Biosynthesis of Porphyrins and Related Macrocycles. Part 10.[1,2] Vitamin B_{12}: Biochemical Derivation of Cobyrinic Acid from Uroporphyrinogen III, Studies with the Corresponding Ring c Methyl Heptacarboxylic Porphyrinogen, and Proof of Seven Intact Methyl Transfers

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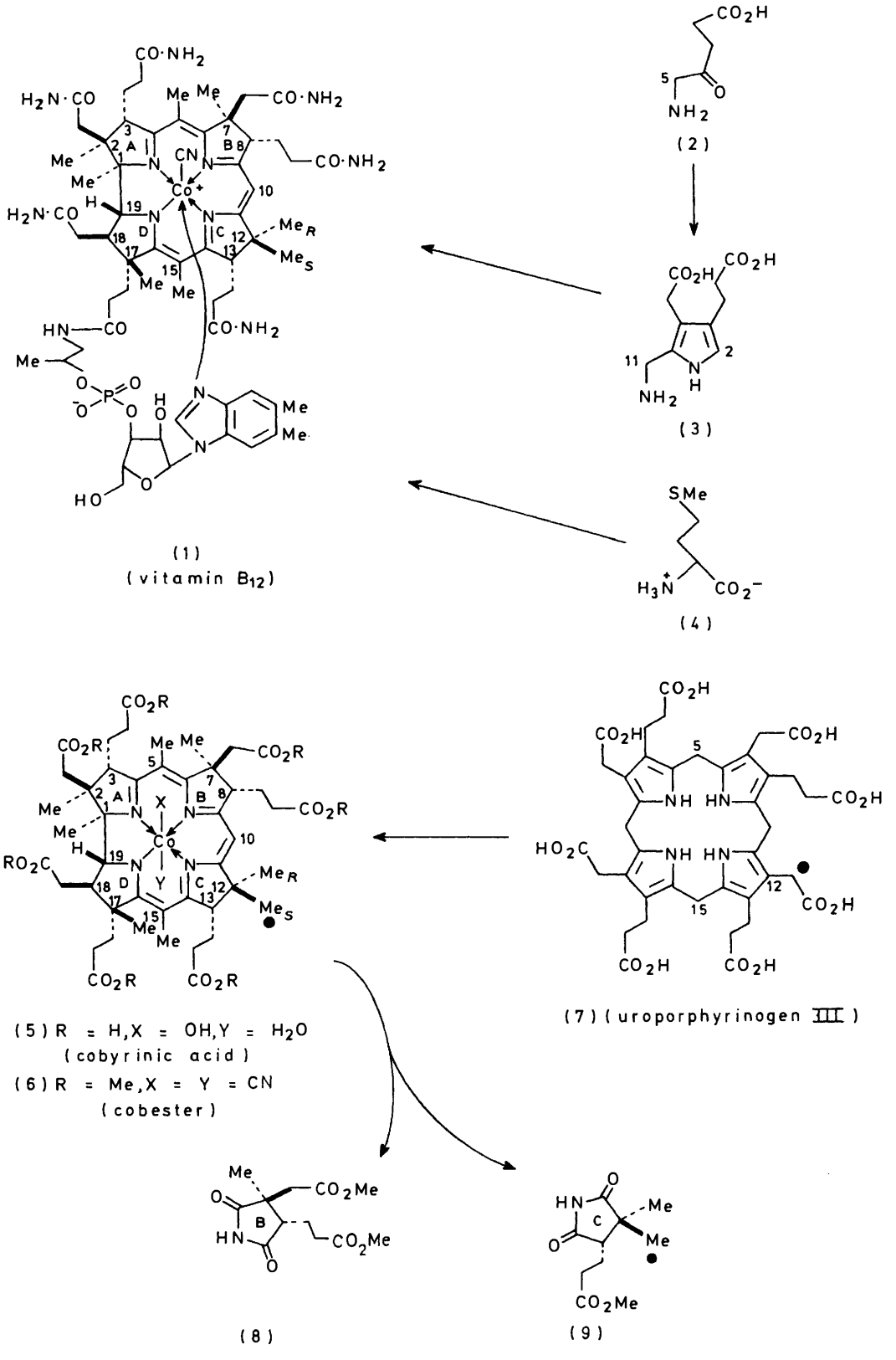
A broken-cell enzyme system is developed from *Propionibacterium shermanii* which converts porphobilinogen into cobyrinic acid in 7—12% yields. This system is used to demonstrate incorporation of unsymmetrically labelled uroporphyrinogen III (7) into cobyrinic acid (5) and retention of specificity of labelling is proved by degradation of (5). Comparative incorporation experiments with the ring c methyl porphyrinogen (13) show that it is 30—50 times less effective than uroporphyrinogen III as a precursor of cobyrinic acid; this finding is discussed.

Double labelling with ^{2}H and ^{13}C is used to prove that all seven methionine-derived methyl groups of vitamin B_{12} are transferred intact without significant exchange of their protons with the medium; the importance of this result for the C-1 methyl group is explained.

EVIDENCE from Chicago, Yale, and Cambridge summarised in the preceding paper [1] showed that the macrocycle of vitamin B_{12} (1) is built from eight molecules of 5-aminolaevulinic acid (ALA) (2) and that seven of the peripheral *C*-methyl groups are derived from methionine

[1] Part 9, A. R. Battersby, M. Ihara, E. McDonald, J. R. Redfern, and B. T. Golding, preceding paper.

[2] Preliminary accounts, A. R. Battersby, M. Ihara, E. McDonald, F. Satoh, and D. C. Williams, *J.C.S. Chem. Comm.*, 1975, 436; A. R. Battersby, E. McDonald, R. Hollenstein, and D. C. Williams, *ibid.*, 1976, 543.



(4). The stereochemistry of the methylation process at C-12 of vitamin B_{12} was also elucidated. These findings were based on experiments in which the ^{13}C-labelled precursors (ALA and methionine) were incorporated into the vitamin from the medium in which *Propionibacterium shermanii* cells were suspended or growing.

A *Broken-cell Enzyme System for Corrin Biosynthesis.*—Our early experiments soon showed that study of the biosynthetic steps beyond ALA (2) and methionine (4) would be severely handicapped by poor penetration of whole bacterial cell walls by the labelled materials of interest. It was obviously necessary to examine porphobilinogen (PBG) (3) as an intermediate on the pathway to corrins. Schwartz and his co-workers [3] had earlier reported the incorporation of ^{13}C-labelled PBG (3) into vitamin B_{12} (1) though the vitamin was not degraded at that time to locate the radioactivity. Our efforts to incorporate small concentrations of [2,11-$^{14}C_2$]-PBG (3) into the corrin macrocycle using whole cells of *P. shermanii*, either growing or washed resting cells, gave incorporations which were too low to permit successful experiments with ^{13}C-labelled materials under the same conditions. Further progress clearly depended on the development of a broken-cell system from *P. shermanii* which retained the enzymes for biosynthesis of the corrin macrocycle.

Several commonly used methods for rupturing bacterial cells were studied and the resulting preparations were incubated with ^{14}C-labelled PBG (3), methionine (4), and Co^{2+} together with a range of co-factors (see Experimental section) in order to test the effectiveness of these broken-cell systems for the synthesis of radioactive corrins. Each system was assayed by adding a radioinactive corrin as carrier material,* and in accord with our initial plan [1] all the corrins present were then converted into heptamethyl cobyrinate (6) (cobester) by methanolysis following the method of Eschenmoser, Keese, and Werthemann.[4] The resultant samples of crude cobester (6) obtained from the methanolysis were often highly radioactive, but after chromatography on silica and recrystallisation to constant specific activity, the incorporation values † fell to $<0.1\%$ in the majority of the early runs. After much experimentation with conditions and additives, a broken-cell preparation was devised which reproducibly converted PBG (3) into cobyrinic acid (5) with incorporations in the range 7—12%. This preparation, which was first described at the E.S.B.O.C. Symposium, Gregynog, in May 1973, involves breaking *P. shermanii* cells by three passes below 0 °C in an X-press, addition of the set of co-factors, and incubation anaerobically with the test precursor at pH 7.6 and 37 °C for 24 h. Cobyrinic acid is then added, the solution is freeze-dried, and the corrins are extracted into aqueous methanol containing potassium cyanide; methanolysis as above then affords crystalline cobester (6). A similar effective cell-free preparation has been developed by Scott *et al.*[5] for use in some of their studies on vitamin B_{12}.

Incorporation of Uroporphyrinogen III into Cobyrinic Acid without Randomisation.—With the problem of penetration into the cells of *P. shermanii* thus overcome, the way was open for the study of likely precursors of cobyrinic acid (5), and so of vitamin B_{12}, which lie on the pathway beyond PBG (3). In particular, it was important to study uroporphyrinogen III (7). There had long been pointers from the work of Porra [6] and Burnham and Plane [7] that this macrocycle (7) is a possible precursor of the corrin nucleus of vitamin B_{12}.

Our plan was to synthesise uroporphyrin III octamethyl ester (10) carrying a *single* ^{14}C-label at the methylene group attached to C-12, then to reduce the corresponding octacarboxylic acid (11) to the porphyrinogen (7) and to examine its incorporation by the broken-cell enzyme system into cobyrinic acid (5). Importantly, specificity of incorporation could be checked by degradation of the isolated cobester (6) as described later to the ring c imide (9).

The preparation of the singly labelled pyrrole (12) required for this synthesis has already been reported,[8] together with its conversion [9] into specifically labelled uroporphyrin III octamethyl ester (10).

