyrin were 5% (after 30 min), 9% (8 h), and 25% (96 h).

(b) *In ethyl acetate–acetic acid.* A solution of (1) (0.47 μmol) in pH 9 buffer (3 ml) was shaken with ethyl acetate–acetic acid (3 : 1 v/v) and the extract was kept at room temperature. After 3 h, the conversion into porphyrin was $<0.4\%$.

(c) *In* 15% *hydrochloric acid.* The solution of (1) (0.46 μmol) in 15% hydrochloric acid (3 ml) gave immediate formation of the porphyrin chromophore which rapidly intensified.

(d) *With boiled enzyme from* E. gracilis. A standard enzyme preparation [15] (250 ml) was boiled for 0.5 h and to the cooled separated solution was added PBG (2 mg) and pyrromethane (1) (8 mg). The mixture was incubated at 37 °C for 35 min and the formation of porphyrin was negligible.

(e) *With aqueous iodine.* Aqueous solutions of pyrromethane (1) on treatment with an excess of 1.3% aqueous iodine produced no porphyrin directly or during the subsequent work-up and esterification steps (see section on 'Quantitative Comparison' below). This result (u.v. analysis) was confirmed with [^{14}C]pyrromethane (total activity 10^6 disint. min^{-1}) [(1a) ^{14}C at ▲] which proved that the formation of porphyrin was $<1.8\%$. Relative to the

enzymic runs described below, this is a maximal figure (probably by a factor of at least 2) since at the end of a 16 h incubation, a considerable part of the original pyrromethane has been converted into porphyrins or porphyrinogens or has chemically decomposed.

(f) *At* pH 7.4. The pyrromethane (1a) (10 mg) in buffer (50 ml) at pH 7.4 at 38 °C for 16 h yielded 11% of uroporphyrins.

(g) *In boiled enzyme from avian blood.* The enzyme [15] (50 ml) was boiled for 10 min and the pyrromethane (1a) (1.27 mg) was incubated with the cooled separated solution at pH 8.1 and 38 °C for 16.5 h to produce a 3.6% yield of uroporphyrins.

Separation of Coproporphyrin Isomers.—(a) *As free acids on cellulose.*[23] T.l.c. plates carrying 0.3 mm and 1.0 mm thickness of cellulose were prepared and dried in air for at least 2 weeks. A mixture of coproporphyrin isomers (*ca.* 40 μg) in pyridine was applied to a 20 cm^2 × 0.3 mm plate and elution with 10 : 7 (v/v) 2,6-lutidine–0.7M-NH_4OH for 12—16 h gave 3 bands identified (^1H n.m.r.) as isomer-II (high R_F), isomers-III and -IV (middle band), and isomer-I (low R_F). The porphyrins, eluted with 0.7M-NH_4OH–pyridine (1 : 1 v/v) for u.v. analysis, were contaminated with a colourless impurity derived from the absorbent.

(b) *As methyl esters on cellulose.* Adsorption of the mixture of esters onto paper sheets or a cellulose column followed by elution with 9 : 1 (v/v) heptane–propanol separated mainly a mixture of isomers-III and -IV with isomers-I and -II remaining at the origin. However, separation seemed to be due more to the lower solubility of the isomers-I and -II rather than to a true chromatography and isomers-I and -II were slowly leached from the column.

(c) *As methyl esters on silica (h.p.l.c.).* A wide range of columns and solvent systems were tested to achieve this important separation and the method used in these studies is given in detail in the following section (see also ref. 6).

Quantitative Comparison of Yield and Isomer Analysis of Uro'gen formed from Pyrromethane (1a) *with Normal and Boiled Deaminase–Cosynthetase from* E. gracilis.—A solution of [^{14}C]pyrromethane (1a) was prepared by hydrolysis of the corresponding lactam trimethyl ester (30.61 mg; 1.7 × 10^5 disint. min^{-1}), (which had been shown to be at least 97% pure by h.p.l.c. analysis) in methanol (5 ml) with 2M-aqueous potassium hydroxide (1 ml) for 50 h at 20 °C under nitrogen. The methanol was removed (N_2 stream) and the solution was adjusted to pH 8.0 by adding aqueous 2M-KH_2PO_4 (0.95 ml). The final volume was adjusted to 2.0 ml and *half* of the solution (*i.e.* 33.3 μmol; 8.5 × 10^4 disint. min^{-1}) was used for each of two incubations (normal and boiled enzyme) run in parallel as follows.

