Biosynthesis of Porphyrins and Related Macrocycles. Part 28.[1,2] Development of a Pulse Labelling Method to Determine the *C*-Methylation Sequence for Vitamin B_{12}.

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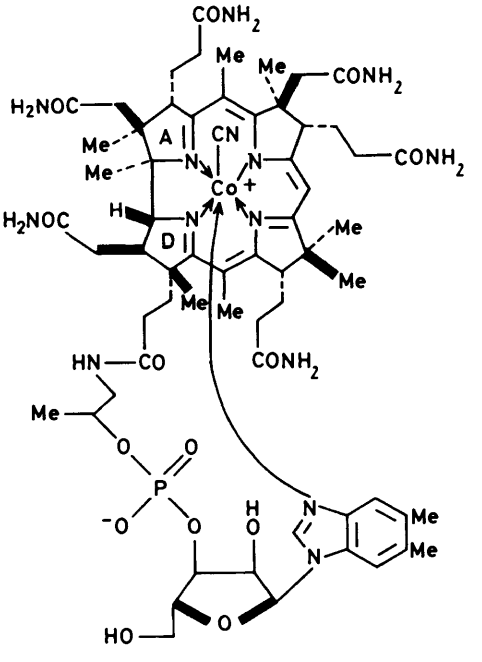
The biosynthesis of vitamin B_{12} involves enzymic *C*-methylation of uroporphyrinogen-III at 8 sites. Earlier work had established that the first 3 methyl groups are introduced at positions 2, 7, and 20 in that order. Pulse-labelling experiments with ^{13}C-labelled and unlabelled *S*-adenosylmethionine show that the remaining 5 *C*-methyl groups are added in the order 17, 12, 1, 15, 5. The pulse labelling approach depends on assaying the biosynthetic product for extent of ^{13}C-labelling by n.m.r.

The results lead to discussion of the late stages of the B_{12} biosynthetic pathway. This highlights the need for a simple scheme of nomenclature for the various biosynthetic intermediates and a systematic one based on the parent names precorrin and corrin is proposed.

Vitamin B_{12} (**1**), the anti-pernicious anaemia vitamin, has a structure which differs strikingly in two ways from most of the other pigments of life: (i) there is a direct link between ring A and ring D so that its corrin macrocycle is smaller by one carbon atom than the macrocycles of protohaem or chlorophyll *a* and (ii) B_{12} is heavily *C*-methylated around its periphery. This paper will report experiments aimed at determining the order in which these *C*-methylations occur.



(**1**) Vitamin B_{12}

The biosynthetic pathway to vitamin B_{12} (**1**) has been intensively studied and the results obtained up to 1981 have been collected in a comprehensive review.[3] Scheme 1 shows the pathway which emerged from these investigations in several laboratories and the main features are as follows. (*a*) The pathway to vitamin B_{12} initially involves the same steps which are used for the biosynthesis of protohaem and chlorophyll *a* as far as uroporphyrinogen-III (**2**), hereafter shortened to uro'gen-III.

(*b*) At this stage, enzymic *C*-methylation of uro-gen-III (**2**), involving *S*-adenosylmethionine (SAM), causes the B_{12}-pathway to branch away from that leading *e.g.* to protohaem. The first methyl group is introduced at position-2 as demonstrated by the isolation of the *C*-methylated chlorin (**4**) from a B_{12}-producing organism. It is presumed that the true biosynthetic intermediate is the tetrahydrochlorin (**3**), or a tautomer of that structure, and that the isolated chlorin (**4**) is formed from it by aerial oxidation.