During the development of the foregoing enzymic and synthetic work, results in this area appeared from three other laboratories. Two groups [10,11] using unbroken cells found that ^{14}C-labelled uroporphyrinogen III (7) was not significantly incorporated into vitamin B_{12} when small amounts of the labelled precursor were used, but the Yale group [12] obtained radioactive vitamin B_{12} by using a large amount (relative to the quantity of whole bacterial cells) of mixtures of uroporphyrinogen isomers which were labelled equivalently in each PBG-derived residue; either isomers I and III or all four isomers I—IV were employed together (see Scheme). Evidence was presented against the incorporation of uroporphyrinogen I present in the labelled materials.

This valuable positive finding was a spur to the efforts in Cambridge outlined above, and as it turned out also in Yale, to study non-equivalently labelled pure uroporphyrinogen III (7) as a precursor of cobyrinic acid (5) in order to establish *intact incorporation* of the porphyrinogen macrocycle into the corrin system, *i.e.* to exclude the possibility of reversal and reincorporation of fragments.

Accordingly, the acid derived from [12-*methylene*-^{14}C]-

* The early experiments involved addition of vitamin B_{12} (1) or cobester (6) but dilution at the earliest point is preferable and cobyrinic acid (5) is now added routinely as the carrier.

† All incorporations reported are absolute values: (total activity of cobyrinic acid formed ×100)/(total activity of administered precursor).

[3] S. Schwartz, K. Ikeda, I. M. Miller, and C. J. Watson, *Science*, 1959, **129**, 40.

[4] A. Eschenmoser, R. Keese, and L. Werthemann, unpublished results; *cf.* L. Werthemann, Diss. No. 4097, E.T.H., Zürich, 1968.

[5] A. I. Scott, B. Yagen, and E. Lee, *J. Amer. Chem. Soc.*, 1973, **95**, 5761.

[6] R. J. Porra, *Biochim. Biophys. Acta*, 1965, **107**, 176.

[7] B. F. Burnham and R. A. Plane, *Biochem. J.*, 1966, **98**, 13C.

[8] A. R. Battersby, D. A. Evans, K. H. Gibson, E. McDonald, and L. Nixon, *J.C.S. Perkin I*, 1973, 1546.

[9] A. R. Battersby, M. Ihara, E. McDonald, J. Saunders, and R. J. Wells, *J.C.S. Perkin I*, 1976, 283.

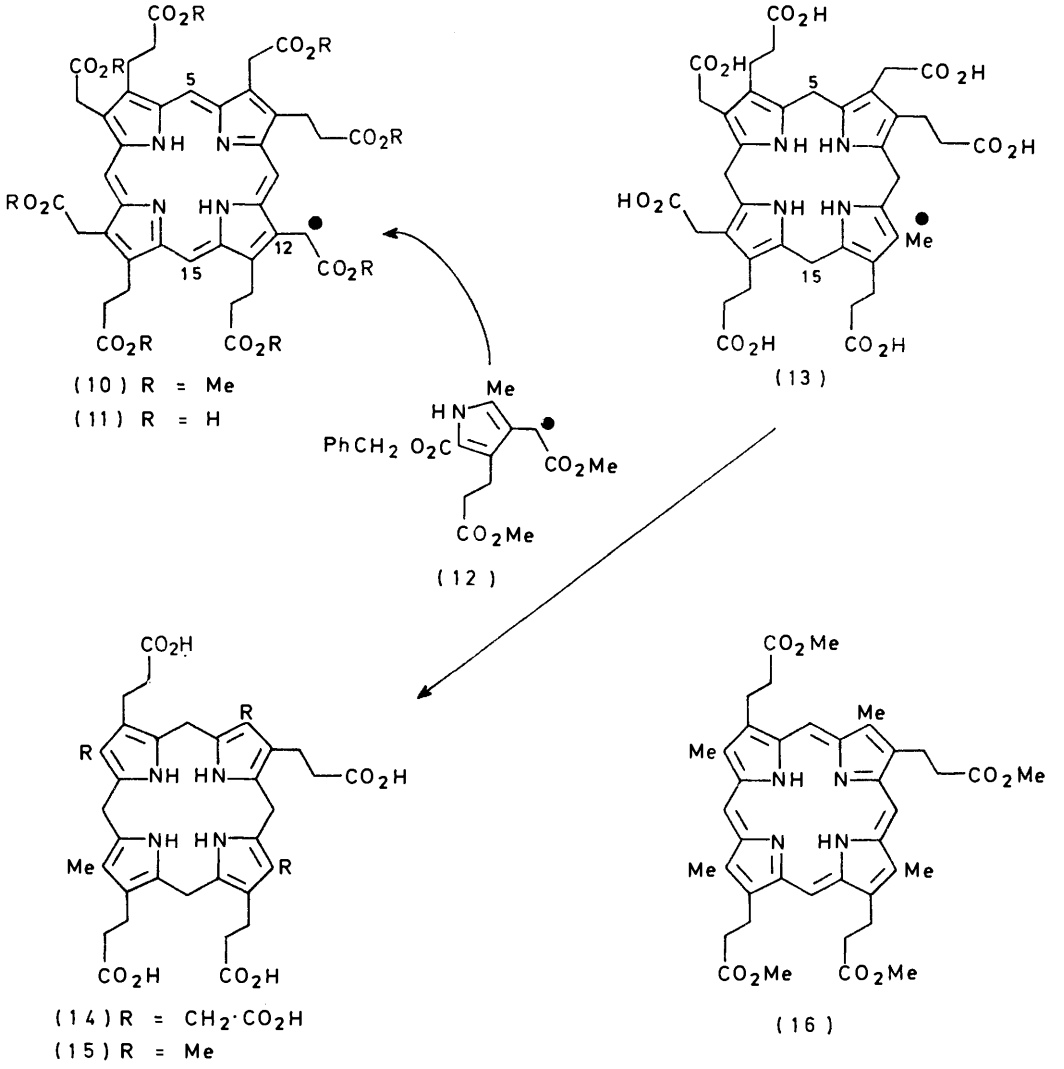
[10] G. Müller and W. Dieterle, *Z. physiol. Chem.*, 1971, **352**, 143.

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

[12] A. I. Scott, C. A. Townsend, K. Okada, M. Kajiwara, and R. J. Cushley, *J. Amer. Chem. Soc.*, 1972, **94**, 8269; A. I. Scott, C. A. Townsend, K. Okada, and M. Kajiwara, *ibid.*, 1974, **96**, 8054.

uroporphyrin III octamethyl ester[9] (10) was reduced with sodium amalgam to [12-*methylene*-^{14}C]uroporphyrinogen III (7). This was incubated in the way described above with the broken cell enzyme preparation from *P. shermanii*, the resultant cobyrinic acid was isolated as cobester (6), and this was purified to constant specific activity. Further evidence for the radiochemical purity of this product was gained by preparative fractionation of it by high-pressure liquid chromatography (h.p.l.c.). Collection of the central section of the single sharp peak followed by recrystalisation of the cobester gave material having the same specific activity as at the outset. The incorporations achieved in this way of uroporphyrinogen III (7) into cobyrinic acid (5) were reproducibly in the range 5—8%.

Eschenmoser and Bogard[13] had earlier developed an important degradation of cobester (6) by ozonolysis which yielded the crystalline ring C imide (9) together with the amorphous imide (8) arising from ring B. The foregoing radioactive cobester was oxidised in this way; the imide (9) from ring C carried, on a molar basis, 90% of the radioactivity of the original cobester (6). In contrast, the activity of the ring B imide (8) corresponded to <3.5% of that originally present in cobester (6). These results establish that uroporphyrinogen III (7) is a specific biosynthetic precursor of cobyrinic acid (5) and so also of vitamin B_{12} itself[2] (1). Parallel work at Yale[14a] showed that [5,15-$^{13}C_2$]uroporphyrinogen III [*cf.* (7)] was converted by resting whole cells of *P. shermanii* into vitamin B_{12} enriched with ^{13}C at C-5 and C-15. The combined results with specifically labelled materials thus firmly establish the intermediacy of uroporphyrinogen III (7) on the pathway to vitamin B_{12}. More recently, Dauner and Müller[14b] have demonstrated high incorporations into cobyrinic acid (5), using a mixture of uroporphyrinogens I, II, III, and IV, which



[13] A. Eschenmoser and T. L. Bogard, unpublished work, referred to by P. Dubs, Diss. No. 4297, E.T.H., Zürich.

[14] (*a*) A. I. Scott, N. Georgopapadakou, K. S. Ho, S. Klioze, E. Lee, S. L. Lee, G. H. Temme, III, C. A. Townsend, and I. A. Armitage, *J. Amer. Chem. Soc.*, 1975, **97**, 2548; (*b*) H.-O. Dauner and G. Müller, *Z. physiol. Chem.*, 1975, **356**, 1353.

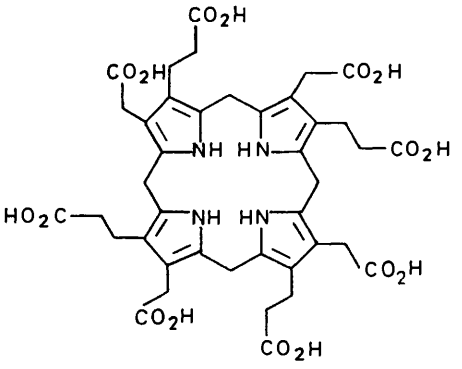
were labelled equivalently at each pyrrolic residue. A cell-free system from *Clostridium tetanomorphum* was used and they have now achieved similar incorporations with this system from [12-*methylene*-^{14}C]uroporphyrinogen III (7) prepared from our synthetic sample of the porphyrin (10); we thank Professor Müller for sending this information to us (March 1976).

