Pyrroles and Related Compounds. Part XXXII.[1] Biosynthesis of Protoporphyrin-IX from Coproporphyrinogen-III [2]

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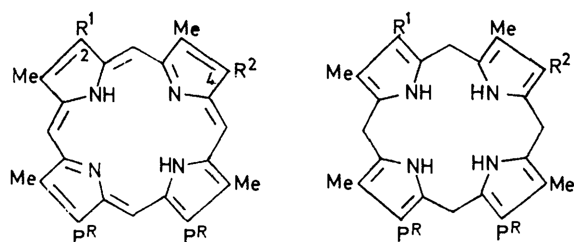
New syntheses of 4,6,7-tris-(2-methoxycarbonylethyl)-1,3,5,8-tetramethyl-2-vinylporphyrin (2c) and its 4-vinyl isomer (2d), from pyrromethanes, are reported. These porphyrins and coproporphyrin-III tetramethyl ester (2b) were labelled at the *meso*-positions with tritium, and incubated in the form of the porphyrinogen carboxylic acids, with a cell-free system from *Euglena gracilis*. Coproporphyrinogen-III (3b), the standard for the system, was incorporated into protoporphyrin-IX (1a) to the extent of 2·0%, whereas the 2- and 4-vinylporphyrinogens gave incorporations of 3·0 and 0·5%, respectively. These results show conclusively that, in the biosynthesis of protoporphyrin-IX from coproporphyrinogen-III in *Euglena gracilis*, the 2-propionic acid group is converted into vinyl before the 4-substituent is modified. Further confirmation was obtained by detection of radiochemically labelled 2-vinylporphyrinogen (3c) [by isolation of the corresponding porphyrin (1c) using dilution analysis] as a product of the coproporphyrinogen-III feeding; the isomeric identity of the porphyrin (1c) was established using high-pressure liquid chromatography.

BIOSYNTHESIS of protoporphyrin-IX (1a) from coproporphyrinogen-III (3b) involves conversion of the 2- and 4-propionic acid functions into vinyl substituents, followed by oxidation of the product (3a) to the



$P^R = CH_2{\cdot}CH_2{\cdot}CO_2R$
$V = CH{:}CH_2$

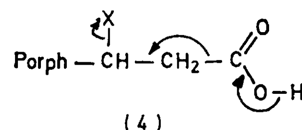
(1) R=H
(2) R=Me
(3) R=H

a; $R^1 = R^2 = V$
b; $R^1 = R^2 = P^R$
c; $R^1 = V$, $R^2 = P^R$
d; $R^1 = P^R$, $R^2 = V$
e: $R^1 = R^2 = CH(OH){\cdot}CH_2{\cdot}CO_2H$
f: $R^1 = H$, $R^2 = P^R$
g: $R^1 = H$, $R^2 = V$

porphyrin state. A great deal is already known about the mechanism of formation of the vinyl groups, which proceeds in each propionic acid group in a stereospecific fashion with loss of only one hydrogen atom;[3] furthermore, this has been identified[3a] as the *pro-S* hydrogen atom. The conclusion is that the process follows a mechanism of the type shown in (4), where X is H, OH, or a good leaving group. Oxygen is the only oxidant used by the enzyme concerned, named coproporphyrinogenase, but the possibility that X is H cannot be excluded on account of the observation of anaerobic coproporphyrinogenase activity in extracts of *R. spheroides* and *Chromatium*.[4]

In the case (4; X = OH), the interpretation is consistent with earlier proposals of Sano[5] involving hydroxylation followed by decarboxylative dehydration; the intermediacy of the porphyrinogen (3e) was demonstrated, suggesting that both propionic acids are converted into hydroxypropionate before any vinyl group is formed. In contradistinction, other work[6] has implicated the intermediacy of species containing one vinyl and three propionic acid groups in the biosynthetic transformation. These apparently conflicting results could be interpreted in terms of non-specificity of the coproporphyrinogenase system; for example, it has been shown[7] that the porphyrinogen (3f) is converted into the vinyldeuteroporphyrin (1g) by a beef-liver mitochondrial extract.

Following our identification[8] of the monovinyl tripropionic porphyrin found in the Harderian gland of the rat as the 2-vinyl isomer (1c) (harderoporphyrin), and because of the co-occurrence of this porphyrin in the gland with coproporphyrin-III (1b) and protoporphyrin-IX (1a), it was postulated[8] that the 2-propionic acid group in (3b) might be transformed into vinyl before



(4)

that at position 4 in the last stages of the biosynthesis of protoporphyrin-IX. We now describe experimental results which confirm this hypothesis.

For this work we required fairly large samples † of the

† A biosynthetic investigation of this type requires not only the relatively insignificant quantity of feeding material, but also substantial amounts to enable dilution analysis to be performed.

[1] Part XXXI, R. V. H. Jones, G. W. Kenner, and K. M. Smith, *J.C.S. Perkin I*, 1974, 531.

[2] Preliminary publication, J. A. S. Cavaleiro, G. W. Kenner, and K. M. Smith, *J.C.S. Chem. Comm.*, 1973, 183.

[3] (*a*) Z. Zaman, M. M. Abboud, and M. Akhtar, *J.C.S. Chem. Comm.*, 1972, 1263; (*b*) A. R. Battersby, J. Baldas, J. Collins, D. H. Grayson, K. J. James, and E. McDonald, *J.C.S. Chem. Comm.*, 1972, 1265; A. R. Battersby, in XXIIIrd Internat. Congress of Pure and Appl. Chem., vol. 5, Butterworths, London, 1971, p. 1.

[4] G. H. Tait, *Biochem. J.*, 1972, **128**, 1159.

[5] S. Sano, *J. Biol. Chem.*, 1966, **241**, 5276.