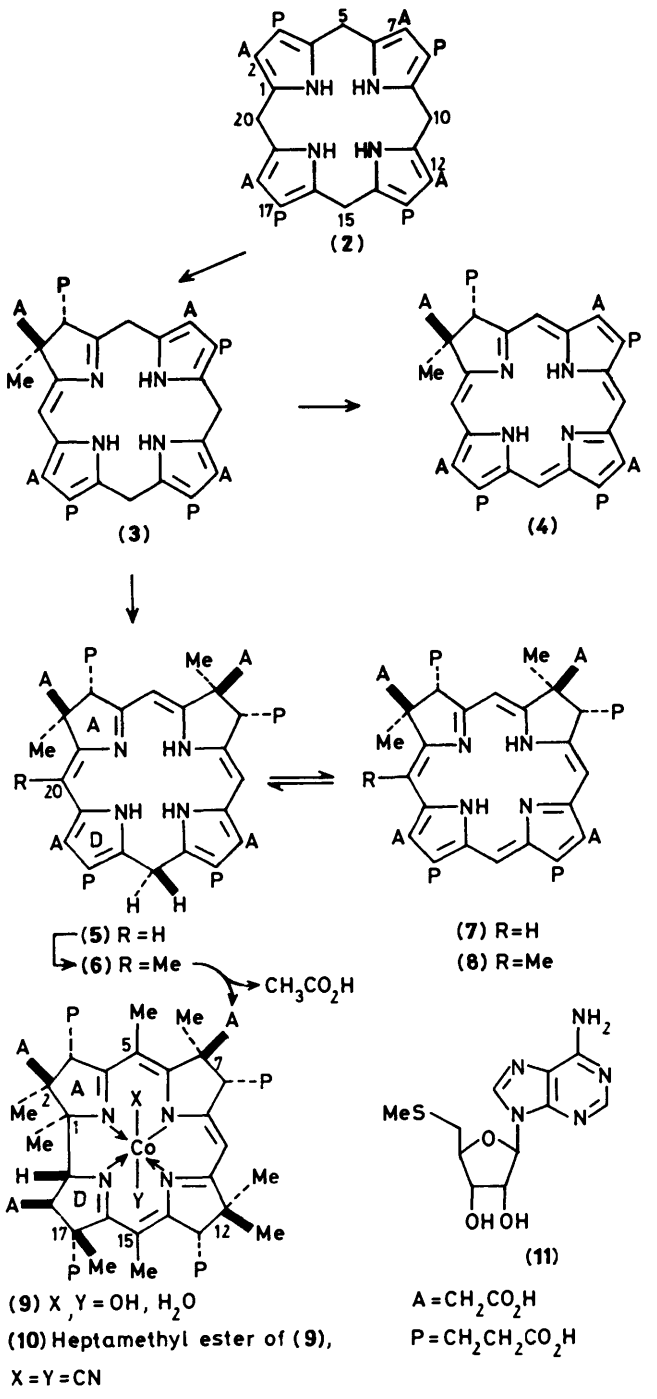
(*c*) *C*-Methylation occurs next at position-7 and the third methyl group, surprisingly at the time, was found to be inserted at position-20. This knowledge depended on the isolation from two B_{12}-producing organisms of the isobacteriochlorin (**7**) called sirohydrochlorin and its relative (**8**) and the determination of their structures. Since the 1981 review,[3] the true dimethylated biosynthetic intermediate has been isolated and its structure was proved to be the dihydroisobacteriochlorin (**5**).[4] It is highly probable that the trimethylated intermediate is also produced and further transformed *in vivo* at the dihydro level of oxidation (**6**), as indicated in Scheme 1. Here again, the reduced intermediates (**5**) and (**6**), undergo aerial oxidation to the aromatic systems (**7**) and (**8**) during isolation.

(*d*) The appropriate incorporation experiments based on isotopically labelled samples demonstrated that the mono-, di- and tri-*C*-methylated macrocycles are converted enzymically into cobyrinic acid (**9**) a proven late precursor of vitamin B_{12} (**1**).

(*e*) At some stage beyond the trimethylated intermediate (**6**), carbon-20 and its attached *C*-methyl group are extruded during the ring-contraction process which generates the direct link between ring A and ring D of cobyrinic acid (**9**). These two carbon atoms were found to be eliminated as acetic acid.

(*f*) As adumbrated under (*d*), cobyrinic acid (**9**) is a late corrinoid precursor of vitamin B_{12} (**1**), and the nucleotide loop and the amide groups present in the vitamin (**1**) are attached enzymically to (**9**) near the end of the whole sequence.

So by 1979, it was known[3] from rigorous isolation work and structure determinations that the biosynthesis of vitamin B_{12} (**1**) involves the first *C*-methyl group being placed at C-2, the second at C-7 and the third at C-20. The order of introduction of the remaining five *C*-methyl groups which appear in the vitamin at positions 1, 5, 12, 15, and 17 remained to be discovered. Two parallel approaches were adopted in Cambridge. One was a continuation of the earlier strategy of seeking new intermediates by isolation and the results of that work will be published in future papers. The second, to be described here, was inspired by the successful use of pulse-labelling methods to discover the order in which the four rings of uro'gen-III (**2**) are assembled.[5]



Scheme 1.