By inspection, the biosynthetic conversion of uroporphyrinogen III (7) into cobyrinic acid (5) requires the following structural changes: (*a*) introduction of seven methyl groups from methionine (4) at C-1, C-2, C-5, C-7, C-12 (*pro-R*), C-15, and C-17, (*b*) decarboxylation of the acetic acid side-chain at C-12, (*c*) extrusion of C-20 from the skeleton of uroporphyrinogen III, (*d*) possible adjustment of the oxidation level, (*e*) insertion of cobalt.

In the absence of biosynthetic evidence concerning the sequence of these steps, a vast number of permutations is possible and it seems premature to suggest detailed hypothetical pathways. Working hypotheses are necessary however for the design of experiments and in this respect, it is helpful to speculate about the *first* step in the overall conversion of uroporphyrinogen III (7) into cobyrinic acid (5). The *last* step is another attractive point for speculative attack.

[15] A. R. Battersby, E. Hunt, M. Ihara, E. McDonald, J. B. Paine, III, F. Satoh, and J. Saunders, *J.C.S. Chem. Comm.*, 1974, 994; *J.C.S. Perkin I*, 1976, 1008.

We reasoned that the two most probable first steps are *C*-methylation *or* decarboxylation of the C-12 side-chain to yield a heptacarboxylic porphyrinogen (13). This latter idea, which has also been considered by several others (*e.g.* ref. 14), is simpler to test and was therefore tackled first. Also we had experience of such systems in that phyriaporphyrinogen III (14) had been shown [15,16] to be an intermediate on the pathway from uroporphyrinogen III (7) to coproporphyrinogen III (15).



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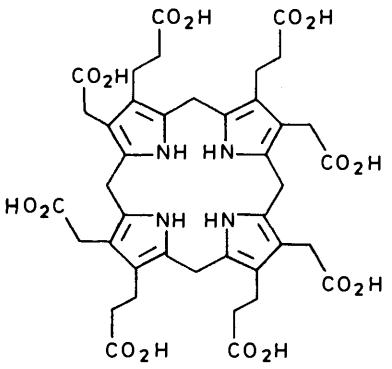

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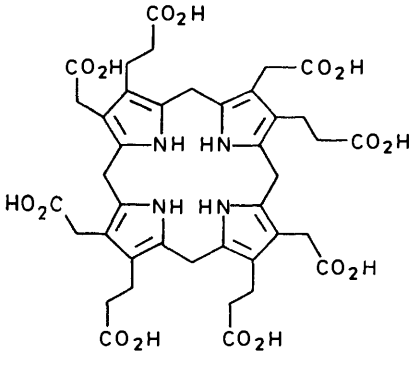

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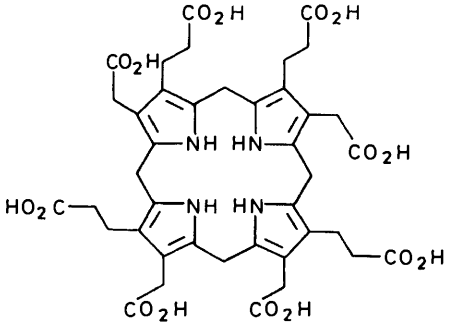


IV

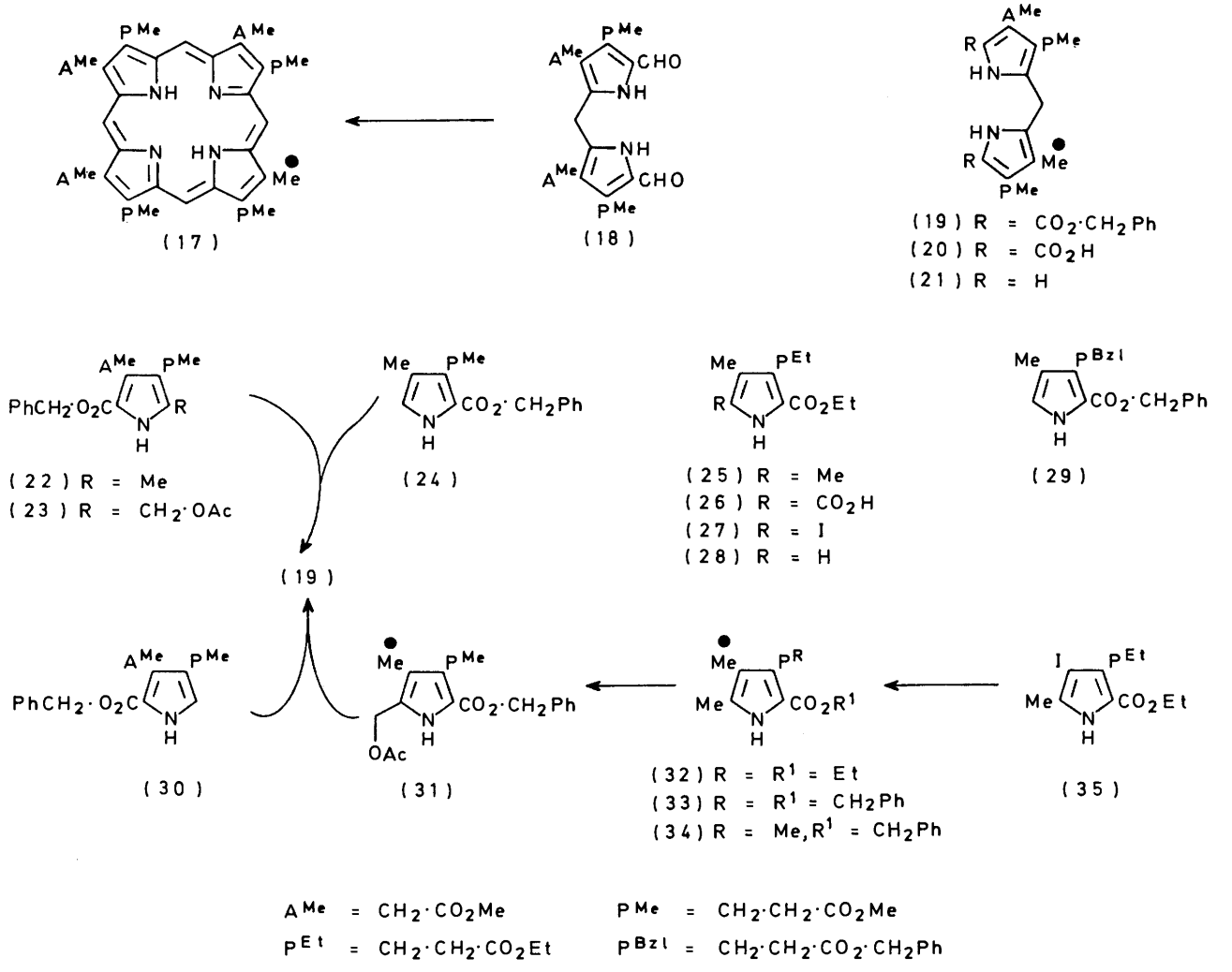
SCHEME The four uroporphyrinogen isomers

Incorporation Experiments with the Ring C *Methyl Heptacarboxylic Porphyrinogen.*—The required porphyrin heptacarboxylic ester (17) was synthesized as follows. First, two routes were developed for synthesis of the unsymmetrical building block (19). For the unlabelled series, the preferred route involved interaction of the pyrroles (23) and (24). The former was obtained by oxidation of the pyrrole (22) with lead tetra-acetate. For the latter (24), a standard conversion of the pyrrole (25) was used *via* the trichloro-derivative to the acid (26), which was converted into the 2-iodopyrrole (27) and then hydrogenated to yield the α-free product (28). Transbenzylation to the dibenzyl ester (29) followed by

[16] (*a*) P. S. Clezy, T. T. Hai, and P. C. Gupta, *Austral. J. Chem.*, 1976, **29**, 393; (*b*) A. H. Jackson, H. A. Sancovich, A. M. Ferramola, N. Evans, D. E. Games, S. A. Matlin, G. H. Elder, and S. G. Smith, *Phil. Trans. Roy. Soc., Ser. B*, 1976, **273**, 191.

selective base-catalysed methanolysis gave the product (24).

The dibenzyl ester (19) obtained by tin(IV) chloride-catalysed reaction of the pyrroles (23) and (24), was hydrogenated to yield the crystalline dicarboxylic acid (20). This was decarboxylated in boiling diethylformamide [15] and the resultant pyrromethane (21) was condensed directly with the symmetrical diformylpyrromethane (18) to yield the porphyrin heptacarboxylic ester (17) in 32—40% yield. The ^{1}H n.m.r. spectrum of this product was clearly different from those of the group for two important reasons: (*a*) in the event of a positive incorporation of the corresponding porphyrinogen (13) into cobyrinic acid, the derived cobester could then be degraded as above to the imides (8) and (9) to determine whether the labelling is specific or scattered; (*b*) it was essential to be certain that even traces (<0.5%) of uroporphyrin III ester [as (10)], which might be formed during the synthesis, would necessarily be *radioinactive*; without this safeguard, a small incorporation of activity from the corresponding porphyrinogen carboxylic acid into cobyrinic acid could be spurious.



isomeric type III porphyrin heptacarboxylic esters [15] [as (17) with the *C*-methyl group respectively on ring A, B, or D]. Also, the purified porphyrin ester (17) was shown by h.p.l.c. on C_{18}-reverse phase [17] to be free of uroporphyrin isomers which can arise [9,18] as by-products.