To deaminase–cosynthetase (38 000 units) from *E. gracilis* [15] in buffer at pH 7.4 (19 ml) was added the foregoing pyrromethane solution (1 ml) to give a pyrromethane concentration of 1.7mM. The solution was adjusted to pH 7.2 with 2M-hydrochloric acid, and nitrogen was bubbled through the solution which was incubated at 37 °C for 16 h in a sealed tube in the dark. This is the Enzymic Run.

An exactly parallel experiment used enzyme solution which had been heated at 96 °C for 5 min, then cooled to 25 °C before adding the pyrromethane solution; this is the Chemical Run.

While hydrolysis of the pyrromethane lactam ester was in progress, at the start of the foregoing experiments, a mixture of the octamethyl esters of uroporphyrins-I (1.00 mg), -II (1.01 mg), -III (1.00 mg), and -IV (1.03 mg) (each >99% by h.p.l.c. analysis) was dissolved in tetrahydrofuran (3 ml) and stirred under nitrogen at 20 °C with 2M-aqueous potassium hydroxide (3 ml) for 24 h. The aqueous layer was freed of tetrahydrofuran by passing a stream of nitrogen while warming to *ca.* 40 °C. The solution was then neutralised with 10% aqueous hydrochloric acid and made slightly alkaline with 0.1M-aqueous potassium hydroxide (volume now *ca.* 8 ml) before adding sodium amalgam (from 0.75 g of sodium in 25 g mercury). Reduction to colourless uro'gens was complete within 15 min and the solution was decanted from the amalgam, filtered through glass wool, adjusted to pH 7.4 by addition of 2M-KH_2PO_4 (0.5 ml) and 2M-aqueous potassium hydroxide and adjusted to a final volume of 10 ml.

At the end of the above incubations (Enzymic and Chemical), *half* of the mixture of uro'gens I—IV (5 ml) was added to each flask, and a 1.3% solution of iodine in 2% aqueous potassium iodide (0.5 ml) was added. After 10 min at 37 °C, the excess of iodine was discharged by adding aqueous 1% $Na_2S_2O_3$ (1 ml) and the solutions were evaporated to dryness. Each residue was esterified using 5% (v/v) concentrated sulphuric acid in methanol (50 ml) and the uroporphyrin octamethyl esters were isolated by neutralisation with aqueous ammonia, extraction into chloroform, and chromatography on alumina (yields 7.5 mg from Chemical, 5.5 mg from Enzymic). Assuming 100% recovery of the added carrier material, yields from the pyrromethane were 32 (Chemical) and 22% (Enzymic). Both yields are minimal since the assumed 100% recovery of diluent would not be achieved; the Enzymic value is further reduced by irreversible adsorption of pigment onto protein which precipitated during the incubation.

An aliquot (0.5 mg) of each of the foregoing uroporphyrin esters was transferred to a separate Carius tube, dissolved in 7.5M-hydrochloric acid (0.8 ml) and then mixed with water (20 ml). The tubes, sealed under nitrogen, were then heated at 180 °C for 3 h and the resultant coproporphyrins were isolated either by neutralising the solution with sodium acetate and exhaustive extraction with ether or preferably by evaporation of the solution to dryness. They were then esterified with 5% sulphuric acid in methanol as usual.[15] The resultant mixture of coproporphyrin esters was filtered in chloroform through a short column of silica, recovered by evaporation and analysed as follows by h.p.l.c.

First column procedure. Two 30 cm 10μ CN Bondapak columns (Waters) were joined in sequence, and were run at 1 ml min^{-1} using a mixture of acetonitrile (60 ml), toluene (200 ml), and hexane (made up to 1 l). The injected solution contained 50—60 μg of coproporphyrin esters and detection was by a Cecil flow cell with u.v. analyser set at *ca.* 390 nm. A typical separation is shown in Figure 1(a).

Each analysis of radio-labelled material was repeated three times, the column being ' flushed ' with a solution of radio-inactive coproporphyrin esters after each run to eliminate cross contamination. The separated fractions (type-I, type-II, and types -III and- IV together) were then assayed for radioactivity (see Table).

