Biosynthesis of Porphyrins and Related Macrocycles. Part 14.[1] Studies with [^{14}C]Isoporphobilinogen and Four Isomeric Labelled Pyrromethanes: Specific Conversion of the Unrearranged Pyrromethane $\overset{+}{N}H_3$.AP.AP into Haem and Protoporphyrin-IX

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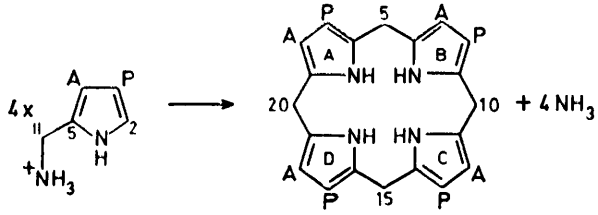
[11-^{14}C]Isoporphobilinogen and ^{14}C-, and ^{13}C-labelled forms of four isomeric pyrromethanes have been synthesised in order to study at what stage in the building process rearrangement occurs to produce the natural porphyrins. The finding that isoporphobilinogen is not a biosynthetic precursor of protoporphyrin-IX points against the rearrangement being at the monopyrrole level. Of the four pyrromethanes, only the unrearranged system $\overset{+}{N}H_3$.AP.AP (5) is significantly converted, in the presence of the necessary enzymes from *Euglena gracilis* or duck's blood, into protoporphyrin-IX or haem. Degradation of these products from ^{14}C-labelled (5) proved that the conversion of $\overset{+}{N}H_3$.AP.AP into the type-III macrocycle occurs without randomisation of the labels; this conclusion was rigorously confirmed by ^{13}C-n.m.r. spectra from samples of ^{13}C-protoporphyrin-IX produced enzymically from ^{13}C-labelled (5).

'The Type-III problem'[2] in porphyrin biosynthesis[3] is concerned with the ubiquitous enzymic conversion of porphobilinogen PBG (1) into uroporphyrinogen-III (uro'gen-III) (2) and ammonia. The transformation, which clearly involves rearrangement, is of fundamental importance because uro'gen-III (2) is the parent macrocycle from which all the haems, chlorins, and corrins are derived.[3]

Biosynthetic studies with *diluted* [2,11-$^{13}C_2$]PBG [as (1)] defined precisely the nature of the rearrangement:[4] the 11-CH_2 of that PBG molecule which becomes ring D migrates *intramolecularly* from C-5 to C-2. Furthermore the same study[4] revealed that the three PBG molecules which are built into ring A (with C-20), ring B (with C-5), and ring C (with C-10) are incorporated with their carbon skeletons intact. Although these studies eliminated the majority of the many mechanisms proposed for the formation of uro'gen-III[3] they conveyed no information regarding the *timing* of the rearrangement step. Earlier experiments showed[5] that uro'gen-III (2) is *not* formed by rearrangement of uro'gen-I (3). Uro'gen-I is the product expected from straightforward head-to-tail assembly of four PBG units.

In principle, the single rearrangement might take place at the monopyrrole, dipyrrole, tripyrrole, or linear tetrapyrrole levels, and it should be possible to distinguish between these possibilities by studying the incorporation of the appropriate precursors, preferably in labelled form. In this paper we describe studies[6] with ^{14}C-labelled isoporphobilinogen, iso-PBG (4), and labelled forms of the four isomeric pyrromethanes (5)—(8).

Results from Enzymic Experiments.—Iso-PBG (4) had earlier[7] been mixed with ^{14}C-PBG [as (1)] and incubated with an haemolysate from chicken blood. No significant diminution of specific activity was found in the isolated porphyrin indicating that iso-PBG (4) was not being used for type-III porphyrin biosynthesis. This approach would certainly detect a large incorporation of iso-PBG



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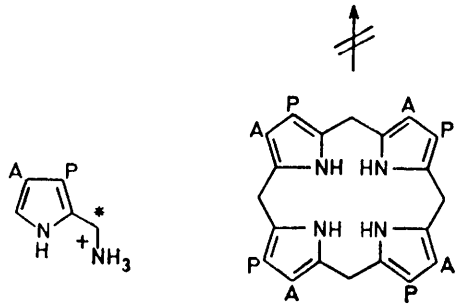

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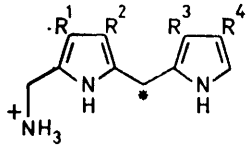


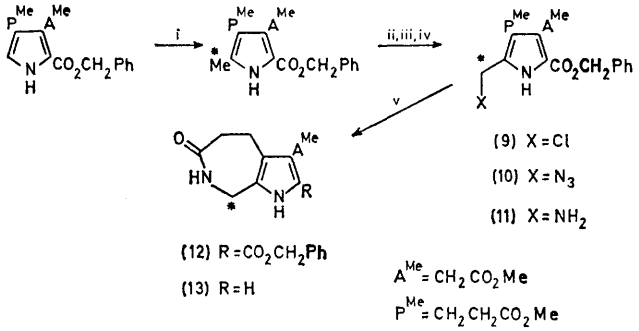
	R^1	R^2	R^3	R^4
(5)	A	P	A	P
(6)	A	P	P	A
(7)	P	A	A	P
(8)	P	A	P	

A = CH_2CO_2H

P = $CH_2CH_2CO_2H$

but to ensure that a small one had not occurred, [11-^{14}C]iso-PBG (4) was synthesised.

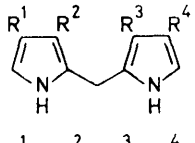
The precursor required for iso-PBG (4) is the lactam (12) which had been prepared [8] by a route involving phenyldiazomethane. This inconvenient step was avoided by using the sequence shown in the Scheme.