Pulse Labelling Approach.—It was decided to use a cell-free enzyme system prepared from *Clostridium tetanomorphum* since cells were available which afforded high incorporations of the labelled dimethylated system, sirohydrochlorin (**7**), into cobyrinic acid (**9**) in the range 15—45%. The plan was as follows: add at the start of the experiment only a small amount of *unlabelled* SAM to the enzyme system* containing added sirohydrochlorin (**7**). Then after a suitable incubation time t_A to allow later methylated intermediates to be produced from the dimethylated precursor (**7**), add a large excess of [*methyl*-^{13}C]SAM. Then continue the incubation on for a further time t_B to produce cobyrinic acid (**9**). This product, to be isolated as cobester (**10**), should have a lower ^{13}C-content in the methyl groups introduced early in the sequence of methylations than in the later ones; labelling levels were to be determined by ^{13}C n.m.r. spectroscopy. All the necessary signal assignments for cobester (**10**) had been made.[6,7]

This way just described for carrying out the pulse-labelling involving addition of unlabelled SAM first followed by [*methyl*-^{13}C]SAM is called the *Normal Pulse*. That to be described later with [*methyl*-^{13}C]SAM added first followed by unlabelled SAM is the *Inverse Pulse*.

Success in the foregoing approach depends on the successful solution of several preliminary problems and our studies of these are outlined below. (i) [*methyl*-^{13}C]SAM was required at a high level of ^{13}C-enrichment. Unlabelled SAM can be prepared enzymically from methionine using baker's yeast[8] but when [*methyl*-^{13}C]methionine is used, there is the danger of dilution with endogenous unlabelled methionine or endogenous unlabelled SAM. This was checked by using ^{1}H n.m.r. spectroscopy as a sensitive assay of ^{13}C-content on the [*methyl*-^{13}C]methionine added as precursor and on the [*methyl*-^{13}C]SAM which was formed. The results showed that there had been an insignificant change in the ^{13}C-enrichment (from 85.5 to 84.9%) so the amounts of endogenous methionine and SAM must be very small. It was also clear that the biosynthesis of methionine in the yeast had been essentially switched off under the conditions of this experiment.

(ii) Since the pulse-labelling approach involves addition of controlled amounts of unlabelled and ^{13}C-labelled SAM to a cell-free enzyme preparation which produces cobyrinic acid (**9**), it is essential to know whether this enzyme preparation itself contains SAM and if so, how much. Any such endogenous SAM would be especially disadvantageous for the Inverse Pulse since the small amount of [*methyl*-^{13}C]SAM added at the beginning of that experiment would be diluted more seriously than the large amount introduced as the second addition for the Normal Pulse.

The amount of SAM present in 100 g of *C. tetanomorphum* cells was determined by adding a known quantity of [*methyl*-^{13}C]SAM to the derived broken cell preparation followed by re-isolation. This product was too impure so it was degraded by the known base-catalysed degradation[9] to yield *S*-methyl thioadenosine (**11**) which was readily purified. ^{1}H N.m.r. spectroscopy then showed that a small dilution of the ^{13}C-labelled material had occurred corresponding to *ca.* 0.6 mg of unlabelled SAM in the original 100 g of cells. This low level was fortunate and could readily be taken into account in planning both Normal and Inverse Pulses.

(iii) The choice of t_A and t_B (see above) was made after following the time-course of incorporation of ^{14}C-labelled sirohydrochlorin (**7**) into cobyrinic acid in the standard cell-free enzyme system. It was found that incorporation ceased after 7—8 h. We reasoned that during the roughly linear part of the time-course (up to *ca.* 4 h) the concentrations of biosynthetic intermediates should be approximately constant and therefore that the value of t_A was not too critical. Periods of 1.5 and 2.25 h were used.

However, t_B is a more sensitive parameter. Too long a t_B in the Normal Pulse would lead to the formation of considerable quantities of cobyrinic acid (**9**) uniformly ^{13}C-labelled at the SAM-derived methyl groups. This would obscure the sought-after differences in labelling levels at those methyl groups which had been generated during the early part of the t_B period. Values of t_B between 30 and 60 min were therefore used.