Second column procedure. The columns were two 30 cm 10μ Porasil (Waters) joined in series, the solvent being heptane (3 parts) and ether, 90% saturated with water (2 parts). Injection and detection were as above and the material fractionated was that recovered from the overlapping type-III and type-IV band from the first column. After 10 recycle stages, the separation was sufficient to allow pure samples of coproporphyrin-III and copropor-

phyrin-IV esters to be assayed for radioactivity. This separation is shown in Figure 1(b); it was repeated twice on the labelled material with the same ' flushing ' precaution as above; the results are collected in the Table.

Methyl 2-{4-(2-*Methoxycarbonylethyl*)-3-*methoxycarbonylmethyl* [5-^{13}C]*pyrrol*-2-*yl*[^{13}C]*methyl*}-5-*oxo*-4,5,6,7-*tetrahydro*-1H-*pyrrolo*[2,3-c]*pyridine*-3-*propionate* (21b).—A suspension of PBG (40 mg) and PBG lactam [24] (120 mg) in distilled water (10 ml) was adjusted to pH 8.2 by addition of 10% aqueous diethylamine. The solution was degassed and heated at 70—75 °C under nitrogen for 2 h, then cooled and freeze-dried. The residue in methanol (5 ml) was treated with an excess of ethereal diazomethane and, after evaporation, the product was chromatographed on a column of neutral alumina packed in chloroform. Elution with 9 : 1 (v/v) chloroform–methanol gave the pyrromethane lactam ester (21a) (44.6 mg; 54%), m.p. 193.5—194.5 °C (from methanol) (lit.,[4] 192—194 °C), δ_C 19.3 and 20.7 (2 × pyrr CH_2CH_2CO), 22.0 (pyrr$_2CH_2$), 29.1, 29.4, and 29.8 (3 × pyrr CH_2CO), 34.4 and 34.9 (2 × pyrr CH_2CH_2CO), 40.9 (CH_2N), 51.3, 51.5, and 52.1 (3 × OMe), 110.1, 110.7, 113.2, 114.0, 118.1, 120.8, 127.7, and 128.0 (8 × pyrr-C), and 171.2, 173.4, 173.3, and 174.6 (4 × CO).

[2,11-^{13}C]PBG (89 mg) was prepared as earlier [15] from [5-^{13}C]ALA (90 atom % ^{13}C) and was isolated by dilution with unlabelled PBG so that the final product carried 19 atom % ^{13}C at each labelled site.

This product was converted as above into the [$^{13}C_2$]-pyrromethane (21b) (98 mg, 54%), m.p. 190—193 °C, δ_H 4.82 (81 : 19 s : d, J 128 Hz, pyrr$_2CH_2$) and 6.44 (81 : 19 s : d, J 185 Hz, pyrr-H); δ_C 22.0 and 114.0 were strongly enhanced.

Incubation of [$^{13}C_2$]*Pyrromethane* (1b) *with Deaminase–Cosynthetase*.—The foregoing pyrromethane lactam ester (32 mg) was hydrolysed and after adjustment of pH as usual was incubated with deaminase–cosynthetase from *Euglena gracilis* [15] (40 ml total vol. 60 000 units) at 37 °C and pH 7.2 for 16 h under nitrogen. The uroporphyrin esters were isolated as before (14.2 mg) and decarboxylated (in 0.5 mg batches) to give coproporphyrin esters (7.8 mg) (h.p.l.c. analysis showed 15% I, 85% III—IV, and negligible II).

After recording the ^{13}C n.m.r. spectrum [Figure 2(a)] the mixture (7.7 mg) in chloroform (20 ml) was adsorbed onto cellulose (25 g; Whatman CF11) by allowing the solvent to evaporate slowly (48 h) in the dark. The impregnated cellulose was added to the top of a column of fresh cellulose (250 g) and eluted with 9 : 1 (v/v) heptane–propan-1-ol to give a mixture of coproporphyrin esters (6.85 mg) now containing 10% of type-I isomer.

The foregoing incubation was repeated under the same conditions with [$^{13}C_2$]pyrromethane (1b) (16 mg) to yield uroporphyrin esters (5 mg) which were converted as before into coproporphyrin esters. Coproporphyrin-I ester was completely removed from this product by preparative h.p.l.c. (first column procedure above) and the resultant mixture of type-III and -IV isomers was added to the product from the first incubation above; the resultant mixture contained 8% (h.p.l.c.) of the type-I system, *i.e. ca.* half of the original value. The ^{13}C n.m.r. spectrum was again determined in the presence of Pr([$^2H_9$]fod)$_3$ [Figure 2(b)].

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