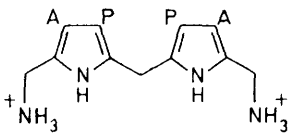
SCHEME *Reagents:* i, *CH_2O–HI–H_3PO_2; ii, SO_2Cl_2; iii, NaN_3; iv, NH_3; v, 2-pyridone, heat

The key step was displacement of azide anion from pyrrole (10) using liquid ammonia; the azide was available in 96% yield from the chloromethyl precursor (9). Conditions could not be found for preparation of the amine (11) directly from the chloromethylpyrrole (9). The remaining steps to the lactams (12) and (13) essentially followed the earlier work [8] but several improvements are given in the Experimental section. Alkaline hydrolysis of lactam (13) then afforded iso-PBG (4). For the labelled series, ^{14}C was introduced from [^{14}C]-formaldehyde by reductive methylation [9] as indicated in the Scheme.

The [11-^{14}C]iso-PBG (4) was then incubated alone and with unlabelled PBG (1) using enzyme preparations from *Euglena gracilis*.[4] The results (expts. 2 and 3, Table 1) confirm the earlier conclusion [7] that the rearrangement which produces uro'gen-III (2) is not at the monopyrrole level. Attention thus turned to di-



	R^1	R^2	R^3	R^4
(14)	A	P	A	P
(15)	P	A	A	P
(16)	A	P	P	A



(17)

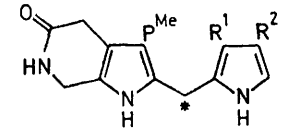
A = CH_2CO_2H

P = $CH_2CH_2CO_2H$

pyrrolic systems. Experiments prior to our studies with pyrromethanes had shown [10] that the headless pyrromethanes (14)—(16) and the double-headed system (17) were not involved in biosynthesis of type-III porphyrins.

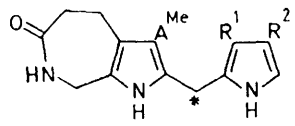
To test rigorously for the involvement of a particular pyrromethane in the biosynthetic pathway to the natural porphyrins, it is important that (*a*) the precursor be specifically labelled and be demonstrated to be chemically and radiochemically pure, (*b*) that the level of its transformation into the isolated porphyrin should be significantly higher with enzyme than that found for a chemical blank, (*c*) that the labelling pattern in the final porphyrin be unambiguously determined to demonstrate intact incorporation.

Accordingly the four pyrromethanes (5)—(8) labelled with ^{14}C at * were synthesised [8,11] *via* the lactam esters (18)—(21). The ^{14}C-labelled specimens of pyrro-



(18) $R^1 = A^{Me}$, $R^2 = P^{Me}$

(19) $R^1 = P^{Me}$, $R^2 = A^{Me}$



(20) $R^1 = A^{Me}$, $R^2 = P^{Me}$

(21) $R^1 = P^{Me}$, $R^2 = A^{Me}$

methanes (7) and (8) were obtained by labelling methods similar to those described [11] for isomers (5) and (6) (see Experimental section). A method was developed based on high pressure liquid chromatography for distinguishing between all four isomeric lactam esters (18)—(21). This showed each sample to be free of isomeric impurities,[8] and it had been established by ^{1}H n.m.r. spectroscopy that alkaline hydrolysis of (18)—(21) to give (5)—(8), respectively, brings about no isomerisation.[8]

The four ^{14}C-labelled pyrromethanes (5)—(8) were then incubated separately with a cell-free enzyme system from *Euglena gracilis* * or alternatively with one from haemolysed duck's blood,* with and without unlabelled PBG (1). Protoporphyrin-IX (23) and, in the case of duck's blood, haemin (25) were isolated. The porphyrin (23) and part of the haemin (25) were converted into protoporphyrin-IX dimethyl ester (26) for rigorous purification to constant specific activity determined by liquid scintillation counting (see Experimental section). As a further check, an aliquot of the purified ester (26) was hydrogenated to its tetrahydro-derivative (mesopor-

* These preparations contain not only deaminase and cosynthetase necessary for formation of uro'gen-III (2) but also enzymes which transform the initially formed uro'gen-III (2) *via* copro'gen-III (22) in protoporphyrin-IX (23) and for duck's blood into haem (24). These steps do not disturb the integrity of the macrocycle [12] and their occurrence is an important advantage for ^{14}C-studies since, in contrast to (23) and (24), there are no known degradations for determining the labelling pattern in uroporphyrin-III (27) or coproporphyrin-III (28).

phyrin-IX dimethyl ester) which was chromatographed and crystallised to constant molar specific activity; within experimental error, this was the same as for the protoporphyrin-IX ester (26). Table 1 collects the results which clearly show that only the *unrearranged* pyrromethane (5) was significantly utilised The incorporations of the isomers (6)—(8) were insignificant and -IXγ, and -IXδ, were separated by chromatography.[13] The IXα-isomer (29) has lost the original C-5, so the radioactivity at this position of the haemin (25) can be estimated by difference, and similarly for C-10, -15, and -20 from the β- (30), γ- (31), and δ-isomers (32). The results, summarised in Table 2, show when [^{14}C]pyrromethane (5) is incubated alone with the enzymes, it

TABLE 1

Enzymic experiments [a] with PBG (1), isoPBG (4), and isomeric pyrromethanes (5)—(8)

Pyrromethane or pyrrole	Expt. no.	Enzyme from *Euglena gracilis*: Total ^{14}C-activity (disint. min^{-1}) (or wt for ^{13}C)	Concn. of pyrromethane or pyrrole (mmol l^{-1})	% Incorpn.[b] into protoporphyrin-IX dimethyl ester (26)
[^{14}C]PBG (1)	1	3.0×10^6	0.11	33
[^{14}C]isoPBG (4)	2	3.9×10^6	0.01	<0.01
+ PBG (1)			0.106	
[^{14}C]isoPBG (4)	3	4.4×10^7	0.087	<0.001
+ PBG (1)			0.053	
[^{14}C]AP.AP (5)	4 *	4.5×10^7	0.026	0.54
[^{14}C]AP.AP (5)				
[^{13}C]AP.AP (5a)	5 *	(27.9 mg)	0.061	7.1
[^{13}C]AP.AP (5b)	6 *	(32 mg)	0.093	5.7
[^{13}C]AP.AP (5b)	7	(84 mg)	0.14	0.57 for ^{13}C
+ [^{14}C]PBG (1)		7.1×10^6	0.071	10 for ^{14}C
[^{14}C]AP.AP (5)	8 *	1.3×10^7	0.024	0.22
+ PBG (1)			0.106	
[^{14}C]AP.AP (5)	9	6.2×10^6	0.012	0.19
+ PBG (1)			0.019	
[^{14}C]PA.AP (7)	10	3.4×10^7	0.087	0.015
+ PBG (1)			0.053	
[^{14}C]PA.AP (7)	11	2.9×10^6	0.01	0.01
+ PBG (1)			0.11	
[^{14}C]PA.PA (8)	12	1.0×10^7	0.087	0.092
+ PBG (1)			0.053	