* It was known that the complex mixture of enzymes in this preparation is capable of reducing sirohydrochlorin (**7**) to the dihydro state (**5**) which is then incorporated into cobyrinic acid (**9**).

(iv) These same differences in labelling levels generated by the pulse labelling would also be obscured if the enzyme system from *C. tetanomorphum* contains relatively large quantities of endogenous cobyrinic acid (**9**) or its amides. In that case, the labelled and unlabelled corrins would all be converted by the final methanolysis step into cobester (**10**). When analysing the ^{13}C spectrum of this ester, one would then face the difficulty of attempting to detect small differences in signal intensity between large signals mainly arising from natural abundance ^{13}C. Accordingly, cobyrinic acid was removed by passing the freshly prepared enzyme-system through DEAE-Sephadex before the incubation with sirohydrochlorin (**7**) and SAM. Then the biosynthesized cobyrinic acid was trapped on a column of DEAE-cellulose which allowed the neutral hepta-amides of cobyrinic acid (present in significant quantities) to pass through.

Normally, labelled cobyrinic acid is isolated after dilution with unlabelled carrier material. For the same reasons just outlined, the amount of carrier had to be kept to the minimum which was practicable. This necessitated the development of improved work-up procedures which are described in the Experimental section.

The Pulse Labelling Experiments.—The knowledge gained from the foregoing studies enabled the Normal and Inverse Pulse labelling experiments to be designed. For the Normal Pulse, unlabelled SAM (20 mg) during t_A was followed during t_B by [methyl-^{13}C]SAM (85 mg) enriched to 85 atom % ^{13}C. This caused a change in ^{13}C level of the SAM from 1.1% (natural abundance) during t_A to *ca.* 70% during t_B.

In order to achieve a similar large change in the ^{13}C-enrichment for the Inverse Pulse, a much greater amount of unlabelled SAM had to be used for the second addition (600 mg) to dilute sufficiently the first aliquot (20.5 mg) of [*methyl*-^{13}C]SAM which was again used at 85 atom % enrichment. Calculation shows that these quantities correspond to a change in the ^{13}C enrichment of the SAM from *ca.* 84% during t_A (slightly lowered from 85% by the endogenous unlabelled SAM) to *ca.* 5% during t_B.

Results

The proton-noise decoupled ^{13}C n.m.r. spectrum of cobester (**10**) isolated from the Normal Pulse is shown in Figure 1. This should be compared with the corresponding standard spectrum, Figure 2, determined under precisely the same conditions, from a sample of cobester (**10**) having uniform ^{13}C-labelling at the SAM-derived methyl groups; the latter sample was prepared by feeding a large quantity of [*methyl*-^{13}C]methionine to growing *Propionibacterium shermanii* cells. Because the precursor for the pulse feeding was unlabelled sirohydrochlorin (**7**), which already carries methyl groups at C-2 and C-7, the spectrum of the isolated product (Figure 1) showed, as expected, only very small signals corresponding to the natural abundance signals from these two methyl groups. The C-2 and C-7 signals could be used as internal standards to estimate the average ^{13}C-enrichment of the labelled *C*-methyl groups. This turned out to be 7—8 atom % for both the Normal Pulse and for the Inverse Pulse to be described later.

Even by visual inspection of Figure 1, it is strikingly clear that the signal from the methyl group at position 17 of the pulse-labelled cobester (**10**) is of considerably lowered intensity (*ca.* 70% of standard). Thus it was established that the fourth methyl group for vitamin B_{12} is introduced at C-17. This discovery[2a] (1982) led to the important knowledge that the next intermediate beyond the trimethylated stage on the biosynthetic pathway is a *pyrrocorphin*, *e.g.* (**12**) because this macrocycle is produced by 17-methylation of the trimethylated intermediate (**6**) (Scheme 2). It is possible that there are two pyrrocorphin