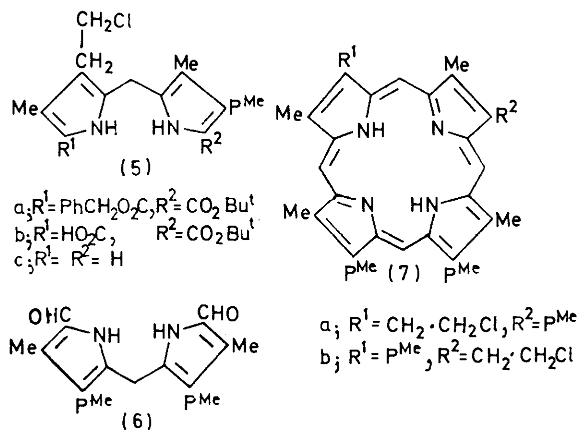
[6] S. Granick and R. D. Levere, *Progr. Hemat.*, 1964, **4**, 1; S. Sano and S. Granick, *J. Biol. Chem.*, 1961, **236**, 1173.

[7] R. J. Porra and J. E. Falk, *Biochem. J.*, 1964, **90**, 69.

[8] G. Y. Kennedy, A. H. Jackson, G. W. Kenner, and C. J. Suckling, *F.E.B.S. Letters*, 1970, **6**, 9; 1970, **7**, 205. Publication of the full paper relating to this work awaits reisolation of the natural material and fresh comparison with higher melting[2] synthetic samples (G. Y. Kennedy, A. H. Jackson, G. W. Kenner, and K. M. Smith, work in progress).

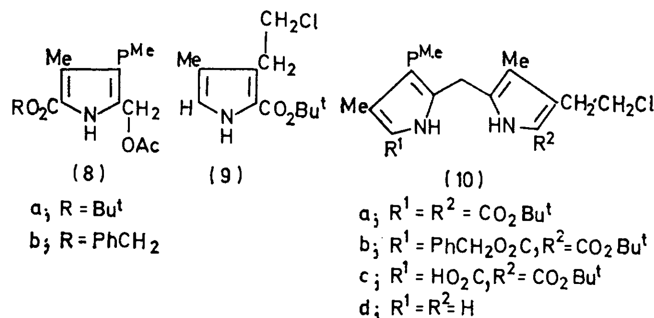
tripropionic porphyrins (1c) and (1d), obtained by a synthesis which would allow, at some stage, tritium labelling of the material. Our earlier syntheses,[8] *via* the *b*-oxobilane route, had this capability at the oxophlorin stage,[9] but the route itself is lengthy, involving several steps after labelling; we therefore chose our recent modification[10] of the MacDonald approach[11] from pyrromethanes. One of the principal factors was our observation[10] that labels (specifically deuterium) could be incorporated into the *meso*-positions during cyclisation, owing to a property of the intermediate phlorin dication. Preliminary experiments showed that tritium could be efficiently incorporated, but in the event we were able to perform the tritiation on the vinylporphyrins themselves, using hexapyridylmagnesium di-iodide and tritiated water in pyridine.[12]

The pyrromethane (5a)[13] was hydrogenated over palladised charcoal and afforded the 5-carboxylic acid (5b), which, without isolation, was treated with cold trifluoroacetic acid, giving the 5,5′-di-unsubstituted pyrromethane (5c) after an alkaline work-up. Treatment with the known[14] 5,5′-diformylpyrromethane (6) in methylene chloride containing toluene-*p*-sulphonic acid hydrate in methanol gave a 35% yield of the porphyrin (7a) after re-esterification with 5% sulphuric acid in methanol. In this reaction, a slight excess of (5c) was used in order to avoid formation of coproporphyrin-II tetramethyl ester by self-condensation of



the dialdehyde (6); such symmetrical by-products are obtained in significant yield if the alkaline work-up of the 5,5′-di-unsubstituted pyrromethane (after the trifluoroacetic acid treatment) is omitted. The zinc chelate of (7a) gave the 2-vinylporphyrin (2c) after treatment with t-butoxide in t-butyl alcohol and demetallation–esterification in sulphuric acid–methanol.

In the synthesis of the 4-vinyl isomer (2d), the pyrromethane (10a) was obtained in 82% yield by condensation of the acetoxymethylpyrrole (8a) with the 2-unsubstituted pyrrole (9) in methanol containing a catalytic

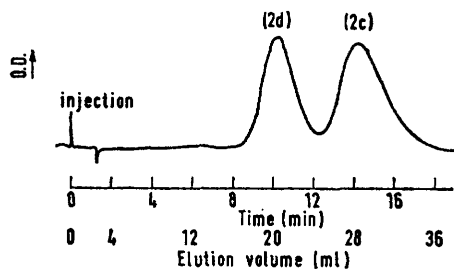


quantity of toluene-*p*-sulphonic acid; this pyrromethane, like many other t-butyl pyrromethanecarboxylates was not crystallised. The pyrromethane (10b) [as isomer of the crystalline compound (5a)] was therefore prepared in a similar way from the pyrroles (8b) and (9). However, this compound, obtained in 85% yield, was also impossible to crystallise. The required 5,5′-di-unsubstituted pyrromethane (10d) was obtained directly from (10a) by trifluoroacetic acid treatment [in preference to the alternative route from (10b) *via* (10c)] and, when cyclised with (6) in the usual way, gave a 30% yield of the 4-(2-chloroethyl)porphyrin (7b); this was further transformed into the 4-vinylporphyrin (2d) as described for the isomer (2c).

The isomeric monovinylporphyrins were identical, by mixed m.p., with new samples obtained[15] by the *b*-oxobilane route. The mass spectra of the isomers (2c) and (2d) showed minor variations in relative intensities of some fragment ions, but as we have found[16] with other type-isomer comparisons, these were not sufficient to allow unambiguous identification under standardised operating conditions. The n.m.r. spectra at similar concentrations ($<0{\cdot}1$M) in deuteriochloroform showed significant differences in both the *meso*-proton and β-methyl resonances, but these did not permit independent structural assignments. No separation was apparent in paper (carboxylic acids; lutidine–ammonia method) or thin-layer chromatography of the isomers, in a variety of solvent systems. Neither were we able to repeat the partial separation by counter current distribution (c.c.d.) that had been earlier observed,[8] and in view of the apparently significant separation after a small number of exchanges (bearing in mind the close K values to be expected for the isomers) we suspect that the anomaly was due to traces of impurities in the original synthetic samples. Some separation by using c.c.d. was, however, apparent from experiments using