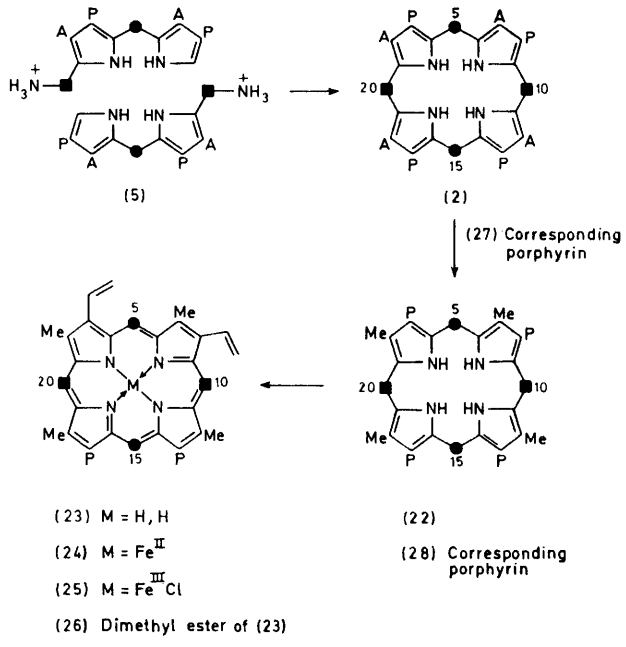
Pyrromethane or pyrrole	Expt. no.	Enzyme from duck's blood: Total ^{14}C-activity (disint. min^{-1}) (or wt for ^{13}C)	Conc. of pyrromethane or pyrrole (mmol l^{-1})	% Incorpn.[b] into protoporphyrin-IX dimethyl ester (26)	% Incorpn.[b] into haemin [c] (25)
[^{14}C]PBG (1)	13	2.2×10^5	0.003	7.4	41
[^{14}C]PBG (1)	14	2.0×10^6	0.027	15	28
[^{14}C]AP.AP (5)	15	1.2×10^7	0.057	0.96	8.1
[^{14}C]AP.AP (5)	16	8.5×10^6	0.039	0.6	2.9
[^{14}C]AP.AP (5)	17	1.2×10^7	0.055	1.6	4.2
+ PBG (1)			0.53		
[^{14}C]AP.AP (5)	18	1.5×10^7	0.07	1.0	4.4
+ PBG (1)			0.66		
[^{14}C]AP.PA (6)	19	2.1×10^7	0.068	0.0	<0.02
[^{14}C]AP.PA (6)	20	2.2×10^7	0.079	<0.01	<0.03
[^{14}C]AP.PA (6)	21	2.2×10^7	0.09	<0.03	<0.02
+ PBG (1)			0.55		
[^{14}C]PA.AP (7)	22	1.4×10^7	0.048	<0.035	0.15
[^{14}C]PA.AP (7)	23	1.4×10^7	0.048	0.06	0.06
+ PBG (1)			0.02		

[a] All incubations with duck's blood enzyme were for 17 h at 37 °C and with *Euglena* enzyme, for 35 min at 30 °C, save the asterisked experiments which were for 17 h. [b] For ^{14}C-experiments, % of total radioactivity in the administered pyrromethane or pyrrole appearing in porphyrin. [c] Converted into protoporphyrin-IX dimethyl ester for purification.

they were too small to permit degradation of the isolated porphyrin to locate its radioactivity.

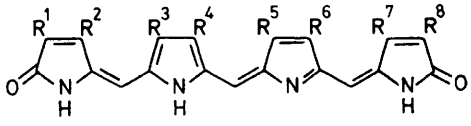
The labelling pattern for the protoporphyrin-IX ester of constant specific activity obtained from the haemin (25) derived from [^{14}C]pyrromethane (5) (expt. 15, Table 1) was determined by conversion back into haemin followed by oxidative degradation using Bonnett's method.[13] The four isomeric biliverdin esters [(29)—(32)] so obtained, respectively, biliverdin-IXα, -IXβ, affords haem (24) [and therefore uro'gen-III (2)] labelled specifically at C-5 and C-15. From expt. 17, Table 1, carried out with [^{14}C]pyrromethane (5) in the presence of unlabelled PBG, significant radioactivity (*ca.* 20%) was found at C-10 in addition to heavy labelling at C-5 and C-15 (see Table 2).

The level of incorporation of the APAP [^{14}C]pyrromethane (5) into protoporphyrin-IX (23) with the *Euglena* enzyme system was not high enough to permit



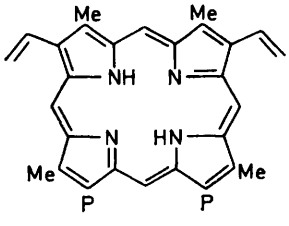
a; ● = ^{13}C, ■ = ^{12}C
b; ● = ^{12}C, ■ = ^{13}C

the labelling pattern to be established *via* the degradation to biliverdins, but this was achieved using ^{13}C-labelled pyrromethane (5a) and (5b). ^{13}C-Labelling allows direct determination of the labelling patterns provided that sufficient product can be isolated at a suitable enrichment.[14] This was made possible by the development [4b] of an improved enzyme system from *E. gracilis* and optimisation [4b] of the incubation conditions.