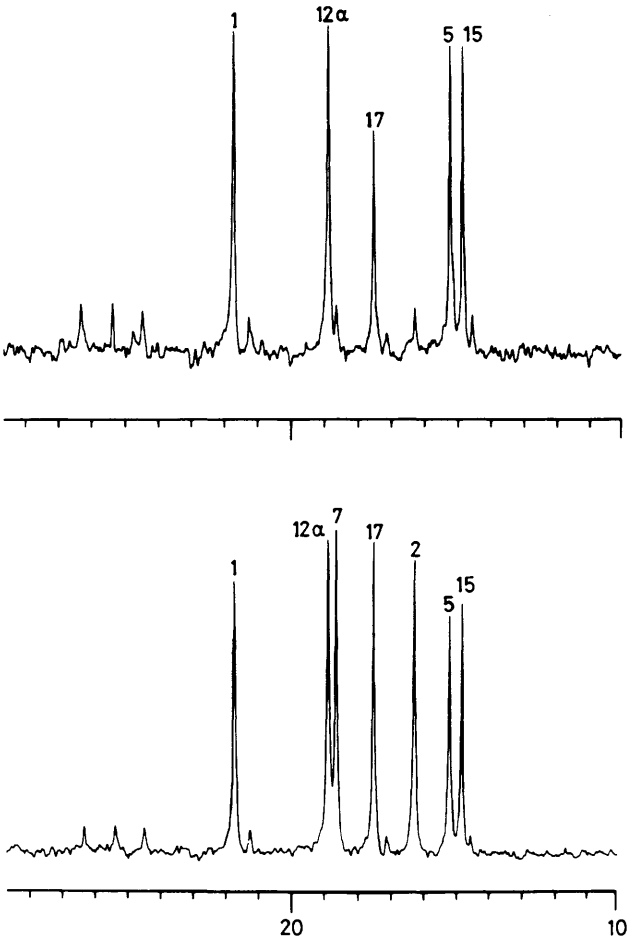


Figure 1 (top). Proton noise decoupled ^{13}C n.m.r. spectrum of cobester (**10**) derived from the Normal Pulse experiment
Figure 2 (bottom). The equivalent ^{13}C n.m.r. spectrum of cobester (**10**) uniformly ^{13}C-labelled at the SAM-derived methyl groups. Both spectra were recorded at 100.6 MHz in C_6D_6

intermediates (**12**) and (**13**) though it must be borne in mind that if the required decarboxylation of the C-12 acetate group preceeds C-17 methylation, then only the 12-methylpyrrocorphin (**13**) would be involved.

The ^{13}C spectrum of cobester (**10**) from the Inverse Pulse,[2b] (Figure 3) now showed by inspection that the signal from the C-17 methyl group was the largest. This gave overwhelming confirmation of the previous finding that the fourth *C*-methylation for vitamin B_{12} occurs at C-17.

Beyond the foregoing important result for the fourth methylation site, there was much valuable information still to be extracted from the spectra in Figures 1 and 3 but this required precise quantitative assays of ^{13}C-signal intensities.

It was decided at the outset to compare all signal intensities from the cobester samples produced by pulse labelling with the corresponding signals from the standard sample of cobester uniformly labelled at the SAM-derived *C*-methyl groups. In this way, and by carrying out all the ^{13}C n.m.r. measurements under *exactly* the same conditions (same instrument, solvent, concentration, temperature, pulse angle, acquisition time) possible errors were avoided arising from *e.g.* different relaxation rates, different efficiencies of n.O.e. or pulse imperfections. The importance of these precautions was emphasised by determining the effect of a large change in the concentration of cobester (**10**) used for n.m.r. measurements on

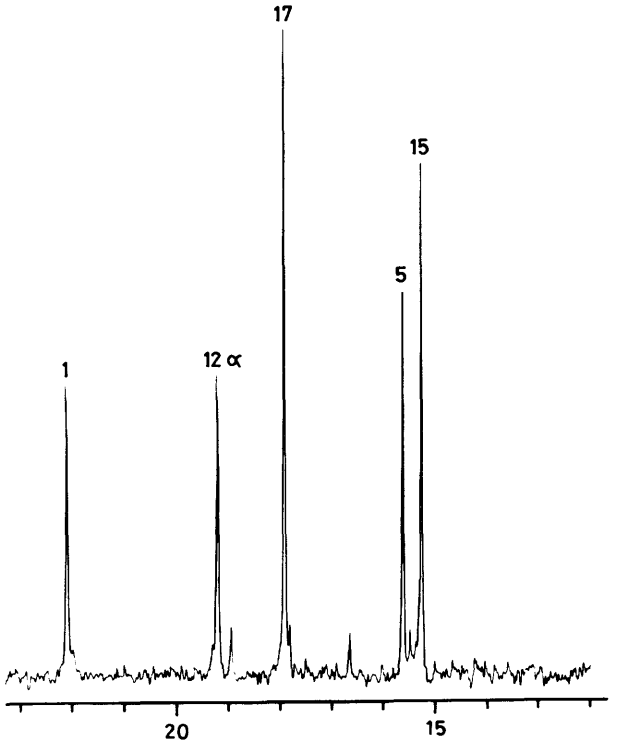


Figure 3. Proton noise decoupled ^{13}C spectrum of cobester (**10**) derived from the Inverse Pulse and recorded at 100.6 MHz in C_6D_6