[9] A. H. Jackson, G. W. Kenner, and K. M. Smith, *J. Chem. Soc. (C)*, 1968, 302.
[10] A. M. d'A. Rocha Gonsalves, G. W. Kenner, and K. M. Smith, *Chem. Comm.*, 1971, 1304.
[11] G. P. Arsenault, E. Bullock, and S. F. MacDonald, *J. Amer. Chem. Soc.*, 1960, **82**, 4384.
[12] G. W. Kenner, K. M. Smith, and M. J. Sutton, *Tetrahedron Letters*, 1973, 1303.
[13] J. A. S. Cavaleiro, A. M. d'A. Rocha Gonsalves, G. W. Kenner, and K. M. Smith, *J.C.S. Perkin I*, 1973, 2471.
[14] R. Chong, P. S. Clezy, A. J. Liepa, and A. W. Nichol, *Austral. J. Chem.*, 1969, **22**, 229.
[15] A. H. Jackson, G. W. Kenner, and K. M. Smith, unpublished results.
[16] A. H. Jackson, G. W. Kenner, K. M. Smith, R. T. Aplin, H. Budzikiewicz, and C. Djerassi, *Tetrahedron*, 1965, **21**, 2913.

tritium labelled samples; in the system aqueous 0·5M-sulphuric acid (lower phase) and methyl isobutyl ketone–t-butyl alcohol (1 : 1) (upper), a mixture of (1c) [2·120 mg inactive and 0·125 active (specific activity $4·8 \times 10^6$ disint. mg^{-1} s^{-1})] and (1d) (1·5 mg inactive) was subjected to 761 transfers in a 96-tube machine, and gave an apparently symmetrical distribution, with the maximum at about tube 325 (*K ca.* 0·75). The specific activities of the porphyrin found in the wings of the distribution were $7·2 \times 10^4$ and $1·1 \times 10^5$ disint. mg^{-1} s^{-1} for the left and right sides, respectively. (Lack of separation would have given equal specific activities.) When the complementary experiment was carried out with labelled (1d)

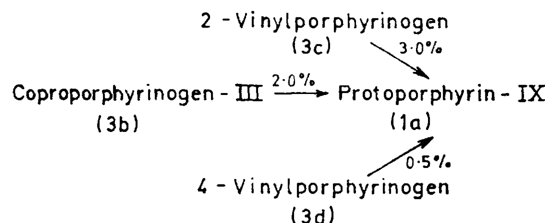


High-pressure liquid chromatographic separation of harderoporphyrin trimethyl ester (2c) and its isomer (2d)

[2·0 mg inactive plus 0·125 mg active (specific activity $3·79 \times 10^6$ disint. mg^{-1} s^{-1})] and inactive (1c) (1·5 mg), after 840 transfers, the specific activities found in the wings of the distribution were $1·1 \times 10^5$ and $6·4 \times 10^4$ disint. mg^{-1} s^{-1} (left and right sides, respectively). These results in conjunction are consistent with a small separation between (1c) and (1d), with the 2-vinylporphyrin having the larger distribution coefficient.* However, almost complete separation of the two isomers was achieved (Figure) using high-pressure liquid chromatography, on Corasil II (4 ft × 1/8 in o.d. column) in solvent mixtures containing chloroform (stabilised with 0·2% alcohol) and cyclohexane.

Coproporphyrin-III tetramethyl ester (2b) was required as a standard in this investigation and this was synthesised as previously reported [18] by a route similar to that used for the synthesis of compounds (2c and d). These three feeding materials were tritiated using hexapyridylmagnesium di-iodide and tritiated water in pyridine (*cf.* ref. 12). The porphyrinogen carboxylic acids (3b—d) necessary for the feeding experiments were obtained by hydrolysis of the labelled porphyrin esters (potassium hydroxide in water–tetrahydrofuran), followed by reduction with sodium amalgam. The hexahydro-derivatives were immediately incubated during 17 h with a divided batch of an enzyme system † from *Euglena gracilis*.[20]

The feeding products were worked up separately,‡ the 2- and 4-vinylporphyrinogen feeding products being diluted with protoporphyrin-IX and the appropriate vinylporphyrin that had been fed; the coproporphyrinogen-III feeding product was diluted with protoporphyrin-IX, the 2-vinylporphyrin (2c), and coproporphyrin-III. Preliminary separations of the esterified fractions were carried out with column chromatography on alumina, and by controlled elution it was possible to separate mixtures of coproporphyrin-III and appropriate monovinylporphyrin and protoporphyrin-IX esters. The protoporphyrin-IX fractions from each feeding were then crystallised to constant activity, and this constancy was confirmed by c.c.d. [aqueous 1·25M-sulphuric acid (lower) and methyl isobutyl ketone–t-butyl alcohol (1 : 1) (upper)] of a mixture of active protoporphyrin-IX and a monovinylporphyrin; a clear separation of these two porphyrins was obtained and the specific activity of the protoporphyrin-IX before and after the distribution was virtually unchanged, indicating no contamination of the product with feeding material.



SCHEME §

§ The overall recovery of administered radioactivity was low (*ca.* 5%); this is apparently a common disadvantage of the *Euglena* system, presumably due to difficulty in extracting porphyrins from the thick enzyme slime, and for this reason our preliminary report [2] gave figures [(3b), 27%; (3c), 33%; and (3d), 4·5%] which were calculated as *specific* incorporations. We regret that this was not made clear in the communication, but, by using the same data, we now report incorporations as more meaningful *real* incorporations (Tables 1—3). The figures have been corrected for the experimentally determined loss of 4% tritium from the *meso*-positions during the reduction–re-oxidation process, which is subject to a favourable primary kinetic isotope effect (Experimental section). Recent unpublished results [A. R. Battersby (Cambridge) and A. H. Jackson (Cardiff), personal communications] suggest that the aromatisation process is enzymic in nature and therefore a 50% loss of label would be expected; our incorporations might therefore be liable to significant upward revision in due course.

The results obtained from the protoporphyrin-IX fractions are summarised in the Scheme, and they

* Assuming a separation of 15 tubes between the two maxima in the distribution (at tube 316, $K = 0·71$; at tube 331, $K = 0·77$) it can be calculated that 2422 transfers would be necessary [17] for a separation of 48 tubes between the maxima in the 96-tube machine used.