	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8
(29)	Me	V	Me	P^{Me}	P^{Me}	Me	Me	V
(30)	Me	P^{Me}	P^{Me}	Me	Me	V	Me	V
(31)	P^{Me}	Me	Me	V	Me	V	Me	P^{Me}
(32)	Me	V	Me	V	Me	P^{Me}	P^{Me}	Me

V = CH=CH$_2$, P^{Me} = [CH$_2$]$_2$ CO$_2$Me



(33)

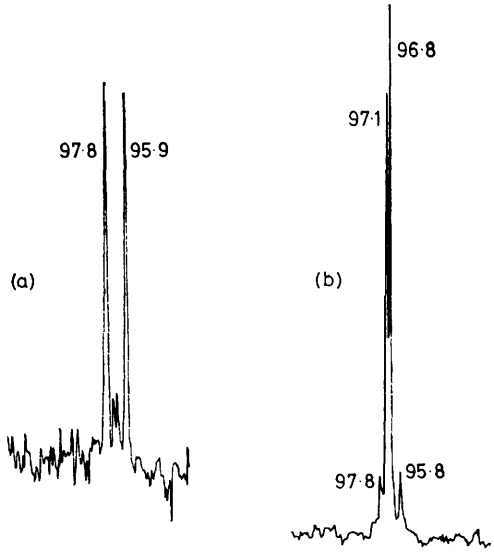
TABLE 2

Degradation of samples of haemin to determine labelling pattern

Expt. no. (see Table 1)	Percentage [a] of total activity at C-5	C-10	C-15	C-20
15	52 ± 5	0 ± 7	51 ± 4	11 ± 7
17	35 ± 5	21 ± 6	56 ± 3	0 ± 7

[a] Calculated from activities of the four derived biliverdins.

Thus [^{13}C]pyrromethane (5a) labelled at ● was synthesised along the same lines [11] as for the ^{14}C-sample and when incubated alone with *E. gracilis* enzyme gave protoporphyrin-IX (23a) enriched only at C-5 and C-15 [Figure (a)]. [*aminomethylene*-^{13}C]Pyrromethane (5b), ^{13}C at ■, similarly gave protoporphyrin-IX (23b) specifically enriched at C-10 and C-20 [Figure (b)]. Furthermore in the presence of ^{14}C-labelled PBG, pyrromethane (5a) ^{13}C-labelled at ● gave protoporphyrin-IX (23a) enriched with ^{13}C mainly at C-5 and C-15



^{13}C N.m.r. signals (proton noise decoupled) from bridge carbon atoms of protoporphyrin-IX dimethyl ester (a) from AP.AP pyrromethane (5a) to label C-5 (δ 97.8) and C-15 (δ 95.9) carbons; (b) from AP.AP pyrromethane (5b) to label C-10 (δ 97.1) and C-20 (δ 96.8) carbons. Both spectra run in $CDCl_3$; SW 5 000 Hz, AT 0.8 s, NT 82 000. The cell-free enzyme system used was from *Euglena gracilis*

but also significantly at C-10 (δ 97.1), exactly as found earlier by the ^{14}C-method. The ^{13}C-spectra were determined in each case on the corresponding dimethyl ester (26a and b) and the unambiguous assignments of the ^{13}C n.m.r. signals from C-5, -10, -15, and -20 depended on synthetic samples of specifically ^{13}C-labelled forms of protoporphyrin-IX dimethyl ester [15] [as (26)].

DISCUSSION

The foregoing facts are thus clear and internally consistent; when pyrromethane (5) is incubated alone with the complete enzyme system, the aminomethylene carbon appears at C-10 and C-20 of uro'gen-III (2) whilst the interpyrrolic methylene forms C-5 and C-15

of (2). But before discussing these facts, mention is needed of other work in this area. Frydman and Frydman [16] did not observe incorporation of the unrearranged pyrromethane (5) into uro'gen-III (2) by the porphyrin-forming enzymes from wheatgerm (see Discussion in following paper) but they found a small incorporation of the rearranged system (7) into uro'gen-III (2) (*ca.* 0.2—0.7% of pyrromethane used). This result was interpreted as indicating that the rearrangement step to produce type-III porphyrins occurs as the first pyrromethane is formed. Later, Scott [17] reported enzymic incorporation of the 'headless' rearranged pyrromethane (10) [7] (0.15—0.89%) into type-III porphyrins, a result thought to prove that the type-III porphyrin synthesis involves rearrangement at the pyrromethane level. The evidence reported in this paper and in subsequent Parts is overwhelming that this is not so (reviewed in ref. 2). It is clear that all these small incorporations of rearranged pyrromethanes are spurious; none of the products has been degraded to establish the labelling pattern. Finally, the suggestion [18] that the labelled porphyrin formed in our enzymic experiments with pyrromethane (5) is not protoporphyrin-IX (23) but really is protoporphyrin-XIII (33) has been shown not to be true.[1,19]

Returning now to the facts summarised at the start of this section, there were two features which warned against the interpretation that the pyrromethane (5) is a true intermediate on the biosynthetic pathway to protoporphyrin-IX (23) and so a substrate for the enzymes producing uro'gen-III (2). First, the strongly preferred process by which protoporphyrin-IX (23) had eventually been produced [by way of uro'gen-III (2)] was clearly by 'dimerisation' of two pyrromethane units (5) *even in the presence of PBG* (1). For the latter conditions, a 2 + 1 + 1 build-up of the tetrapyrrole system would have been expected had (5) been a true intermediate. Secondly, the level of apparent incorporation of pyrromethane (5) into the type-III macrocycle (1.6—9.0%) was significantly less than that found for PBG (*ca.* 40%) in comparable experiments. Since the enzyme system is cell-free, problems of transport cannot be invoked to dismiss the discrepancy.