Table 1. Relative ^{13}C-incorporations[a] into *C*-methyl groups of cobyrinic acid (**9**)

	Position of methyl group				
	1	5	12α	15	17
Set A					
Cobester (**10**) from Inverse Pulse (IP)	100	95	106	102	155
Cobester (**10**) from Normal Pulse (NP)	100	109	93	107	72
Difference[b] (IP-NP)	0	−14	13	−5	83
Set B					
Cobester (**10**) from Inverse Pulse (IP)	100	93	102	99	150
Cobester (**10**) from Normal Pulse (NP)	100	108	94	105	70
Difference[b] (IP-NP)	0	−15	8	−6	80

[a] The relative amounts of ^{13}C at each methyl group were obtained by comparison of the peak areas (obtained by line-fitting, Set A, or by averaging all methods for intensity measurement, Set B) with those from a standard sample of (**10**) uniformly labelled at all the SAM-derived methyl groups. The results are expressed as percentages of the ^{13}C relative value for the C-1 methyl group. Statistical analysis of many intensity determinations gave the worst error for each peak as <2%. For each set of ^{13}C-determinations, the 3 spectra (IP, NP, and standard) were recorded consecutively under identical conditions. [b] The more positive this difference value, the earlier the corresponding methyl group is added in the biosynthetic sequence.

Table 2. Values of ^{13}C T_1 (in s, ±0.045 s) for the methyl groups of cobester (**10**)

Concn. of cobester (**10**) in C_6D_6	Position of methyl group						
	1	12α	7	17	7	5	15
0.0066[a] M	0.19	0.27	0.35	0.49	0.49	1.51	1.53
0.188[b] M	0.23	0.22	0.33	0.40	0.40	0.81	0.82

[a] ^{13}C-Enriched to *ca.* 50 atom % at the methyl groups. [b] Sample at natural abundance.

the values of ^{13}C T_1 for its *C*-methyl groups. The results collected in Table 2 show widely differing changes.

Four long term ^{13}C n.m.r. runs of up to 100 000 scans were carried out on each of the three samples (Normal Pulse, Standard, Inverse Pulse). The intensities of the signals from the ^{13}C-labelled methyl groups were then measured repeatedly by integration, cutting the paper and weighing, and by line-fitting. Table 1 gives in Set *A* the data from one set of determinations based on the Lorentzian line-fitting programme. Since the Inverse Pulse enhances the signals from the 'early' methyl groups whereas the Normal Pulse enhances the signals from the 'later' ones, by taking the differences between the values from the two pulses, the discrimination of the approach is increased. The more positive this difference value, the earlier the corresponding methyl group is added in the biosynthetic sequence. Thus from Table 1 the later sequence is revealed as 17, 12, 1, 15, 5.*

The data in Set *B* in Table 1 were derived by averaging many intensity determinations by all the methods mentioned above. Even the relative intensity values are close to those in Set *A* and the difference values are even more similar leading to the same sequence as from Set *A*, *viz* 17, 12α, 1, 15, 5. Indeed, the difference values invariably led to this same methylation sequence from all four ^{13}C runs on the three samples and from all methods of intensity measurement.†