† Kindly made available by Professor A. R. Battersby. The successful incorporation of labelled coproporphyrinogen-III into protoporphyrin-IX using this system has already been described.[19]

‡ An apparently significant observation was that the incubation mixture from the 4-vinyl feeding was the only one with porphyrinic colour prior to the photochemical oxidation step (Experimental section). This seems to suggest that this porphyrinogen was not accepted by the enzyme system, being left to oxidise slowly to the porphyrin level during the aerobic incubation.

[17] J. R. Weisiger, in ' Organic Analysis,' vol. II, eds. J. Mitchell, I. M. Kolthoff, E. S. Proskauer, and A. Weissberger, Interscience, New York, 1954, p. 277.
[18] R. J. Abraham, G. H. Barnett, E. S. Bretschneider, and K. M. Smith, *Tetrahedron*, 1973, **29**, 553.
[19] A. R. Battersby, J. Staunton, and R. H. Wightman, *J.C.S. Chem. Comm.*, 1972, 1118.
[20] E. F. Carell and J. S. Kahn, *Arch. Biochem. Biophys.*, 1964, **108**, 1.

conclusively confirm the postulate that the biosynthesis of protoporphyrin-IX from coproporphyrinogen-III proceeds by modification of the 2-propionic acid substituent before that at position 4.

A significant amount of radioactivity was found in the 2-vinylporphyrin used in the dilution analysis of the coproporphyrinogen-III feeding product. This fraction was further diluted with a little of the 4-vinyl isomer (3d) and the mixture of trimethyl esters was subjected to high-pressure liquid chromatographic separation (*cf.* Figure). All radioactivity was shown, in this way, to be localised in the 2-vinyl fraction, adding confirmatory weight to the biosynthetic sequence outlined above.

This work appears to eliminate the possibility that the biosynthetic sequence involves the bis-hydroxylated compound (3e), but, in view of the non-specificity of the coproporphyrinogenase system under certain conditions, final solution of these problems requires the synthesis and feeding of the appropriate mono- and bis-hydroxypropionate porphyrins to similar enzyme preparations.

EXPERIMENTAL

M.p.s were measured on a Kofler hot-stage apparatus. Neutral alumina (Merck; Brockmann Grade III) was used for all chromatographic separations, and reactions were followed by t.l.c. and by spectrophotometry, as described in earlier parts of this series. Electronic absorption spectra were determined (solutions in methylene chloride) with a Unicam SP 800 spectrophotometer, ^1H n.m.r. spectra (solutions in methylene chloride with tetramethylsilane as internal standard) with a Varian HA-100 instrument, and mass spectra with either an A.E.I. MS902 or MS12 spectrometer (at 50 μA and 70 eV; direct inlet with source temperature 200—230°).

High-pressure liquid chromatography was carried out with a Waters Associates ALC 202—401 instrument (6000 pumping system), with a 254 nm u.v. detector. Samples were introduced with a syringe onto two 2 ft × 1/8 in (o.d.) columns containing Corasil-II (Waters Assoc.) and elution was carried out with chloroform (containing 0·2% alcohol)–cyclohexane mixtures.

Pyrromethanes

Di-t-butyl 4'-(2-Chloroethyl)-3-(2-methoxycarbonylethyl)-3',4-dimethylpyrromethane-5,5'-dicarboxylate (10a).—t-Butyl 2-acetoxymethyl-3-(2-methoxycarbonylethyl)-4-methylpyrrole-5-carboxylate [21] (0·339 g) and t-butyl 4-(2-chloroethyl)-3-methylpyrrole-5-carboxylate [13] (0·249 g) in distilled methanol (5 ml) were treated with toluene-*p*-sulphonic acid hydrate (10 mg) and heated with stirring at 40° during 7 h. Methylene chloride (50 ml) and water (50 ml) were then added and the organic phase was washed with aqueous sodium hydrogen carbonate and then water. The methylene chloride layer was dried (Na_2SO_4) and evaporated to dryness to give an oil, which was chromatographed on alumina; elution was carried out with light petroleum (b.p. 60—80°) containing increasing proportions of benzene and then with pure benzene to give the *pyrromethane* as an oil which could not be induced to crystallise (0·430 g, 82%), τ 0·90br and 1·43br (each 1H, 2NH), 6·10 (2H, s, CH_2), 6·35 (3H, s, OMe), 6·90 (2H, t, CH_2Cl), 7·2—7·7 (6H, m, $CH_2{\cdot}CH_2{\cdot}CO$ and $CH_2{\cdot}CH_2Cl$), 7·78 and 7·98 (each 3H, s, 2 Me), and 8·46 (18H, s, 2 Bu^t).

Benzyl 4'-(2-Chloroethyl)-3-(2-methoxycarbonylethyl)-3',4-dimethyl-5'-t-butoxycarbonylpyrromethane-5-carboxylate (10b).—Similarly, benzyl 2-acetoxymethyl-3-(2-methoxycarbonylethyl)-4-methylpyrrole-5-carboxylate [22] (0·373 g) and t-butyl 4-(2-chloroethyl)-3-methylpyrrole-5-carboxylate [13] (0·245 g) in methanol (5 ml) were treated with toluene-*p*-sulphonic acid hydrate (11 mg), and afforded the *pyrromethane* (0·473 g, 85%) as a pale oil which could not be crystallised, τ 0·88br and 1·17br (each 1H, 2 NH), 2·70 and 4·78 (5H, s and 2H, s, $PhCH_2$), 6·15 (2H, s, CH_2), 6·38 (3H, s, OMe), 6·90 (2H, t, CH_2Cl), 7·2—7·7 (6H, m, $CH_2{\cdot}CH_2{\cdot}CO$ and $CH_2{\cdot}CH_2Cl$), 7·75 and 8·0 (each 3H, s, 2 Me), and 8·48 (9H, s, Bu^t).