These features, puzzling at the time, led to a quantitative study of the extent to which uro'gen-III (2) might be formed *chemically* from pyrromethane (5). The results are described in the following paper [20] and their interpretation opened the way to solving the type-III problem.

EXPERIMENTAL

Apart from exceptions noted below, general directions are in refs. 4*b* and 11. In addition, all evaporations were carried out *in vacuo* at <40 °C.

Benzyl 5-Azido[^{14}C]*methyl-4-(2-methoxycarbonylethyl)-3-methoxycarbonylmethylpyrrole-2-carboxylate* (10).—The corresponding 5-methyl compound [11] (4.0 g) in dry ether (220 ml) and dry CH_2Cl_2 (30 ml) at 0 °C was treated with freshly prepared 5% SO_2Cl_2 in ether (20 ml). After 20 min at 0 °C, the solvents were removed leaving chloromethylpyrrole (9) (CH_2Cl at δ 4.65) as a crystalline residue. Without purification, a solution of (9) in acetone (100 ml) was treated under nitrogen with NaN_3 (4.5 g) in water (75 ml). After 30 min at 20 °C the solvents were removed and the residue was partitioned between CH_2Cl_2 and water. The organic layer was washed, dried, and evaporated, and trituration of the residue with ether gave the crystalline *azidomethylpyrrole* (10) (4.26 g, 96%), m.p. 62—63.5 °C (from ether–hexane) (Found: C, 57.65; H, 5.45; N, 13.6. $C_{20}H_{22}N_4O_6$ requires C, 57.95; H, 5.35; N, 13.5%); M^+ 414; $\nu_{max.}$ 3 290, 2 110, 1 735, and 1 680 cm^{-1}; $\lambda_{max.}$ 276 nm (ε 17 790) δ 2.60 (4 H, m, $[CH_2]_2$), 3.54 and 3.61 (each 3 H, s, 2 × OMe), 3.79 (2 H, s, CH_2CO), 4.35 (2 H, s, CH_2N_3), 5.26 (2 H, s, CH_2Ph), 7.3 (5 H, m, Ph), and 9.6br (1 H, s, NH). [^{14}C]-Labelled azide (437 mg; 0.85 μCi) was similarly prepared from [^{14}C]methylpyrrole.[11]

2-Benzyloxycarbonyl-3-methoxycarbonylmethyl-4,5,7,8-tetrahydropyrrolo[2,3-c]*azepin-6*(1H)*-one* (12).—(*a*) The foregoing azide (10) (426 mg) was added to liquid NH_3 (200 ml) at −78 °C. The solution was allowed to reach room temperature (during *ca.* 30 min), when all the ammonia had evaporated. A filtered solution of the residue in CH_2Cl_2 was evaporated to an orange oil containing mainly the amine (11). The oil and 2-pyridone (450 mg) in dry dioxan (30 ml) were heated at reflux under N_2 for 16 h. The solvent was removed and the residue partitioned between water and CH_2Cl_2. The organic layer was washed (0.1M-HCl, then H_2O), dried, and evaporated, and the residue was crystallised from chloroform–hexane to afford the lactam ester (12) (164 mg), m.p. 224.5—225.5 °C (lit.,[8] 225—225.5 °C). Chromatography of the mother-liquors on Kieselgel GF_{254} (15 g) in acetone gave a further 25 mg of the lactam (12) (total 50%).

The [^{14}C]lactam (12) (225 mg, 57%; 0.5 μCi), m.p. 223.5—224.5 °C, was obtained as above from (10) (437 mg).

(*b*) Hydrogenation of the azide (10) (200 mg) in dry methanol (15 ml) over 5% Rh–Al_2O_3 gave the amine (11) and cyclisation as above gave the lactam (12) (70 mg, 41%), identical with the foregoing material.

3-Methoxycarbonylmethyl-6-oxo-1,4,5,6,7,8-hexahydropyrrolo[2,3-c]*azepine-2-carboxylic Acid.*—The foregoing benzyl ester (12) (1.0 g) in methanol (75 ml) was hydrogenated over 10% Pd–C (200 mg) at ambient temperature and pressure. After 2 h, t.l.c. showed the reaction to be complete and the catalyst was removed by filtration through acid-washed Celite. The pad was washed with hot methanol and the combined filtrate was evaporated to give the crystalline *lactam acid* (740 mg, 99%), m.p. 211—213.5 °C (from MeOH) (Found: C, 54.05; H, 5.5; N, 10.5. $C_{12}H_{14}N_2O_4$ requires C, 54.15; H, 5.3; N, 10.5%); M^+ 266; $\nu_{max.}$ 3 320—3 000, 1 730, 1 690, and 1 640 cm^{-1}; $\lambda_{max.}$ 272 and 247 nm (ε 14 790 and 7 063); δ ([$^2H_5$]pyridine) 2.90 (4 H, s, $[CH_2]_2$), 3.61 (3 H, s, OMe), 4.22 (2 H, s, CH_2CO), and 4.50 (2 H, m, CH_2N). The [^{14}C]-acid (51 mg) was obtained from 65 mg of the corresponding benzyl ester (0.14 μCi).

5-Amino[^{14}C]*methyl-4-(2-carboxyethyl)-3-carboxymethylpyrrole* ('*iso-PBG*') (4).—The foregoing [^{14}C]acid (51 mg) was suspended in degassed water (15 ml) and heated under N_2 at 125—130 °C (sealed tube) for 3¼ h. The solution was cooled, mixed with Et_3N (0.06 ml), and extracted with CH_2Cl_2 to give a residue which crystallised from MeOH–ether to give the α-free lactam (13) (19.4 mg; 63 μCi), m.p. 166—168 °C (lit.,[8] 156—157.5 °C). Dilution of the mother-liquor with radioinactive (13) gave a further 18

mg (12.7 μCi) (total radiochemical yield, 52.5%). Both samples were homogeneous (t.l.c.–radioscan).