A specifically labelled sample of [^{14}C]porphyrin ester (17) was now required for the biosynthetic experiments and we chose to introduce the label at the C-12 methyl

To minimise the number of transformations of radioactive materials, a slightly modified route to the pyrromethane (19) was used. The β-iodopyrrole (35) was reductively methylated [19] with [^{14}C]formaldehyde and the resultant diethyl ester (32) was transesterified as usual, first to the dibenzyl ester (33) and then to the mixed ester (34). Lead tetra-acetate converted the latter into the labelled pyrrole (31), which with the

[17] A. R. Battersby, D. G. Buckley, G. L. Hodgson, R. E. Markwell, and E. McDonald in 'High Pressure Liquid Chromatography in Clinical Chemistry,' eds. P. F. Nixon, C. H. Gray, C. K. Lim, and M. S. Stoll, Academic Press, London, 1976, p. 63.

[18] J. A. S. Cavaleiro, G. W. Kenner, and K. M. Smith, *J.C.S. Perkin I*, 1974, 1188.

[19] M. W. Roomi and S. F. MacDonald, *Canad. J. Chem.*, 1970, **48**, 139.

ester [9] (30) in a reaction catalysed by tin(IV) chloride gave the ^{14}C-labelled pyrromethane (19). The derived acid (20) was identical with that prepared above and it was converted as earlier into the [12-*methyl*-^{14}C]porphyrin heptamethyl ester (17); its purity was again confirmed by h.p.l.c. For clarity, this product will be referred to as the ring C methyl porphyrin.

When the first incorporation experiments based on this material (see later) showed very low radioactivity in the isolated cobyrinic acid, a second complete synthesis of the labelled ring C methyl porphyrin ester (17) was carried out. This synthesis was run at *ca.* 10 times the specific activity of the first and it allowed accurate studies to be made.

A sample of the foregoing [^{14}C]porphyrin (17) was hydrolysed to the heptacarboxylic acid and the product was reduced as usual with sodium amalgam to the [12-*methyl*-^{14}C]ring C methyl porphyrinogen (13). Simultaneously were prepared (i) a sample of [12-*methylene*-^{14}C]uroporphyrinogen III (7) (see above), and (ii) a single batch of our broken-cell preparation from *P. shermanii* cells. The enzyme preparation was divided, one portion being incubated as already described with the labelled uroporphyrinogen III (7) and an equal portion was incubated strictly in parallel with the labelled ring C methyl porphyrinogen (13). Each experiment was worked up as previously to give cobester (6), which was rigorously purified to constant specific activity. Two further pairs of exactly parallel experiments were carried out in which the methodology was gradually improved. The results are collected in Table 1 and they show that uroporphyrinogen III (7) is 30—50 times more effective as precursor of cobyrinic acid (5) than is the ring C methyl porphyrinogen (13). Indeed, the ' incorporation ' of the ring C methyl porphyrinogen was so low that the activity of the isolated cobester was insufficient to allow its degradation for determination of the labelling pattern.

As always, every conceivable explanation of such a finding must be considered before concluding that the tested precursor is probably not on the biosynthetic pathway. The common problem of penetration is not a difficulty here since a broken cell system is being used. However, two other possibilities must be examined. First, the ring C methyl porphyrinogen (13) appears to be autoxidised to porphyrin more rapidly than is uroporphyrinogen III (7), as expected on chemical grounds. If oxidation were extensive during incubation, despite our precautions, then low incorporations would result. To counter this possibility, we examined the incorporations from uroporphyrinogen III and the ring C methyl porphyrinogen into coproporphyrinogen III (15); it is known [16b,20] that the decarboxylases on this pathway will accept isomeric porphyrinogen carboxylic acids. The coproporphyrinogen III (15) was converted into coproporphyrin III tetramethyl ester (16) for isolation and purification. This work was carried out on the same strictly parallel set of experiments which form the basis of Table 1. The results (Table 2) show that uroporphyrinogen III and the ring C methyl porphyrinogen give the same order of incorporation into coproporphyrinogen III (within a factor of two). Since it is known [20] that the enzymic decarboxylation steps leading to coproporphyrinogen III only occur on the *porphyrinogens*, it follows that the ring C methyl porphyrinogen (13) survives the incubation conditions

TABLE 1

Incorporations of [^{14}C]porphyrinogens into cobyrinic acid by broken *P. shermanii* cells

	Absolute incorporation (%)			
Precursor	Expt. 1	Expt. 2	Expt. 3	Expt. 4
[12-*methyl*-^{14}C]Ring C methyl porphyrinogen (13)	0.02	0.063	0.18	0.20 [b]
[12-*methylene*-^{14}C]Uroporphyrinogen III (7)	*a*	3.4	6.2	7.6 [b]

[a] The parallel control was [^{14}C]PBG, which gave an incorporation of 12.1%. [b] Precursor added slowly over 5 h.

TABLE 2

Incorporations of [^{14}C]porphyrinogens into coproporphyrinogen III [a] (15) by broken *P. shermanii* cells

	Absolute incorporation (%)			
Precursor	Expt. 1	Expt. 2	Expt. 3	Expt. 4
[12-*methyl*-^{14}C]Ring C methyl porphyrinogen (13)	8.7	14.7	*c*	19.6 [b]
[12-*methylene*-^{14}C]Uroporphyrinogen-III (7)	*c*	12.3	*c*	25.0 [b]

[a] Isolated as coproporphyrin III tetramethyl ester (16). [b] Precursor added slowly over 5 h. [c] Not examined.

sufficiently to be enzymically decarboxylated roughly as efficiently as uroporphyrinogen III (7) itself. It is thus also available to the enzyme systems producing cobyrinic acid (5).

A second possibility concerns the concentration of ring C methyl porphyrinogen (13) in the enzymic experiments. If in fact, this porphyrinogen does follow uroporphyrinogen III on the pathway to cobyrinic acid, it might be formed steadily at low concentration and be converted rapidly into the next intermediate in the sequence. It is thus conceivable that by adding all the ring C methyl porphyrinogen at once, too high a concentration could be present, causing some inhibition mechanism to operate. Accordingly, a very slow addition of the porphyrinogen solution was used for expt. 4 but the result was the same as before (Table 1).

The ring C methyl porphyrinogen [as (13)] has also been studied by Scott *et al.*,[14] who observed a 0.1% incorporation of [5,15-$^{14}C_2$]ring C methyl porphyrinogen into cobyrinic acid, of unknown labelling pattern, in a cell-free system from *P. shermanii*. On this basis, it was concluded that the hepta-acid [as (13)] is an intermediate in corrin synthesis, that uroporphyrinogen III suffers decarboxylation *prior* to the reductive methylation process and that this acid [as (13)] represents the branch-point at which the haem and corrin pathways diverge.

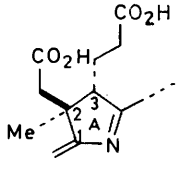
[20] A. R. Battersby and E. McDonald, ' Porphyrins and Metalloporphyrins,' ed. K. M. Smith, Elsevier, Amsterdam, 1975, p. 61; see also ref. 16*b*.

Our comparative study outlined above on uroporphyrinogen III (7) and the ring C methyl porphyrinogen (13) does not lead us to draw such conclusions. Though there must obviously be caution in interpretation, it seems likely that the ring C methyl porphyrinogen (13) is not a normal intermediate in corrin biosynthesis. However, because the incorporations in Table 1 are very low but *not zero*, it seems that the hepta-acid is inefficiently metabolised towards the corrin system by enzyme(s) which normally operate on molecules with an acetic acid side-chain at C-12.*

This interpretation is currently being further tested by mutation and trapping experiments and by the synthesis in labelled form of macrocyclic and open-chain substances for study as precursors of cobyrinic acid.

Studies on the Biological C-Methylation Steps.—It seemed possible that the seven *C*-methyl groups, introduced into the corrin macrocycle from methionine might be sources of hidden information about these later biosynthetic stages. The methyl group at C-1 is particularly important in this respect because of the unsolved problem of bond formation between C-1 and C-19 in the biosynthesis of cobyrinic acid (5). This may involve precursors in which the C-1 methyl group has temporarily become a methylidene residue, with ring A for example as in (36). Alternatively, the C-1 methyl group may be unaffected throughout the process which bonds C-1 to C-19 with ring A remaining for example as in (37). Elegant ring closures of the corrin macrocycle *in vitro* involving a methylidenepyrrolenine [as (36)] have recently been carried out.[21]

Our earlier approach for the study of intact methyl transfer in this series [1,22] could not be used for the C-1 methyl group because methods are not available for the isolation of ring A by degradation of cobester (6). Nor could we feed ^2H-labelled methionine and then study the isolated vitamin B_{12} or cobester by n.m.r. or mass spectrometry. The reason is that growing *P. shermanii* cells use labelled methionine to produce vitamin B_{12}



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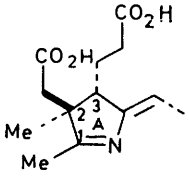


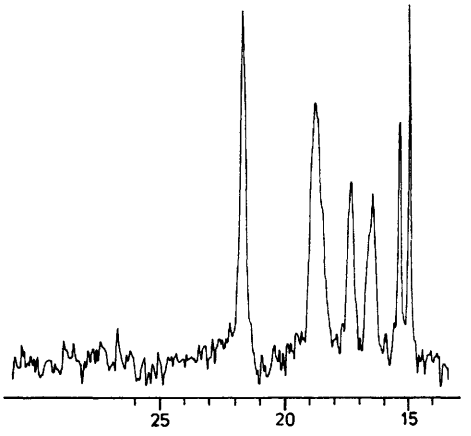
(37)

molecules which variously carry from zero to seven labelled methyl groups. As a result, estimation of the ^2H content of individual methyl groups by direct spectroscopic methods becomes impossibly complex.