Porphyrins

2-(2-Chloroethyl)-4,6,7-tris-(2-methoxycarbonylethyl)-1,3,5,8-tetramethylporphin (7a).—Benzyl 3-(2-chloroethyl)-4'-(2-methoxycarbonylethyl)-3',4-dimethyl-5'-t-butoxycarbonylpyrromethane-5-carboxylate [13] (0·699 g) in tetrahydrofuran (100 ml) containing triethylamine (2 drops) and palladised charcoal (70 mg) was hydrogenated at room temperature and atmospheric pressure until uptake had ceased (30 min). The catalyst was filtered off on Celite, which was washed thoroughly with methylene chloride and then tetrahydrofuran. The combined filtrates were evaporated to dryness and the residue was kept at 0·1 mmHg during 30 min before being treated with trifluoroacetic acid (10 ml) under a stream of nitrogen during 40 min. The solvent was evaporated off and the residual oil was taken into methylene chloride and washed successively with water, aqueous sodium hydrogen carbonate, and finally water. The organic phase was dried (Na_2SO_4) and the solvent was partially removed. To the solution was added 3,3'-bis-(2-methoxycarbonylethyl)-4,4'-dimethylpyrromethane-5,5'-dicarbaldehyde [14] (0·402 g) and the volume was made up to 400 ml with methylene chloride in a darkened flask. Methanolic toluene-*p*-sulphonic acid (1·440 g in 24 ml) was then added and after stirring for 6 h in the dark the solution was treated with saturated methanolic zinc acetate (24 ml); it was then set aside overnight. The mixture was washed with water, aqueous sodium hydrogen carbonate, and then water again, dried (Na_2SO_4), and evaporated to dryness. The residue was treated with 5% v/v sulphuric acid in methanol (100 ml) overnight in the dark and then poured into methylene chloride (200 ml) and water (500 ml). The organic phase was successively washed with aqueous sodium acetate, aqueous sodium hydrogen carbonate, and water, dried (Na_2SO_4), and evaporated to dryness. The residue was chromatographed on alumina (elution with methylene chloride); evaporation of the porphyrinic eluates gave a residue, which crystallised from methylene chloride–n-hexane to give the *chloroethylporphyrin* (240 mg, 35%), m.p. 173—174°. Admixture with a sample prepared [8] by the *b*-oxobilane route caused no depression of the m.p. (Found: C, 66·4; H, 6·3; N, 8·4. $C_{38}H_{43}ClN_4O_6$ requires C, 66·4; H, 6·3; N, 8·15%), τ (*ca.* 0·06M) 0·01, 0·04, 0·15, and 0·29 (each 1H, s, 4 *meso*-H), 5·5—5·9 (10H, m, $CH_2{\cdot}CH_2Cl$ and 3 $CH_2{\cdot}CH_2{\cdot}CO$), 6·31 and 6·33 (6H, s and 3H, s, 3 OMe), 6·43, 6·51, and 6·61 (3H, s, 6H, s, and 3H, s, 4 Me), 6·6—6·9 (6H, m, 3 $CH_2{\cdot}CH_2{\cdot}CO$), and 14·1br (2H, 2 NH), $\lambda_{max.}$ 400 (log ε 5·23), 500 (4·15), 532 (3·98), 568 (3·83), and 621 nm (3·54), *m/e* 686 (100%), 652 (17), and 613 (23).

[21] C. J. Suckling, Ph.D. Thesis, Liverpool, 1970.
[22] A. W. Johnson, I. T. Kay, E. Markham, R. Price, and K. B. Shaw, *J. Chem. Soc.*, 1959, 3416.

4-(2-*Chloroethyl*)-2,6,7-*tris*-(2-*methoxycarbonylethyl*)-1,3,5,8-*tetramethylporphin* (7b).—Di-t-butyl 4′-(2-chloroethyl)-3-(2-methoxycarbonylethyl)-3′-4-dimethylpyrromethane-5,5′-dicarboxylate (0·400 g) in trifluoroacetic acid (10 ml) was set aside during 40 min under a stream of dry nitrogen. The solution was then evaporated to dryness and the residual oil was dissolved in methylene chloride and washed successivly with water, sodium hydrogen carbonate solution, and water. The organic phase was dried (Na_2SO_4) and the solvent partially removed before addition of 3,3′-bis-(2-methoxycarbonylethyl)-4,4′-dimethylpyrromethane-5,5′-dicarbaldehyde [14] (0·236 g); the solution was then made up to 235 ml with methylene chloride. Toluene-*p*-sulphonic acid hydrate (0·960 g) in methanol (16 ml) was then added and after stirring for 6 h in the dark, saturated methanolic zinc acetate (16 ml) was added and the solution was set aside overnight. The mixture was then worked-up as described for the isomer, and gave the *chloroethylporphyrin* (120 mg, 30%), m.p. 190—191° (from methylene chloride–n-hexane); no depression of m.p. was observed when this material was mixed with a sample prepared by the *b*-oxobilane route [8] (Found: C, 66·6; H, 6·3; N, 8·2. $C_{38}H_{43}ClN_4O_6$ requires C, 66·4; H, 6·3; N, 8·15%), τ (*ca.* 0·04M) −0·05, 0·01, and 0·08 (1H, s, 2H, s, and 1H, s, 4 *meso*-H), 5·5—5·8 (10H, m, CH_2·CH_2Cl and 3 C*H*$_2$·CH_2·CO), 6·35 (9H, s, 3 OMe), 6·40, 6·42, and 6·44 (3H, s, 3H, s, and 6H, s, 4 Me), and 6·6—6·9 (6H, m, 3 CH_2·C*H*$_2$·CO), $\lambda_{max.}$ 400 (log ε 5·24), 500 (4·16), 532 (3·99), 569 (3·84), and 620 nm (3·59), *m*/*e* 686 (100%), 652 (23), and 613 (59), *m** 548 (686 ⟶ 613).