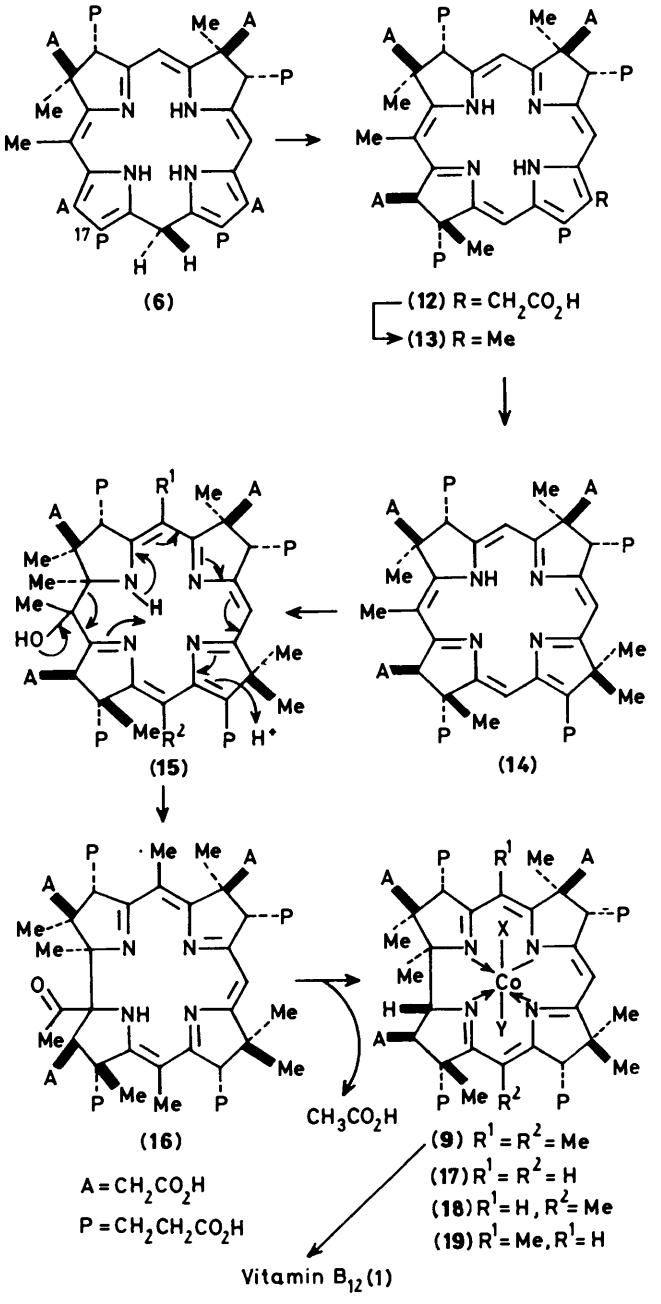
As a final check on the foregoing conclusions, the ^1H n.m.r. spectra of the samples from the Normal and Inverse Pulses and the standard sample were determined. The runs were long enough to reduce the noise level to *ca.* 1% of the signal. Now the intensities of the ^1H satellites of the signals from the *C*-methyl groups, which arise by one-bond ^1H-^{13}C coupling, were measured to give an indirect assay for the ^{13}C-enrichment of the various methyl groups. The difference values (Inverse-Normal) again led to the now familiar methylation order 17, 20α, 1, 15, 5.

Two points should be added at this stage. Firstly, a spin-echo technique was successfully developed[10] to obtain ^1H n.m.r. signals selectively only from protons attached to ^{13}C. In this way, the information gained by direct ^{13}C n.m.r. measurements, and illustrated in Figure 1, was confirmed by a completely different method. Secondly, the foregoing pulse-labelling method was subsequently taken up by others[11] to confirm our earlier finding[2a] that the fourth methylation site is at C-17 and to extend this to indicate the later order as 12 then 1 followed by 5 and 15 in unknown sequence.

Late Stages of the Biosynthetic Pathway to Vitamin B_{12}.— Knowledge of the sequence of the last 5 *C*-methylation steps for the biosynthesis of vitamin B_{12} allows many of the main features of the pathway beyond the trimethylated stage (**6**) to be discussed. The point on the pathway where cobalt is inserted is unknown but experiments based on *Propionibacterium shermanii* indicate that this step is not the last one.[12] Also, Nussbaumer and Arigoni[13] found that 5,15-bisnorcobyrinic acid (**17**) and the 5-nor (**18**) and 15-nor (**19**) analogues are not incorporated into cobyrinic acid (**9**) in an active enzyme system from *P. shermanii*. These two studies together suggest that cobalt is inserted at some earlier stage so our proposals in Scheme 2 leave open the timing of cobalt insertion. However, it

* This result could also be explained in principle if (*a*) the last 5 methyl groups of vitamin B_{12} are introduced in a random order; (*b*) the enzyme which introduces the C-17 methyl group binds its substrates more strongly than that which introduces the C-12 methyl group and similarly for the others. This possibility seems so remote as to be neglectable.