A new method was therefore developed based upon double labelling of methyl groups with deuterium and carbon-13. The deuterium is the probe for checking hydrogen retention or loss and the carbon-13 provides the analytical capability, by n.m.r., to establish retention or loss of deuterium.

Accordingly, [*methyl*-$^{13}C^2H_3$]methionine (90 atom % ^{13}C; >98 atom % ^2H) was synthesised as in the preceding paper from $^{13}C^2H_3I$ and homocysteine. This product was then incorporated by growing *P. shermanii* cells into vitamin B_{12} which was isolated and purified as earlier. The ^{13}C n.m.r. spectrum was determined on the corresponding dicyanocobalamin [as (1) with dimethylbenzimidazole ligand replaced by CN] using noise-decoupling of deuterium and ^1H lock. Under these conditions $^{13}C^2H_3$ groups will give rise to singlets, $^{13}C(^2H)_2H$ to doublets (J *ca.* 130 Hz), $^{13}C(^2H)H_2$ to triplets, and $^{13}CH_3$ to quartets. An additional advantage of this approach is that the natural abundance signals from *C*-methyl groups will all be of the last type and so will be depressed into the background by splitting.

The Figure shows the observed spectrum and it is



Methyl region of ^{13}C Fourier transform spectrum of ^{13}C-enriched dicyanocobalamin [as (1) with CN replacing dimethylbenzimidazole ligand] biosynthesised from [*methyl*-$^{13}C^2H_3$]methionine; spectrum run on Varian XL-100 spectrometer with ^1H lock, noise decoupling of deuterium, in 0.1M-KCN in H_2O, 84 785 transients; chemical shifts in p.p.m. downfield from Me_4Si

clear that all seven signals from the ^{13}C-enriched methyl groups are unsplit; thus none carries protium directly bonded to ^{13}C. Long-range ^1H–^{13}C couplings are present, however, as is evident from the broadening of five of the signals. This causes two signals to merge forming the large peak at δ_C *ca.* 18.7.† Attention is drawn to the sharpness of the two high-field signals, in agreement with their earlier assignment [24-26] to the

* Our results were sent to Professor Scott, who kindly told us (April 1976) that his group also finds reproducibly low incorporations of labelled ring C methyl porphyrinogen into cobyrinic acid and that the interpretation is now uncertain.

† The signals are shifted slightly upfield from their normal positions because of replacement of hydrogen by deuterium.[23]

[21] A. Pfaltz, B. Hardegger, P. M. Müller, S. Farooq, B. Kraütler, and A. Eschenmoser, *Helv. Chim. Acta*, 1975, **58**, 1444; B. Krautler, A. Pfaltz, R. Nordmann, K. O. Hodgson, J. D. Dunitz, and A. Eschenmoser, *ibid.*, 1976, **95**, 924.

[22] A. R. Battersby, M. Ihara, E. McDonald, J. R. Stephenson, and B. T. Golding, *J.C.S. Chem. Comm.*, 1974, 458.

[23] G. C. Levy and G. L. Nelson, ' Carbon-13 Nuclear Magnetic Resonance for Organic Chemists,' Wiley–Interscience, New York, 1972, p. 23.

[24] D. Doddrell and A. Allerhand, *Proc. Nat. Acad. Sci. U.S.A.*, 1971, **68**, 1083.

[25] A. R. Battersby, M. Ihara, E. McDonald, J. R. Stephenson, and B. T. Golding, *J.C.S. Chem. Comm.*, 1973, 404.

[26] A. I. Scott, C. A. Townsend, K. Okada, M. Kajiwara, R. J. Cushley, and P. J. Whitman, *J. Amer. Chem. Soc.*, 1974, **96**, 8069.

C-5 and C-15 methyl groups; the nearest ^1H atom is four bonds away.

When the ^{13}C spectrum of cobester (6) derived from the ^{13}C-enriched vitamin B_{12} was recorded under the same decoupling conditions in benzene, essentially the same result was obtained. The quality of the spectrum was lower, however, for computing reasons (overload of signal from benzene).

These results [27] show that all seven methyl groups of vitamin B_{12} (1) which are derived from methionine are transferred intact and that none of them undergoes significant exchange of its hydrogens with the medium during the biosynthesis; our earlier result [1,22] for the methyl groups at C-7 and C-12 is thus confirmed. Further, these findings point against the intermediacy of any system in which the methyl group eventually appearing at C-1 of vitamin B_{12} has temporarily been a methylene residue * [*e.g.* as (36)]. The same results and conclusions were independently reached by the identical double labelling method in Zürich [28] and by a different approach at Yale.[29]

In addition to the above conclusion, the sum of the foregoing results establish the intermediacy of uroporphyrinogen III (7) on the pathway to vitamin B_{12}. They also show that the ring C methyl porphyrinogen (13) is 30—50 times less effective than uroporphyrinogen III as a precursor of cobyrinic acid (5) and so is probably not on the direct pathway. Work on possible intermediates beyond uroporphyrinogen III is in hand.

EXPERIMENTAL

General directions are given in the preceding paper [1] and in refs. 30 and 31. All substances were rigorously protected against light and were stored at 4 °C or lower under nitrogen, in containers wrapped in aluminium foil.

Broken-cell Enzyme System from Propionibacterium shermanii (NCIB 10585).—Growth of the cells proceeded as described earlier [1] with the following changes for work on the broken-cell system: (*a*) the growth batches were scaled up to 12 l; (*b*) purging with nitrogen was omitted; and (*c*) the cells were grown only for 3 days anaerobically, *i.e.* the 2-day period of aerobic growth was omitted. The cells were harvested by centrifugation as before.

Part of the cell paste so obtained (*ca.* 200 ml) was mixed with the minimum volume of pH 7.6 0.1M-phosphate buffer necessary to give a consistency the same as that of thick cream. This was poured into the chamber of an A.B. Biotech X-press (model X-25) and after sealing, the chamber was cooled by ethanol–solid CO_2 to *ca.* −20 °C. The frozen mass was forced three times through the orifice of the press, then just thawed, poured from the press, and stored in 20 ml portions in sealed vessels at −20 °C until used. Normally this broken-cell system was prepared just before use but it has been stored at −20 °C for several weeks without appreciable loss of activity in forming cobyrinic acid.

We also made extensive studies of other methods using two different strains of *P. shermanii* (NCIB 8099 and 10585). These included breaking the cells by sonication under a wide variety of conditions, disruption in a French press, and attempts to render the cell walls permeable by enzymic attack or by detergents with and without dimethyl sulphoxide. In addition, many incorporation experiments were carried out with resting *P. shermanii* cells (harvested and transferred to buffer containing co-factors and other additives) but though they gave positive incorporations of [2,11-$^{14}C_2$]PBG into the isolated cobester, the values were lower (range 0.1—1.0%) than those achieved as described in the next section. Details of these many studies are not given because they were all less satisfactory than the procedure above. The strain NCIB 10585 was used for all the subsequent work.

Incubation of Precursors with the Broken-cell System.—The procedure will first be described for the experiments with [2,11-$^{14}C_2$]PBG (3). A solution was prepared of the following co-factors, thiols, and salts in pH 8.0 0.2M-phosphate buffer (10 ml) containing 0.6mM-ethylenediaminetetra-acetic acid: ATP (20 mg), cysteine hydrochloride (8 mg), β-mercaptoethanol (50 μl), glutathione (25 mg), NADPH (30 mg), *S*-adenosylmethionine (20 mg), 5,6-dimethylbenzimidazole (7mg), $CoCl_2,6H_2O$ (9 mg), $MgCl_2,6H_2O$ (36 mg).

To this solution was added the foregoing broken-cell preparation (20 ml), freshly thawed, followed by deionised, distilled water (20 ml) and the pH of the solution was adjusted to 8. After flushing the mixture with nitrogen, [2,11-$^{14}C_2$]PBG (1 mg) was added, and the flask was sealed and incubated at 37 °C in the dark for 24 h. The mixture at this stage showed pH 5.5—6.0; potassium cyanide (200 mg) and cobyrinic acid (*ca.* 30 mg) were then added and the mixture was freeze-dried.

The residue was extracted with a mixture of methanol (200 ml) and aqueous 1% potassium cyanide (200 ml). Centrifugation normally gave a pellet containing no corrins; in those cases where it was still coloured, the extraction was repeated with methanol (100 ml) and aqueous 1% potassium cyanide (100 ml). The total aqueous methanolic solution of corrins was freed from methanol by evaporation and the aqueous solution was freeze-dried. Extraction of the now small residue with methanol usually took up all the corrins but occasionally a small extraction with the above aqueous methanolic potassium cyanide was necessary. Evaporation of the corrin-containing solution, which had been clarified by centrifugation, yielded a purple residue. This was heated for 20 h under reflux and nitrogen in absolute methanol (100 ml) containing concentrated sulphuric acid (10 ml). After the solution had been evaporated to *ca.* 30 ml, water (50 ml) was added (cooling) followed by solid sodium hydrogen carbonate to basify the mixture. Potassium cyanide (200 mg) was added and the mixture was extracted with carbon tetrachloride (total 250 ml). When occasionally the aqueous layer remained coloured, this indicated incomplete esterification and these corrins were extracted into dichloromethane for re-esterification as above.