Specimens of lactam (13) were hydrolysed [8] to iso-PBG (4) by stirring a suspension (*ca.* 0.5 mg) in 2M-KOH (0.5 ml) for 24 h under N_2 at 20 °C. The solution was adjusted to pH 7.6 immediately prior to incubation.

Synthesis of ^{14}C-*Labelled Pyrromethanes* (5)—(8) *and* ^{13}C-*Labelled Pyrromethane* (5a) *and* (5b).—The synthesis of the [^{14}C]pyrromethanes (5) and (6) from [^{14}C]formaldehyde was described in Part 1.[11] The [^{14}C]pyrromethanes (7) and (8) were prepared from the same intermediate [^{14}C]-methylpyrroles by reaction with lead tetra-acetate to afford the corresponding acetoxy[^{14}C]methylpyrroles (*cf.* refs. 8 and 11) which were used to alkylate lactam (13) (*cf.* refs. 8 and 11). The syntheses were then completed as described in Part 4.[8]

The ^{13}C-labelled pyrromethane in the differently labelled forms (5a) and (5b) was prepared in a strictly analogous way from the available ^{13}C-labelled monopyrrolic building blocks; [11,15] the enrichment was 90 atom %.

Separation of Isomeric Pyrromethanes by H.p.l.c.—A mixture of the four pyrromethane lactams (18)—(21) was completely resolved on a 60 cm × 4 mm 10μ Porasil column eluting with 1 : 16 (v/v) MeOH–Et_2O at 2 ml min^{-1}. With the u.v. detector set at 250 nm, the order of elution (and elution volume) was (i) (20) (77 ml), (ii) (18) (85 ml), (iii) (21) (99 ml), and (iv) (19) (120 ml).

The precursors of (18)—(21) having a CO_2Bz group in place of the α-H were also fully resolved on the same column using 1 : 1 (v/v) dioxan–heptane at 2 ml min^{-1}. The elution volumes were (20)–CO_2Bz, 77 ml; (18)–CO_2Bz, 88 ml; (21)–CO_2Bz, 98 ml, and (19)–CO_2Bz, 121 ml.

The crystalline α-free lactams and their crystalline precursor benzyl esters used in the following biosynthetic studies were all shown by h.p.l.c. to be homogeneous and free of isomeric impurities.

Purification of [^{14}C]*PBG and Protoporphyrin-IX and Proof of Absence of Endogenous Porphyrinogens in the* Euglena *Enzyme System.*—Samples of PBG are often pink or develop colour on standing and are purified, prior to incubation, by chromatography on Amberlyst A-21. The column of resin (1.5 × 10 cm) was thoroughly washed with water, methanol, glacial acetic acid, 1M-potassium hydroxide, water to neutrality, and finally methanol. PBG (200 mg) in 1M-Tris buffer (1—2 ml) was run onto the column which was washed with water (5 ml) and methanol (50 ml). Elution with 2% (v/v) acetic acid in methanol gave 15 ml fractions; colourless PBG·H_2O crystallised at 0 °C from the rich fractions. Concentration of the mother-liquor gave a second crop which was slightly pink (overall recovery *ca.* 80%).

For the final purification of protoporphyrin-IX dimethyl ester prior to counting, *ca.* 1 mg was applied to a column of Al_2O_3 (Woelm grade III) (3 g) which had been well washed with CH_2Cl_2 and benzene (both freshly distilled). A large volume of benzene was then passed through the column before eluting the porphyrin ester in CH_2Cl_2. The sample was recrystallised several times from 1 : 2 (v/v) $CHCl_3$–MeOH (Craig tube) and dried overnight at 20 °C and 0.1 mmHg over P_2O_5.

Using these techniques, [^{14}C]PBG (74.3 μCi mmol^{-1}) gave, after incubation with the *Euglena* enzyme system, protoporphyrin-IX dimethyl ester (74.2 μCi per 0.25 mmol by weight; 72.0 μCi per 0.25 mmol by u.v. estimation). The specific activity was constant over 4 successive recrystallisations. Thus, the enzyme preparation used here contained no endogenous porphyrinogens, or their precursors, capable of yielding significant amounts of protoporphyrin-IX.

Incubations with Enzymes from Euglena gracilis.—*Euglena gracilis* was grown and harvested as described in Part 6.[4b] Frozen packed cells in 0.05M-phosphate buffer (250 ml) were thawed, then centrifuged at 17 000 G, and the supernatant was adjusted to pH 7—8 and made 0.02M in EDTA as before to provide '*E. gracilis* enzymes' (250 ml). Incubations were carried out by adding a partially neutralised solution of precursor (*ca.* pH 8) (usually obtained by alkaline hydrolysis of the corresponding lactam ester) to an aliquot (50—75 ml) of *E. gracilis* enzymes in a 250 ml Erlenmeyer flask. The flask was sealed with a loose cotton plug, wrapped in aluminium foil, and continuously swirled in a thermostatted bath at 30 °C. At the end of the incubation, porphyrins were isolated as described earlier.[4]

Preparation of Duck Blood Haemolysate.[21]—Blood (40 ml) was withdrawn from a vein beneath the duck's wing using a heparinised syringe (to prevent clotting). All subsequent operations were conducted in the cold room.

The whole blood was centrifuged and the plasma was discarded. The red cells were resuspended in isotonic saline (0.9% NaCl) to the original volume, and recentrifuged. After four such washings, red cells and the last saline wash were treated with an equal volume of distilled water and allowed to stand at 0 °C for *ca.* 3 h. To the haemolysed blood (80 ml) was added M-Tris buffer (pH 7.8) (10 ml) and a solution of KCl (686 mg) and $MgCl_2$·$6H_2O$ (42 mg) in water (4 ml), and the entire 'duck blood enzyme preparation' (including cell debris) (94 ml) was used for incubations with labelled precursors. Incubations were run at 37 °C for 17 h using essentially the same procedures as that for the *Euglena* enzyme system (see above).