4,6,7-*Tris*-(2-*methoxycarbonylethyl*)-1,3,5,8-*tetramethyl*-2-*vinylporphin* (*Harderoporphyrin Trimethyl Ester*) (2c).—2-(2-Chloroethyl)-4,6,7-tris-(2-methoxycarbonylethyl)-1,3,5,8-tetramethylporphin (60 mg) in methylene chloride (25 ml) was treated with saturated methanolic zinc acetate (5 ml). After slight warming, the solution was poured into water and the zinc chelate was extracted with more methylene chloride (50 ml). The organic phase was washed with water, dried (Na_2SO_4), and evaporated to dryness; the residue was further dried under high vacuum. Tetrahydrofuran (25 ml) was added, followed by M-potassium t-butoxide in t-butyl alcohol (50 ml), and the solution was set aside, under nitrogen, in the dark, during 70 h; acetic acid (1 ml), chloroform (50 ml), pyridine (2 ml), and water (20 ml) were then added and the organic phase was washed with water, dried (Na_2SO_4), and evaporated to dryness. The residue was treated with 5% v/v sulphuric acid in methanol (80 ml) and left overnight in the dark at room temperature. The solution was diluted with methylene chloride and washed with aqueous sodium acetate solution, sodium hydrogen carbonate solution, and finally water. The organic layer was dried (Na_2SO_4) and evaporated to dryness, to give a residue which was chromatographed on alumina (elution with methylene chloride). Evaporation of the porphyrinic eluates and crystallisation of the residue from methylene chloride–n-hexane gave the *vinylporphyrin* (40 mg, 70%), m.p. 203—204°, identical with a sample prepared by the *b*-oxobilane route [8] (Found: C, 70·0; H, 6·4; N, 8·7. $C_{38}H_{42}N_4O_6$ requires C, 70·1; H, 6·5; N, 8·6%), τ (*ca.* 0·05M) −0·02, 0·02, and 0·08 (1H, s, 1H, s, and 2H, s, 4 *meso*-H), 1·7—2·1 and 3·6—4·0 (1H, m, and 2H, m, CH:CH_2), 5·6—5·9 (6H, m, 3 C*H*$_2$·CH_2·CO), 6·35 and 6·37 (6H, s, and 3H, s, 3 OMe), 6·45, 6·49, and 6·53 (6H, s, 3H, s, and 3H, s, 4 Me), 6·6—7·0 (6H, m, 3 CH_2·C*H*$_2$·CO), and 14·15br (2H, 2 NH), $\lambda_{max.}$ 405 (log ε 5·26), 507 (4·24), 540 (4·11), 574 (4·01), and 630 nm (3·73), *m*/*e* 650 (100%), 577 (41), and 504 (8), *m** 512 (650 ⟶ 577).

2,6,7-*Tris*-(2-*methoxycarbonylethyl*)-1,3,5,8-*tetramethyl*-4-*vinylporphin* (*Isoharderoporphyrin Trimethyl Ester*) (2d).—This porphyrin was prepared in an analogous manner to the foregoing isomer, from 4-(2-chloroethyl)-2,6,7-tris-(2-methoxycarbonylethyl)-1,3,5,8-tetramethylporphin (61 mg). The *vinylporphyrin* (40 mg, 70%), m.p. 216—218° (from methylene chloride–n-hexane), was identical with a sample prepared by the *b*-oxobilane method [8] (Found: C, 70·0; H, 6·4; N, 8·9. $C_{38}H_{42}N_4O_6$ requires C, 70·1; H, 6·5; N, 8·6%), τ (*ca.* 0·05M) −0·19, 0·05, and 0·11 (1H, s, 1H, s, and 2H, s, 4 *meso*-H), 1·6—2·0 and 3·6—4·0 (1H, m, and 2H, m, CH:CH_2), 5·6—6·0 (6H, m, 3 C*H*$_2$·CH_2·CO), 6·35 (9H, s, 3 OMe), 6·39, 6·47, and 6·51 (3H, s, 6H, s, and 3H, s, 4 Me), 6·6—7·0 (6H, m, 3 CH_2·C*H*$_2$·CO), and 14·04br (2H, 2 NH), $\lambda_{max.}$ 405 (log ε 5·27), 505 (4·18), 539 (4·07), 574 (3·94), and 628 nm (3·71), *m*/*e* 650 (100%), 577 (45), and 504 (14), *m** 512 (650 ⟶ 577).

Radioactivity Experiments

By using the procedure described in Part XXIII,[23] and dilution of the highly active products with inactive material, samples of 4,6,7-tris-(2-methoxycarbonylethyl)-1,3,5,8-tetramethyl-2-vinylporphin (2c) (sp. act. $4·8 \times 10^6$ disint. mg^{-1} s^{-1}), 2,6,7-tris-(2-methoxycarbonylethyl)-1,3,5,8-tetramethyl-4-vinylporphin (2d) ($3·79 \times 10^6$), and coproporphyrin-III tetramethyl ester (2b) ($2·77 \times 10^6$) were prepared.

Euglena *Feeding: General Procedure*.—A sample (*ca.* 1 mg; accurately weighed) of each porphyrin ester was taken separately into tetrahydrofuran (3 ml) and then treated with aqueous 2N-potassium hydroxide (3 ml) and stirred overnight in the dark. Total hydrolysis was indicated by the colourless tetrahydrofuran layer. Aqueous N-hydrochloric acid (2·5 ml) was added; a change in colour to brown and the appearance of a precipitate accompanied addition of the last few drops. Addition of one drop of 2N-potassium hydroxide redissolved the precipitate.

Sodium amalgam (2%) was prepared by addition of small pieces of sodium (128 mg) to mercury (5·6 g); it was then kept in a desiccator before use (<30 min). The three porphyrinogens were prepared by treatment of each solution with *ca.* one third of the sodium amalgam, shaking vigorously with slight warming in each case (*ca.* 5 min). The clear solutions were decanted from the mercury residues, which were washed with distilled water and added to the porphyrinogen solutions. These were added separately to equal portions (*ca.* 80 ml) of a divided batch of a cell-free extract from *Euglena gracilis*; [19] details of the development and refinement of this system in Cambridge for investigation of protoporphyrin-IX biosynthesis will be reported elsewhere [24] and we thank Professor Battersby for access to this preparation before its publication. The solutions were incubated with swirling at 30° in the dark during 17 h.

Ethyl acetate–acetic acid (3 : 1; 166 ml) was added to each solution and, after shaking, they were left in daylight during 30 min (the solution from the 4-vinylporphyrinogen feeding had a pronounced red colour prior to this step).

[23] J. A. S. Cavaleiro, G. W. Kenner, and K. M. Smith, *J.C.S. Perkin I*, 1973, 2478.

[24] A. R. Battersby, J. Baldas, J. Collins, D. H. Grayson, K. J. James, and E. McDonald, manuscript in preparation.