* A methylene residue could only be formed and still satisfy the spectroscopic results if deuterium removed from the C-1 methyl group is *returned to the same carbon atom without exchange with the medium.*

[27] Preliminary account, A. R. Battersby, R. Hollenstein, E. McDonald, and D. C. Williams, *J.C.S. Chem. Comm.*, 1976, 543.

[28] M. Imfeld, C. A. Townsend, and D. Arigoni, *J.C.S. Chem. Comm.*, 1976, 541.

[29] A. I. Scott, M. Kajiwara, T. Takahashi, I. M. Armitage, P. Demou, and D. Petrocine, *J.C.S. Chem. Comm.*, 1976, 544.

[30] A. R. Battersby, G. L. Hodgson, E. Hunt, E. McDonald, and J. Saunders, *J.C.S. Perkin I*, 1976, 273.

[31] A. R. Battersby, J. Saunton, and M. C. Summers, *J.C.S. Perkin I*, 1976, 1052.

The solution in carbon tetrachloride was filtered and evaporated and the residue was fractionated on a silica column made by intimate mixing of the silica (7.5 g) with freshly, finely ground potassium cyanide (200 mg). The column was eluted with benzene containing increasing amounts of methanol. Pure benzene developed the column, 1% methanol in benzene eluted coproporphyrin III tetramethyl ester, which was further purified as below. Benzene with 2% methanol eluted cobester (usually *ca.* 20 mg), which was crystallised from benzene (2—3 ml) and n-hexane (2—3 ml) as purple needles (usually 14—18 mg). Recrystallisation was continued until constant specific activity was reached.

Radioassay was carried out by solution scintillation counting with internal standardisation and ^{14}C efficiencies of *ca.* 90% were obtained when the sample of cobester weighed 0.25 mg or less.

TABLE 3

Incorporations of [2,11-$^{14}C_2$]PBG into cobyrinic acid

Expt. no.	Incubation time (h)	Cobyrinic acid added (mg)	Incorp. into cobyrinic acid (%)
1	24	50	8.8
2	48	50	7.3
3 [a]	24	50	4.9
4	24	26 [b]	12.1

[a] The supernatant obtained by centrifugation of 40 ml of broken cells was used. [b] This quantity was used for all later experiments.

Purification of the labelled coproporphyrin III tetramethyl ester was carried out, after dilution with an appropriate quantity of radioinactive synthetic material, by preparative layer chromatography (p.l.c.) on silica with dichloromethane–ether (8 : 12) and recrystallisation to constant specific activity from dichloromethane–methanol (see Table 2).

Incubation experiments with [12-*methylene*-^{14}C]uroporphyrinogen III (7) and with [12-*methyl*-^{14}C]ring C methyl porphyrinogen (13) also involved 1 mg of the labelled substrate with the enzyme preparation described above. Normally, the products from two such simultaneous runs (*i.e.* 2 mg of labelled substrate) were combined for work-up (see following section and Tables 1 and 2).

Incorporation of [12-methylene-^{14}C]*Uroporphyrinogen III* (7) *into Cobyrinic Acid* (5).—A suspension of [12-*methylene*-^{14}C]uroporphyrin III octamethyl ester [9] (2.45 mg; total activity 9.56×10^6 disint. min^{-1}) in aqueous 2N-potassium hydroxide (3 ml) and tetrahydrofuran (3 ml) was stirred at 20 °C in the dark under nitrogen for 21 h. The organic layer was then removed; the aqueous solution was washed twice with fresh tetrahydrofuran (3 ml each) and then was acidified with 10% hydrochloric acid before adjustment to *ca.* pH 8 with potassium hydroxide. Small pieces of *freshly prepared* sodium amalgam were added to the solution, which quickly became colourless (<5 min); it was then filtered, the organic material was washed with water (2×3 ml), and the combined aqueous solution was halved and each half was incubated as in the foregoing example with one batch of the broken-cell enzyme preparation (20 ml) from *P. shermanii*.

After addition of cobyrinic acid (32 mg) as carrier, the combined mixture was worked up by esterification with absolute methanol (100 ml) and concentrated sulphuric acid (10 ml) in the dark under reflux in nitrogen for 19 h. Isolation and crystallisation of the cobester as previously gave purple needles (12.5 mg). The (constant) specific activity of this product was 1.45×10^4 disint. min^{-1} mg^{-1}, corresponding to an incorporation of 5.2%.

Five similar incorporation experiments were carried out with [12-*methylene*-^{14}C]uroporphyrinogen III (7) and the incorporations ranged from 5 to 8%.

Degradation of Labelled Cobester (6) *derived from* [12-methylene-^{14}C]*Uroporphyrinogen III* (7).—The crystalline cobester (total 20 mg) combined from two of the foregoing runs was diluted with radioinactive cobester (80 mg) and recrystallised from benzene (10 ml) and n-hexane (10 ml + 3 ml + 3 ml) to give needles (92 mg; 2 840 disint. min^{-1} mg^{-1}).

The mother liquors from which the *original* cobester (20 mg) had been obtained were mixed with radioinactive cobester (200 mg) and the ester was carefully repurified by chromatography and recrystallisation of the appropriate fractions to yield more labelled cobester (199 mg; 660 disint. min^{-1} mg^{-1}).

The high level (86 mg) and low level (194 mg) samples were mixed with unlabelled cobester (870 mg); recrystallisation from benzene (115 ml) and n-hexane (115 ml + 60 ml + 60 ml) gave the sample for degradation (1 128 mg; 315 disint. min^{-1} mg^{-1}).

Ozonised oxygen (generated at 210 V; 30 l h^{-1}) was passed into a solution of the cobester (1 g) in absolute methanol (100 ml) at −78 °C for 40 min.[13] The colourless solution was then treated at 20 °C with 50% hydrogen peroxide (25 ml) and after the mixture had been heated at reflux for 15 min it was freed from methanol by evaporation. Further work-up essentially as previously [1] gave a gum (902 mg), which was fractionated on silica gel (18 g) (benzene and then chloroform as eluant). The main fraction (170 mg) was separated by p.l.c. on silica (purified ether) to yield the ring C imide (9) (39 mg) and the ring B imide (8) (34 mg). Crystallisation of the former from ether–n-hexane gave needles (16 mg), m.p. 71—72°, identical (n.m.r.) with that obtained earlier.[1] After sublimation at 125 °C and 0.2 mmHg, the ring C imide had an activity of 1 442 disint. min^{-1} mg^{-1} (90% of the original activity of cobester on a molar basis).

The ring B imide was mixed with an approximately equal weight of radioinactive ring C imide and the two were separated by p.l.c. as before, to give ring B imide of activity 59 disint. min^{-1} mg^{-1} (*ca.* 3.5% of the original molar activity).

Synthesis of [12-methyl-^{14}C]*Ring* C *Methyl Porphyrin* (10). *Ethyl* 3-(2-*Ethoxycarbonylethyl*)-4,5-*dimethylpyrrole*-2-*carboxylate* (25).—(a) via β-*Free pyrrole*. Acetic anhydride (1 ml) was added dropwise to a stirred, cooled mixture of 56% hydriodic acid (1 ml; stored over phosphonium iodide) and phosphinic acid (0.2 ml). When the mixture became pale yellow, paraformaldehyde (6.6 mg) was dissolved in it followed by ethyl 3-(2-ethoxycarbonylethyl)-5-methylpyrrole-2-carboxylate [8] (50.6 mg). The solution was stirred at 20 °C for 4 h, then at 40—45 °C for 40 min, and finally evaporated at 30—35 °C. Water (3 ml) was added to the residue and after the mixture had been kept for 16 h the solid was collected, washed with a little cold water, dried (P_2O_5) *in vacuo*, and dissolved in saturated absolute ethanolic hydrogen chloride (3 ml). After 20 h, the solution was evaporated and the residue crystallised from n-hexane to give the dimethylpyrrole (36 mg), m.p.

83—85°, identical with an earlier sample [32] (lit.,[32] m.p. 83—85 and 90—91°).

When the above reaction was carried out at a lower temperature the dimethylpyrrole was contaminated by ethyl 4-acetoxymethyl-3-(2-ethoxycarbonylethyl)-5-methylpyrrole-2-carboxylate, which was isolated by p.l.c. on silica [1 : 1 chloroform–ether] and crystallised from chloroform–n-hexane; m.p. 110—112°, δ_H 4.98 (2 H, s, CH_2·OAc), 4.30 and 4.14 (each 2 H, q, *J* 7 Hz, O·CH_2Me), 2.30 and 2.02 (each 3 H, s, 5-Me and OAc), and 1.34 and 1.24 (each 3 H, t, *J* 7 Hz, O·CH_2*Me*); *m/e* 325 (M^+, 32%), 311 (9), 280 (20), 266 (40), 265 (85), 238 (38), 219 (20), and 192 (100).

(b) via *The* β-*iodopyrrole* (35). To a stirred solution at 20 °C of the iodopyrrole [15] (35) (100 mg) in acetic acid (1 ml) was added 56% hydriodic acid (0.5 ml as above), then 50% phosphinic acid (0.5 ml). When the mixture was pale yellow, paraformaldehyde (8.7 mg) was added in portions to the stirred solution, which was then heated at 60 °C for 4 h. Further work-up involved only minor variations of method (a) to give the dimethylpyrrole (52 mg, 73%), m.p. 85—89°, identical with an authentic sample.