Isolation of ^{13}C-*Enriched Protoporphyrin-IX from Experiments with* Euglena *Enzymes.*—Experiment No. 7, Table 1, is described as an example. Pyrromethane lactam ester corresponding to (5b) (84 mg) was stirred at 20 °C in 2M-aqueous potassium hydroxide (2 ml) under N_2 for 40 h. The solution was adjusted to pH 7.6 by carefully adding 1M-hydrochloric acid, and then added to a standard *Euglena* enzyme preparation (2.6 l). After the two had been thoroughly mixed, a solution of [^{14}C]PBG (21 mg; 17.7 μCi mmol^{-1}) (pH 7.6) was added, and the solution was divided among 18 × 250 ml Erlenmeyer flasks which were shaken at 30 °C for 15 min. A further equal quantity of [^{14}C]PBG was then added (divided equally among the flasks) and shaking was continued for 20 min, when 90% of the PBG had been consumed (Ehrlich test). The incubates were then combined, worked up as usual for porphyrins, to afford protoporphyrin-IX dimethyl ester (3.0 mg; 57.8 μCi mmol^{-1}). This figure is 18% less than that expected for incorporation of only the PBG.

Isolation of Haemin and Protoporphyrin-IX from Experiments with Duck Blood Enzymes.—The incubation solution (*ca.* 50 ml; equivalent to 20 ml whole blood) was added dropwise to hot glacial acetic acid (60 ml), containing saturated brine (0.4 ml), on a steam-bath. Concentrated HCl (0.1 ml) was added and heating was continued for 1 h. On cooling, crystalline haemin separated, was collected by centrifugation and was washed with two successive portions of 1 : 1 (v/v) aqueous HOAc, then with ethanol and finally ether.

To the supernatant was added a solution of protoporphyrin-IX (0.2—0.3 mg) in glacial acetic acid (0.5 ml)

containing a few drops of concentrated HCl. Protein, precipitated by adding EtOAc (180 ml), was collected by centrifugation and washed with 3 : 1 (v/v) EtOAc–HOAc until the washings were colourless. The combined supernatant from the centrifugation and washings was extracted with saturated aqueous NaOAc (2 × 200 ml) and 3% aqueous NaOAc (40 ml) and finally with 5M-HCl until the extracts no longer showed an absorption at 400 nm (Soret band). The porphyrins in the acidic extracts were extracted into ether after neutralisation in the usual way [4b] (solid K_2CO_3), and were esterified in 7% BF_3–MeOH (5 ml) at 20 °C for 16 h. Radioinactive protoporphyrin-IX dimethyl ester (5 mg) was added to this solution as diluent, and the total ester was then isolated as earlier [4b] for purification by chromatography on Al_2O_3 and recrystallisation to constant specific radioactivity.

Conversion of Haemin into Protoporphyrin-IX Dimethyl Ester.[22]—The haemin (30—50 mg) without further purification from the foregoing isolation was dissolved in pyridine (0.2—1.0 ml) and the filtered solution was diluted with chloroform (5.0 ml) and methanol (5.0 ml). Anhydrous iron(II) sulphate [obtained by carefully heating the hydrate (3.0 g) in a stream of dry HCl with a Bunsen flame to drive off the water and cooling the residue in a desiccator] was added and dry HCl gas was passed through the solution, excessive heating being avoided by use of a 20 °C water-bath. When the colour changed from red-brown to green-violet (501, 624 nm ⟶ 560, 601 nm), the solution was shaken with chloroform and the organic layer was quickly washed (water, dilute aqueous NH_3, and water), dried, and evaporated to give protoporphyrin-IX dimethyl ester, which was recrystallised ($CHCl_3$–MeOH) to constant activity.

Further proof that this product was radiochemically pure was obtained by conversion into the corresponding tetrahydro-derivative (mesoporphyrin-IX dimethyl ester) as follows.

Protoporphyrin-IX dimethyl ester (10 mg) in formic acid (10 ml) was hydrogenated over 10% palladium–charcoal (20 mg) for 1.5 h. The catalyst was removed by filtration and washed thoroughly with chloroform. The filtrate was neutralised by adding solid $NaHCO_3$ and the chloroform solution was evaporated to give crude mesoporphyrin-IX dimethyl ester.

After preparative t.l.c. on silica with chloroform, the product was recrystallised ($CHCl_3$–MeOH) and dried *in vacuo* over P_2O_5 before counting.

The relative molar specific activity was within experimental error (±3%) the same as that for the original protoporphyrin-IX dimethyl ester.

Assay of Radioactive Porphyrins and Biliverdins by Scintillation Counting.—(a) *Using* Na–Hg *Reduction.* The radioactive porphyrin or biliverdin ester (see later) (*ca.* 1.0 mg) was accurately weighed into a counting-vial and suspended in 1 : 1 (v/v) methanol–toluene (2 ml). Sodium amalgam (2.5%; *ca.* 100 mg) was added and the vial, wrapped in aluminium foil, was sealed with a rubber septum, degassed, and filled with N_2. Gentle swirling of the contents completely discharged the colour (*ca.* 2 h). Deoxygenated organic scintillator solution (10 ml) was added *via* a hypodermic syringe and the vial was immediately transferred to the refrigerated chamber of the scintillation counter.

Solutions prepared in this manner reoxidise slowly to porphyrin, and so the changing counting efficiency was therefore determined by the channel ratio method.

(b) *Direct counting.* When the specific activity of the radioactive porphyrin was sufficiently high, an accurately weighed specimen (*ca.* 5 μg) was counted directly with acceptable counting efficiency.

(c) *Using free-radical oxidation.* Accurately weighed porphyrin ester (*ca.* 0.1 mg) and dibenzoyl peroxide (4 mg) were dissolved in tetrahydrofuran (0.5 ml) and the solution was diluted with organic scintillator (6.5 ml). The solution was allowed to stand in bright light until the colour was that of pale straw (*ca.* 6—16 h) before counting was started.