Each sample was then worked up as follows: the top layer was decanted and the lower layer was centrifuged. The supernatant layer was taken off and added to the original solution. Ethyl acetate was added to the enzyme slime from the centrifugation and after vigorous shaking the slime was removed by filtration on Celite; the slime was discarded and the filtrate (A) set aside. The original ethyl acetate fraction was washed with saturated sodium acetate solution (100 ml) and separated and the aqueous phase was back-extracted with solution (A), which was then combined with the bulk of the ethyl acetate. This was then washed with aqueous 3% sodium acetate (*ca.* 50 ml) and after careful separation, the ethyl acetate layer was extracted with small portions of 1 : 1-hydrochloric acid–water (40 ml total). A visible absorption spectrum was run at this stage; the solution was then diluted with protoporphyrin-IX (*ca.* 1·5 mg) in a small amount of acetic acid, and measurement of the visible absorption spectrum, followed by comparison of the Soret band intensity, allowed calculation of the dilution factor (D_1). The porphyrin was then extracted into ether by careful neutralisation of the two-phase system with solid sodium carbonate. The extract was evaporated to dryness and the residue was further dried under high vacuum.

Each crude fraction was then treated as follows.

(a) *Coproporphyrinogen*-III *feeding*. The products were esterified in 5% v/v sulphuric acid in methanol (10 ml) and, after measurement of the visible absorption spectrum, the solution was diluted with inactive protoporphyrin-IX dimethyl ester (19·3 mg). Remeasurement of the absorption spectrum gave the dilution factor (D_2). Further dilution with coproporphyrin-III tetramethyl ester (7·11 mg) was followed by chromatography, which served to separate coproporphyrin-III tetramethyl ester (5·8 mg) from other, less polar porphyrins; this was crystallised to constant activity. The fore-running fraction was then diluted with harderoporphyrin trimethyl ester (2c) (5·5 mg) and careful re-chromatography (elution with methylene chloride–benzene) gave a protoporphyrin-IX dimethyl ester (14·1 mg) fraction as well as a harderoporphyrin trimethyl ester (3·1 mg) fraction. The 14·1 mg of protoporphyrin-IX was crystallised to constant activity and then subjected to c.c.d.,* with no significant decrease in the specific activity. The harderoporphyrin fraction was first shown to be pure tripropionic porphyrin by c.c.d.* and then, after dilution with a little (*ca.* 1 mg) 4-vinylporphyrin trimethyl ester (2d), was separated by analytical high-pressure liquid chromatography (*cf.* Figure). Counting of 13 fractions collected showed activity to be localised entirely in the 2-vinylporphyrin band. The radioactivities during recrystallisation and specific incorporations for this feeding are shown in Table 1.

(b) 2-*Vinylporphyrinogen feeding*. Similarly, the crude fraction was esterified, diluted with protoporphyrin-IX dimethyl ester (18·6 mg) and harderoporphyrin trimethyl ester (8·8 mg), and chromatographed. Details of the crystallisations to constant activity are shown in Table 2.

(c) 4-*Vinylporphyrinogen feeding*. The crude fraction was again esterified, diluted with protoporphyrin-IX dimethyl ester (20·6 mg) and 4-vinylporphyrin (2d) (15·2 mg), and then separated. Full details are given in Table 3.

Blank Determination of Primary Isotope Effect.—Coproporphyrin-III tetramethyl ester (1·035 mg; sp. act. 2·77 ×

* Porphyrin carboxylic acids used; for hydrolysis, see later.

TABLE 1

Recrystallisations and incorporation of coproporphyrinogen-III into protoporphyrin-IX

Recrystallisation	Specific activities (disint. mg^{-1} s^{-1})		
	Protoporphyrin-IX dimethyl ester	Coproporphyrin-III tetramethyl ester	Tripropionic fraction
1	(1·35 × 10³)	(6·72 × 10³)	1·0 × 10³
2	3·10 × 10³	1·10 × 10⁴	
3	2·97 × 10³	9·26 × 10³	
4	2·90 × 10³	9·40 × 10³	
5	3·00 × 10³		
Final activity	2·95 × 10³	9·33 × 10³	1·0 × 10³
Recovery	2·95 × 10³	9·33 × 10³	1·0 × 10³
(disint. s^{-1})	× 20·9	× 7·11	× 5·5
	= 6·17 × 10⁴	=66·34 × 10³	=5·5 × 10³

Dilution factor = 249·3 (D_1 = 13·55, D_2 = 18·4). Tritium retention = 96%. Material fed = 1·164 mg (2b) (sp. act. = 2·77 × 10⁶ disint. mg^{-1} s^{-1}).

Incorporation

$$= \frac{\text{total disintegrations isolated in pure sample}}{\text{total disintegrations fed}} \times \frac{100}{96} \times 100\%$$

$$= \frac{6{\cdot}17 \times 10^4 \times 10^4}{1{\cdot}164 \times 2{\cdot}77 \times 10^6 \times 96} = 2{\cdot}0\%$$

TABLE 2

Recrystallisations and incorporation of harderoporphyrinogen (3c) into protoporphyrin-IX

Recrystallisation	Specific activities (disint. mg^{-1} s^{-1})	
	Protoporphyrin-IX dimethyl ester	Harderoporphyrin trimethyl ester (2c)
1	(8·09 × 10³)	7·63 × 10³
2	7·23 × 10³	7·40 × 10³
3	7·12 × 10³	8·0 × 10³
4	(7·46 × 10³)	
5	7·08 × 10³	
Final activity	7·14 × 10³	7·68 × 10³
Recovery	7·14 × 10³	7·68 × 10³
(disint. s^{-1})	× 20·2	× 8·8
	=1·44 × 10⁵	=67·58 × 10³

Dilution factor = 217·3 (D_1 = 10·65, D_2 = 20·4). Tritium retention = 96%. Material fed = 1·048 mg (2c) (sp. act. = 4·80 × 10⁶ disint. mg^{-1} s^{-1}).

$$\text{Incorporation} = \frac{1{\cdot}44 \times 10^5 \times 10^4}{1{\cdot}048 \times 4{\cdot}80 \times 10^6 \times 96} = 3{\cdot}0\%$$

TABLE 3

Recrystallisations and incorporation of isoharderoporphyrinogen (3d) into protoporphyrin-IX