A large-scale run using iodopyrrole (10 g), acetic acid (60 ml), hydriodic acid (15 ml), phosphinic acid (18 ml), and paraformaldehyde (1.8 g) gave 71% of the dimethylpyrrole (5.01 g).

(c) ^{14}C-*Labelled material*. Method (b) was modified as follows. The β-iodopyrrole (35, 511 mg) in acetic acid (3 ml), 56% hydriodic acid (2.5 ml), and 50% phosphinic acid (3.0 ml) was treated with radioinactive paraformaldehyde (5 mg), followed by a suspension of [^{14}C]paraformaldehyde (0.6 mCi; 1.64 mg) in the minimum of acetic acid, and lastly unlabelled paraformaldehyde (35 mg). The reaction and work-up as above gave the labelled dimethylpyrrole (267 mg, 74%; 3.38×10^6 disint. min^{-1} mg^{-1}).

A second synthesis at 10 times the specific activity was carried out using β-iodopyrrole (102 mg) and [^{14}C]paraformaldehyde (1.0 mCi).

Benzyl 3-(2-*Methoxycarbonylethyl*)-4-*methylpyrrole*-2-*carboxylate* (24).—The foregoing unlabelled pyrrole was taken through standard steps [33] to yield the corresponding α-free pyrrole (28). This (1.14 g) in benzyl alcohol (5 ml) containing 0.31M-sodium benzyl oxide (10 drops) was heated in nitrogen under reflux for 1 h. The cooled mixture was poured into 1 : 1 water–methanol (20 ml) and the crystals were collected and washed with 1 : 1 water–methanol (20 ml) then water (10 ml) before drying to give the dibenzyl ester (29) (1.58 g), m.p. 64—65° (from ether–n-hexane), δ_H 8.85br (1 H, NH), 7.30 (10 H, 2Ph), 6.60 (1 H, d, *J* 3 Hz, pyrrole H), 5.25 and 5.06 (each 2 H, s, CH_2Ar), 3.08 and 2.55 (each 2 H, m, CH_2·CH_2·CO), and 2.00 (3 H, s, pyrrole Me).

A solution of the dibenzyl ester (2 g) in absolute methanol (100 ml) containing 0.35M-sodium methoxide (3 ml) was heated at 60 °C for 5 min, and after 3 h at 20 °C the solution was evaporated to 30 ml. It was mixed with aqueous 0.1M-acetic acid–sodium acetate buffer (pH 4.7) and extracted with chloroform, from which a gum was obtained after washing, drying, and evaporation. This crystallised from ether–n-hexane to give the *pyrrole ester* (24) (1.29 g), m.p. 57.5—58.5° (Found: C, 67.9; H, 6.3; N, 4.7. $C_{17}H_{19}NO_4$ requires C, 67.8; H, 6.4; N, 4.7%), δ_H 9.0br (1 H, NH), 7.34 (5 H, Ph), 6.60 (1 H, d, *J* 3 Hz, pyrrole H), 5.26 (2 H, s, CH_2Ar), 3.06 (3 H, s, OMe), 3.05 and 2.48 (each 2 H, m, CH_2·CH_2·CO), and 2.02 (3 H, s, pyrrole Me).

Dibenzyl 3,4′-*Bis*(*methoxycarbonylethyl*)-4-*methoxycarbonylmethyl*-3′-*methylpyrromethane*-5,5′-*dicarboxylate* (19) *and the Corresponding Acid* (20).—*Route A*. The pyrrole (22) (2.5 g) was converted as earlier [15] into the acetoxymethylpyrrole (23, 2.31 g), which crystallised from benzene–n-hexane; δ_H 9.40br (1 H, NH), 7.37 (5 H, Ph), 5.28 (2 H, s, CH_2Ar), 5.06 (2 H, s, CH_2·OAc), 3.81 (2 H, s, CH_2CO), 3.63 and 3.57 (each 3 H, s, OMe), 2.3—2.9 (4 H, m, CH_2·CH_2·CO), and 2.04 (3 H, s, OAc).

A stirred solution of the foregoing α-free pyrrole (24) (100 mg) in anhydrous dichloromethane (5 ml) containing tin(IV) chloride (0.1 ml) at −12 °C was treated with the above acetoxymethylpyrrole (144 mg) in dry dichloromethane (5 ml). After 10 min, 2N-hydrochloric acid (10 ml) was added and the aqueous layer was extracted with dichloromethane (50 ml). The combined organic solutions were washed with saturated brine, saturated sodium hydrogen carbonate, and then brine, and dried. Evaporation gave the pyrromethane as a gum (233 mg), δ_H 7.24 (10 H, 2Ph), 5.22 and 5.20 (each 2 H, s, CH_2Ar), 3.87 (2 H, s, pyrr$_2CH_2$), 3.76 (2 H, s, CH_2·CO), 3.60 and 3.55 (6 H and 3 H, s, 3OMe), 3.10—2.40 (8 H, m, 2CH_2·CH_2·CO), and 2.00 (3 H, s, 3′-Me).

A solution of this pyrromethane (165 mg) in tetrahydrofuran (25 ml) containing triethylamine (4 drops) and 10% palladised charcoal (80 mg) was shaken with hydrogen at 21 °C and 755 mmHg until uptake ceased (1 h). The filtered solution was evaporated and the residue was treated with water (3 ml) and neutralised with acetic acid. Chloroform then extracted the *pyrromethanedicarboxylic acid* (20), which was crystallised from aqueous methanol (110 mg); m.p. 150—152° (decomp.) (Found: C, 56.7; H, 5.9; N, 5.6. $C_{23}H_{28}N_2O_{10}$ requires C, 56.1; H, 5.7; N, 5.7%), δ_H [$(CD_3)_2SO$] 12.0br (2 H, CO_2H), 11.30 and 11.09 (each 1 H, s, 2NH), 3.80 and 3.72 (each 2 H, s, pyrr$_2CH_2$ and CH_2CO), 3.56 (9 H, s, 3 OMe), and 1.91 (3 H, s, 3′-Me).

Route B. To a stirred solution at −20 °C of the α-free pyrrole (30) (100 mg) in dry dichloromethane (5 ml) containing tin(IV) chloride (0.1 ml) was added dropwise a solution of the acetoxymethylpyrrole (31) (104 mg) in dichloromethane (5 ml). After the mixture had been stirred for 10 min at −20 °C, dichloromethane (20 ml) was added and further work-up as above gave the pyrromethane (19) (179 mg), identical with the foregoing product, *m/e* 672 (M^+) and 581 ($M^+ - C_7H_7$). It was debenzylated as before to yield the pyrromethanedicarboxylic acid, again identical with the earlier preparation.

The source of the monopyrroles for route B is given below.

Benzyl 4-(2-*Methoxycarbonylethyl*)-3-*methoxycarbonylmethylpyrrole*-2-*carboxylate* (30).—This had previously been prepared [8,9] by other routes. Here, the pyrrole [15] (22) (1 g) in dry ether (50 ml) was stirred with sulphuryl chloride (1 ml) and then heated under reflux for 3 h. The residue from evaporation was heated at 80 °C in 4 : 1 acetone–water (50 ml) for a few min, then sodium acetate (2 g) in water (5 ml) was added. After the mixture had been heated at 80 °C for 5 min, it was freed from acetone and the precipitate was dissolved in saturated aqueous sodium hydrogen carbonate. This solution was washed with ether, then acidified, and extracted with chloroform to give the *pyrrolecarboxylic acid* (830 mg), m.p. 146—148° (decomp.)

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

[33] F. Morsingh and S. F. MacDonald, *J. Amer. Chem. Soc.*, 1960, **82**, 4377.

(Found: C, 59.15; H, 5.25; N, 3.25. $C_{20}H_{21}NO_8$ requires C, 59.55; H, 5.25; N, 3.45%).

To a stirred mixture of this acid (470 mg), sodium hydrogen carbonate (295 mg), water (10 ml), and 1,2-dichloroethane (10 ml) was added a solution of iodine (330 mg) in aqueous sodium iodide (5 ml). After the stirring had been continued at 60—65 °C for 2 h, the organic layer was shaken with a little aqueous sodium disulphite and then evaporated to yield the *iodopyrrole* (515 mg), m.p. 123—125° (from ether–n-hexane) (Found: C, 47.0; H, 4.2; N, 2.7. $C_{19}H_{20}INO_6$ requires C, 47.05; H, 4.15; N, 2.9%).

The iodopyrrole (0.5 g) in methanol (100 ml) containing sodium acetate (0.5 g) and platinum oxide (5 mg) was shaken with hydrogen at normal temperature and pressure until uptake ceased (1 h). The filtered solution was evaporated and the residue in chloroform was washed with water; then the solution was dried and evaporated. Crystallisation from ether–n-hexane gave the α-free pyrrole (30) (328 mg), m.p. 60°, identified by direct comparison.[8,9]

Benzyl 3-(2-*Methoxycarbonylethyl*)-4-*methyl*-5-*acetoxymethylpyrrole*-2-*carboxylate* (31).—*Route A*. The pyrrole (32) was converted essentially by standard steps [34] *via* (33) into (34), δ_H 8.65br (1 H, NH), 7.33 (5 H, Ph), 5.26 (2 H, s, CH_2Ar), 3.60 (3 H, s, OMe), 3.10—2.95 and 2.57—2.40 (each 2 H, m, CH_2·CH_2·CO), and 2.14 and 1.92 (each 3 H, s, OAc and 4-Me). This product gave the acetoxymethylpyrrole (31) by oxidation with lead tetra-acetate [35] in 93% yield; m.p. 97—98° (lit.,[35] 83—84°).