Degradation of Labelled Protoporphyrin-IX Dimethyl Ester.—(a) *Formation of haemin.*[23] A suspension of protoporphyrin-IX dimethyl ester (100 mg) in methanol (20 ml) was heated at reflux under N_2 for 24 h with KOH (300 mg) in water (10 ml). The pH was adjusted to 3—4 by adding 2M-HCl and the precipitated protoporphyrin-IX was collected by centrifugation, dried, and then dissolved in pyridine (50 ml). Glacial acetic acid (50 ml) containing NaCl (*ca.* 10 mg) was added and, after flushing with N_2, the solution was heated to 90 °C (bath) and finely-ground $FeSO_4 \cdot 7H_2O$ (1 g) was added. After 5 min a second portion (0.5 g) was added and the suspension was stirred at 90 °C for 30 min. The flask was opened to the atmosphere, allowed to cool during 30 min, and the solvents were partially evaporated (to *ca.* 10 ml). Pyridine (5 ml) was added, followed by ethyl acetate (200 ml) and 1M-HCl (100 ml). The organic layer was washed sequentially with 1M-HCl (3 × 100 ml), 12% HCl (2 × 100 ml) and water (2 × 50 ml), filtered, and evaporated to dryness to yield labelled haemin ready for degradation under (b).

(b) *Oxidation to biliverdins.* Haemin (300 mg) was dissolved in pyridine (AnalaR 100 ml) at 100 °C and the solution was filtered to remove insoluble matter (29 mg). Distilled water (300 ml) was added and the solution was stirred whilst a stream of oxygen was passed through a sinter (2.2 cm) into the solution at 38—40 °C. After 30 min, a solution of ascorbic acid (3.6 g) in water (15 ml) was added and passage of oxygen and stirring (at 38—40 °C) was continued for 20 min. The solution was then extracted with chloroform (1 × 100 ml, 3 × 25 ml) and the combined extracts were washed with water (100 ml), filtered, and evaporated to a moist residue of verdohaemochrome.

The above procedure was repeated and the combined products in chloroform were divided between two 50 ml centrifuge tubes, the volume in each was reduced to 3—4 ml and then light petroleum, b.p. 60—80 °C (*ca.* 40 ml) was added to each. After thorough mixing, the tubes were centrifuged, the supernatant was pipetted off, and the precipitate was washed with light petroleum by centrifugation and then dried.

This verdohaemochrome was dissolved in MeOH (25 ml) containing 2M-KOH–MeOH (2 ml) and after *ca.* 1 min was treated with 14% BF_3–MeOH (25 ml) under dry nitrogen. After the solution had been heated under reflux for 15 min, it was kept at 20 °C for 16 h. It was then diluted with chloroform (100 ml) and shaken with aqueous $NaHCO_3$ (2 × 20 ml) and then with brine (3 × 20 ml). The combined aqueous extracts were extracted with $CHCl_3$ (2 × 20 ml), and these organic extracts were washed with water (100 ml) and then added to the main chloroform solution which was filtered and evaporated. The residue in chloroform (10 ml) was added to a pad of Kieselgel GF_{254} (3.5 cm diameter, 2.5 cm thickness) which was saturated with chloroform and held in a sintered glass funnel. The pigments were washed into the Kieselgel with small portions

of chloroform. Without delay, the biliverdin esters were eluted from the Kieselgel with 20% acetone in $CHCl_3$ (50 ml) and the eluant was evaporated to give 172 mg of mixed isomers.

(c) *Separation and dilution of biliverdins.*[13] A column (7.5 cm i.d.) was prepared from Kieselgel GF_{254} (150 g) slurried in benzene (400—500 ml). Once the adsorbent had settled to allow a drip-rate of *ca.* 2 drops s^{-1}, pressure (1—3 lbf in^{-2}) from a N_2 cylinder was applied. Ether–benzene (1 : 9 v/v) was then passed through the column until (*a*) the column was saturated with the new eluant, and (*b*) the surface was sufficiently firm not to be disturbed when the column was gently moved. Since the chromatography took 8—10 h, the column was prepared the previous day.

The foregoing biliverdin esters (172 mg) in benzene (2—4 ml) were added carefully and evenly to the column which was then overlayered with acid-washed sand (*ca.* 1 cm depth). The column was protected from light and eluted with ether–benzene mixtures [10% (1 l); 15% (1 l); 20% (1 l); 25% (1 l); 100% ether (750 ml)], and fractions (250 ml) were collected as soon as the biliverdin colour appeared in the eluate. The early fractions contained the β-isomer of sufficient purity for direct crystallisation by rapidly cooling to −15 °C a solution in chloroform (1—2 ml), methanol (3—4 drops), and light petroleum (b.p. 60—80 °C) (25—30 ml). The late fraction containing mainly the δ-isomer was rechromatographed on 14 preparative t.l.c. plates (each Kieselgel GF_{254}, 10 cm^2) eluting 3 times with 2% (v/v) acetone–chloroform. The middle fractions were rechromatographed on 28 preparative t.l.c. plates eluting 5 times with 2% acetone–chloroform. Recrystallisation of each isomer as above provided the β-isomer (15.3 mg, 3%), m.p. >300 °C, α-isomer (16 mg, 3.1%); γ-isomer (5.5 mg, 1.1%), and δ-isomer (15.3 mg, 3%).

The radioactive haemin was degraded as above and the separated esters were recrystallised. They were then diluted with the appropriate pure unlabelled biliverdin esters obtained above, the dilution factor (usually in the range 2—6) was accurately determined by dissolving the labelled and unlabelled samples in a standard volume of chloroform and measuring absorption at the long-wavelength band (*ca.* 650 nm). The recovered diluted samples were then recrystallised to constant specific activity. In a typical example (experiment 15, Table 1) the final specific activities in dpm mg^{-1} were: α-isomer (857 ± 4), β-isomer (1 366 ± 5), γ-isomer (472 ± 5), δ-isomer (1 861 ± 12). The corresponding dilution factors were 2.69, 3.54, 5.00, 2.29.

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