Recrystallisation	Specific activities (disint. mg^{-1} s^{-1})	
	Protoporphyrin -IX dimethyl ester	Isoharderoporphyrin trimethyl ester (2d)
1	1·29 × 10³	6·19 × 10³
2	1·20 × 10³	5·85 × 10³
3	1·07 × 10³	6·15 × 10³
4	1·23 × 10³	
5	1·16 × 10³	
Final activity	1·18 × 10³	6·06 × 10³
Recovery	1·18 × 10³	6·06 × 10³
(disint. s^{-1})	× 22·3	× 15·2
	=2·63× 10⁴	=92·11 × 10³

Dilution factor = 136·56 (D_1 = 6·54, D_2 = 20·88). Tritium retention = 96%. Material fed = 1·322 mg (2d) (sp. act. = 3·79 × 10⁶ disint. mg^{-1} s^{-1}).

$$\text{Incorporation} = \frac{2{\cdot}63 \times 10^4 \times 10^4}{1{\cdot}322 \times 3{\cdot}79 \times 10^6 \times 96} = 0{\cdot}5\%$$

10^6 disint. mg^{-1} s^{-1}) was hydrolysed and reduced with sodium amalgam as described in the foregoing feeding experiment. The colourless porphyrinogen solution was decanted into a conical flask containing phosphate buffer (150 ml; pH 7·4) and kept overnight in the dark at 30°. The flask was then exposed to daylight for 3 h and the solution treated with acetic acid (5 drops) and ethyl acetate (50 ml). The porphyrin was extracted into the organic phase, which was washed with water and dried (Na_2SO_4). The extract was evaporated to dryness and the residue was treated overnight with 5% v/v sulphuric acid in methanol (10 ml). A visible absorption spectrum was measured before and after addition of coproporphyrin-III tetramethyl ester (6·5 mg) and a dilution factor of 15·04 was calculated. Methylene chloride (30 ml) was added and the mixture was poured into aqueous sodium acetate. After extraction, the organic phase was washed with sodium hydrogen carbonate solution, water, and then dried (Na_2SO_4) and evaporated to dryness. The residue, crystallised from methylene chloride–n-hexane (yield 4·2 mg), had specific activity $1·83 \times 10^5$ disint. mg^{-1} s^{-1} (*i.e.* 96% retention of tritium).

Typical Countercurrent Distribution Experiment.—Protoporphyrin-IX dimethyl ester (2·10 mg), harderoporphyrin trimethyl ester (2c) (1·440 mg), and isoharderoporphyrin trimethyl ester (2d) (1·750 mg) in tetrahydrofuran (9 ml) were treated with methanol (7 ml) and aqueous N-sodium hydroxide (3 ml) and left overnight in the dark. Acetic acid was added and the solution was evaporated to dryness. A 1 : 1 mixture of t-butyl alcohol and isobutyl methyl ketone was equilibrated with an equal volume of aqueous sulphuric acid (of a concentration pre-determined by separatory funnel experiments) by shaking together for 5 min and then leaving for 10 min to separate. The sulphuric acid solution (3 ml per tube) (the bottom phase) was pipetted into tubes 1—96 and 3 ml more was added to tubes 1, 25, 49, and 73. Also, the upper phase mixture was pipetted into tubes 1, 2, and 3 (3 ml each time). Top phase (2 ml) and bottom phase (1 ml) were added to the porphyrin acids, and this solution was placed in tube 0; 1 ml of top and 2 ml of bottom phase were used for washing. The reservoir was fitted and the distribution started with a robot and the programme: shake (2 min), settle (2 min), and with the top phase running off the end. When the distribution was completed, the optical densities at 404 nm were measured for the top phase (Unicam SP 500). Distribution curves were obtained by plotting these measurements *vs.* tube number. The porphyrins were then removed from the machine, or if recycling (with harderoporphyrin and isoharderoporphyrin) was required, only the protoporphyrin-IX was removed. In the latter case, bottom phase (3 ml) was added again to each emptied tube, followed by top phase (3 ml), the reservoir was removed, and the recycling attachment was connected. After completion of the distribution, ethyl acetate (50 ml) was added to the phases from the machine and this solution was washed with aqueous sodium acetate, water, and dried (Na_2SO_4). After evaporation and esterification, the compounds were obtained. In cases of radioactive separations, appropriate dilutions were then carried out and the compounds were crystallised to constant activity if necessary.

Procedure for Counting Active Porphyrins.—For high activity samples, a dilution technique was employed. The porphyrin (0·1—1·0 mg, accurately weighed) was dissolved in tetrahydrofuran (*ca.* 10 ml) and diluted to 100 ml with benzene. A sample (1·0 ml) of this solution was pipetted into a counting vial, organic scintillator solution [10 ml; 0·6% w/v solution of 't-butyl PBD' (Ciba) in AnalaR toluene] was added, and the sample was counted in a Packard Tri-Carb liquid scintillation counter. Ten 100 s counts were taken after the sample had been equilibrated in the counter and the average count was calculated.

Standard tritiated n-hexadecane (200—350 mg; sp. act. 2·588 disint. mg^{-1} s^{-1}) was weighed in, and the count redetermined. The background count was determined on a blank, taking the average of thirty 100 s counts. The specific activity of the sample was then calculated. For samples with activity less than 10^4 disint. mg^{-1} s^{-1}, a direct counting process was required, and the sample had to be bleached before counting, in order to avoid quenching of the scintillator. The porphyrin (0·2—0·5 mg) was dissolved in tetrahydrofuran (1 ml) in a counting vial. Dibenzoyl peroxide (*ca.* 100 mg) was added and the vial was sealed and then left in sunlight until decolourised. Organic scintillator solution (10 ml) was added, and the sample was left in the counter for 2—3 h (spuriously high counts were often obtained from samples just after removal from the light, but the activity was observed to level off during several hours). When a steady count was obtained, activities were calculated as above. In the bleaching–counting procedure, lower efficiencies were observed, probably owing to the generation of carbonyl compounds derived from photodecomposition of the porphyrin and dibenzoyl peroxide.

We thank Professor A. R. Battersby F.R.S. and Dr. E. McDonald for making the *Euglena* preparation available and for the provision of laboratory facilities in Cambridge during the feeding experiments. We also acknowledge financial assistance from the Calouste Gulbenkian Foundation and financially supported leave of absence (to J. A. S. C.) from the University of Lourenço Marques, Moçambique.

[3/2562 *Received, 17th December,* 1973]