Route B. A solution of the dimethylpyrrole (2 g) in ether was added to a stirred solution of sulphuryl chloride (1.88 g) in ether (20 ml) and the mixture was then heated under reflux for 1 h. More sulphuryl chloride (0.2 ml) was added and after the solution had been heated under reflux for 30 min more, it was evaporated and the residue was hydrolysed by heating at 80—90 °C for 10 min in 2 : 1 water–acetone (120 ml) containing sodium acetate (3 g). The acetone was evaporated off and extraction with chloroform yielded *benzyl* 5-*formyl*-3-(2-*methoxycarbonylethyl*)-4-*methylpyrrole*-2-*carboxylate* (2.07 g), m.p. 108—110° (from dichloromethane–n-hexane) (Found: C, 65.3; H, 5.85; N, 4.0. $C_{18}H_{19}NO_5$ requires C, 65.6; H, 5.8; N, 4.2%), δ_H 9.70 (1 H, s, CHO), 7.32 (5 H, Ph), 5.29 (2 H, s, CH_2Ar), 3.60 (3 H, s, OMe), 2.30—2.67 (4 H, 2 × t, CH_2·CH_2·CO), and 2.30 (3 H, s, 4-Me).

This aldehyde (50 mg) in stirred dry methanol was treated at 0 °C with a slight excess of sodium borohydride for 15 min, and the solvent was then evaporated off. A solution of the residue in chloroform was washed with water, dried, and evaporated to yield a solid which was recrystallised from ether–n-hexane to give *benzyl* 5-*hydroxymethyl*-3-(2-*methoxycarbonylethyl*)-4-*methylpyrrole*-2-*carboxylate*, m.p. 83—85° (50 mg) (Found: C, 64.8; H, 6.2; N, 4.05. $C_{18}H_{21}NO_5$ requires C, 65.2; H, 6.4; N, 4.25%), δ_H 7.26 (5 H, Ph), 5.21 (2 H, s, CH_2Ar), 4.52 (2 H, s, CH_2·OH), 3.56 (3 H, s, OMe), 2.31—3.11 (4 H, m, CH_2·CH_2·CO), and 1.92 (3 H, s, 4-Me).

The hydroxymethylpyrrole (50 mg) in the minimum of dry benzene was treated with acetic anhydride (0.1 ml) and pyridine (1 drop) at 20 °C for 18 h. Standard work-up gave the 5-acetoxymethylpyrrole (31) (52 mg), identical with the sample from route A.

[34] A. Hayes, G. W. Kenner, and N. R. Williams, *J. Chem. Soc.*, 1958, 3779.

3,8,13,17-*Tetrakis*-(2-*methoxycarbonylethyl*)-2,7,18-*tris*-(*methoxycarbonylmethyl*)-12-*methylporphin* (*Ring* c *Methylporphyrin*).—(a) *Unlabelled material*. A solution of the dicarboxylic pyrromethane (20) (0.3 g) in diethylformamide (30 ml) was heated in nitrogen under reflux for 2.5 h and then was evaporated to a gum (D), δ_H 6.50 and 6.39 (each 1 H, d, 2 × pyrrole α-H), 3.79 (2 H, s, pyrr$_2$CH_2), 3.67 (6 H, s, 2 OMe), 3.62 (3 H, s, OMe), 3.43 (2 H, s, CH_2·CO), 2.66 (8 H, m, 2 × CH_2·CH_2·CO), and 1.99 (3 H, s, pyrrole Me). The diformylpyrromethane (18) was prepared as earlier [9] and it (221 mg) was added to the gum (D); both were dissolved in acetic acid (100 ml) and treated with hydriodic acid (1 ml; stored over PH_4I) at 20 °C for 30 min under nitrogen. A solution of sodium acetate (4.97 g) in acetic acid (70 ml) was then added and air was passed through the solution for 16 h. Evaporation, and extraction of the residue with chloroform gave a solution which was washed with saturated brine and aqueous sodium thiosulphate solution, then filtered and evaporated. The dried residue was esterified for 18 h at 20 °C in absolute methanol (150 ml) containing concentrated sulphuric acid (3 ml). After the mixture had been evaporated to *ca.* half volume, it was poured into saturated brine (200 ml) and the resulting mixture was extracted with chloroform (3 × 80 ml). The residue from the chloroform was chromatographed on silica gel (25 g) in dichloromethane containing increasing amounts of chloroform. Combination of the appropriate fractions and crystallisation from chloroform–methanol gave the *ring* c *methyl porphyrin ester* (150 mg, 40%), m.p. 237—240° (Found: C, 62.2; H, 5.9; N, 6.0%; M^+, 884. $C_{46}H_{52}N_2O_{14}$ requires C, 62.5; H, 5.9; N, 6.2%; *M*, 884), δ_H (45μM in $CDCl_3$) 3.32 (8 H, t) and 4.26 (8 H, m) (4 × CO·CH_2·CH_2porph), 3.56 (3 H, s), 3.62 (3 H, s), 3.66 (9 H, s), 3.76 (3 H, s) and 3.78 (6 H, s) (7 × OMe and porphin Me), 4.90, 5.02, and 5.08 (each 2 H, s, 3 × CH_2), and 9.84 (1 H, s), 9.94 (2 H, s), and 10.02 (1 H, s) (4 porphin H).

(b) ^{14}C-*Labelled material*. The dimethylpyrrole (32) (267 mg) prepared earlier and labelled at the 4-methyl group was converted as for the unlabelled series into the monobenzyl ester (34) (187 mg). Half this product was used to afford the pyrromethanedicarboxylic acid (20) (48 mg) by the earlier steps. Decarboxylation and condensation with the diformylpyrromethane (18) gave [12-*methyl*-^{14}C]ring c methyl porphyrin as its heptamethyl ester (38 mg; 1×10^6 disint. min^{-1} mg^{-1}).

A second synthesis was carried out in the same way at ten times the specific activity of ^{14}C; the product activity was 1×10^7 disint. min^{-1} mg^{-1}.

The purity of these synthetic samples of ring c methyl porphyrin heptamethyl ester was confirmed by h.p.l.c. on a column 30 cm × 6 mm (o.d.) of 10 μ C_{18}-Porasil, using acetonitrile (350 ml) made up with water to 500 ml.[17]

Attempted Incorporation of [12-*methyl*-^{14}C]*Ring* c *Methyl Porphyrinogen* (13) *into Cobyrinic Acid*.—Each enzymic experiment with the labelled ring c methylporphyrinogen (13) was run alongside a strictly parallel one involving labelled uroporphyrinogen III (7) as described in the text. The details which follow refer to Expt. 3 in Table 1.

The [12-*methyl*-^{14}C]porphyrinogen (13) was prepared essentially as described earlier for uroporphyrinogen III using the labelled porphyrin ester (10) (2.045 mg; total

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

activity 1.43×10^7 disint. min^{-1}). The aqueous solution of the porphyrinogen was equally divided between two 20 ml batches of the broken-cell enzyme system and the mixture was incubated for 24 h under nitrogen as previously. The work-up through to cobester was exactly as earlier.

For Expt. 4, Tables 1 and 2, the labelled ring c methyl porphyrinogen (13), prepared from the porphyrin ester (10) (2.045 mg; total activity 1.43×10^7 disint. min^{-1}) was contained in aqueous solution (10 ml) and to each of the two 20 ml batches of the broken-cell enzyme system was added one portion (2 ml) of this porphyrinogen solution at the outset. Further portions (2 ml and 1 ml + 0.5 ml wash) were added 2 and 5 h after the start of the experiment. Further incubation and work-up were as usual.

Synthesis and Incorporation into Vitamin B_{12} of (2S)-[methyl-$^{13}C^2H_3$]*Methionine.*—The doubly-labelled amino-acid was synthesised from commercially available $^{13}C^2H_3I$ (1 g) (90 atom % ^{13}C; >98 atom % ^2H) by the method used earlier [1] to give the (2*S*)-methionine (0.8 g). N.m.r. showed absence of SCH_3 groups and the mass spectrum showed a strong peak 4 mass units higher than the parent ion of unlabelled material.

Incorporation of this material (500 mg) into vitamin B_{12} by *P. shermanii* followed the previously developed method [1] save that a larger volume (10.5 l) of medium and cells was used. After the 5 day growth regime,[1] the cells (200—300 g wet weight) were harvested and worked up as before [1] to yield pure crystalline vitamin B_{12} (17.4 mg). A second similar experiment (400 mg of [$^{13}C^2H_3$]methionine) yielded more vitamin B_{12} (12.6 mg).

The combined quantity of vitamin B_{12} (30 mg) was used for ^{13}C n.m.r. and the spectrum was obtained by dissolving the vitamin in aqueous 0.1M-potassium cyanide (1.5 ml) (*i.e.* using H_2O not D_2O). High-power noise decoupling of ^2H was applied at *ca.* 15.4 MHz, and acquisition time was 0.6 s with 84 785 transients and ^1H lock. The resultant spectrum is shown in the Figure.

The total recovered vitamin B_{12} was converted by the standard method [1] into cobester (22 mg) and the ^{13}C n.m.r. spectrum was run in C_6H_6 again with noise decoupling of ^2H and ^1H lock. Because the computer became overloaded with signal from the solvent, the spectrum was of lower quality than that in the Figure but it fully confirmed the illustrated pattern of signals.

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