† In the unlikely event that the errors accumulated in one direction for all 4 runs, then the discrimination between the methylation order at C-1 and C-15 would become doubtful.



Scheme 2.

must constantly be borne in mind that the transformations at or beyond the pyrrocorphins (**12**) and (**13**) could, in principle, be occurring on cobalt-containing species.

Methylation of the dihydro-trimethylated system (**6**) at C-17 generates the pyrrocorphin (**12**) which by decarboxylation produces the second pyrrocorphin (**13**); only the latter may be involved if C-12α decarboxylation preceeds C-17 methylation. That decarboxylation precedes the fifth methylation step is probable on the grounds that decarboxylation is far more readily rationalised mechanistically before rather than after 12α-methylation. Introduction of the fifth *C*-methyl group at the 12-position then produces the corphin * (**14**) and the appearance of this structure in the Scheme interlocks with Eschenmoser's earlier speculations.[14] Methylation of the corphin (**14**) at C-1 leads to a cation at C-20 which could be trapped by water to produce the tertiary alcohol (**15**) as illustrated or by the C-18 acetic acid residue to form a δ-lactone. Ring-contraction of either one by essentially the same mechanism leads to the corrin (**16**). The illustrated ring-contraction mechanism is based on Eschenmoser's important biomimetic model studies[15] together with the knowledge, already available,[16,17] that during ring-contraction, C-20 and its attached methyl group are extruded as acetic acid. It is not yet possible in any rational way to suggest what the methylation state is at C-5 and C-15 over the stages (**15**) and (**16**) but by having both methyl groups in place on the 19-acetylcorrin (**16**), the final hydrolytic step directly yields acetic acid and cobyrinic acid (**9**) ready for conversion into vitamin B_{12}. Work aimed at the preparation and/or detection of several of the intermediates in Scheme 2 is in progress.

Nomenclature.—Substances (**4**), (**7**) and (**8**) have often been called Faktors I, II and III, respectively, which is unfortunate because these names have already been used in the B_{12}-literature for quite different materials.[18] For example, Faktor III is the long established synonym for 5-hydroxybenzimidazolylcobinamide and Faktor V_{1a} is cobyrinic acid. Clearly to extend this 'Faktor system' in the future to other substances on or near the B_{12}-pathway would add more confusion and would be wrong. Moreover, the so-called Faktors I, II and III are *not* biosynthetic precursors of vitamin B_{12} so names are needed for the true mono-, di-, and trimethylated intermediates and for the intermediates still to be isolated.

We suggest, and will use henceforth, a logical nomenclature where the name of the intermediate makes evident that it is a precursor of the corrin ring system and the name will carry a number *corresponding to the number of C-methyl groups which have been introduced from SAM into uro'gen-III* to generate that intermediate. Thus the first three intermediates beyond uro'gen-III (**2**) are now named precorrin-1 (**3**), precorrin-2 (**5**), and precorrin-3 (**6**). Structure (**12**) would be precorrin-4A and structure (**13**) would be precorrin-4B. Further extension as far as the immediate precursor of the first-formed corrin macrocycle is obvious. The intermediates which are corrins are then simply named corrin with an attached number having the same meaning as for the earlier intermediates. For example, if the 5 and 15 methyl groups of vitamin B_{12} are introduced *after* corrin formation, then the 5,15-bisnor analogue of structure (**16**) could be involved; that would be corrin-6. But if the 5 and 15 methyl groups are in place before corrin formation, then structure (**16**) seems likely to be produced and its name would be corrin-8.

The value of this logical system is that it is flexible and we have satisfied ourselves that it can cope with a wide variety of structural permutations. This is necessary because earlier experience with the B_{12}-pathway suggests that there could still be further surprises. Importantly, we shall not give the numbered precorrin or corrin name to any intermediate prior to satisfactory evidence for its structure.

Experimental

General directions are given in refs. 19 and 20. In addition all water used in the biological work was glass distilled. Large-scale preparative t.l.c. was carried out on plates coated to 2mm with Kieselgel H60. All t.l.c. plates used for purification of cobester (**10**) were developed once in methanol saturated with potassium cyanide and then dried before loading with the sample. All solvent systems used for chromatography of cobester (**10**) were saturated with potassium cyanide. Diethylaminocellulose was

* Ref. 11 also included a biosynthetic sequence which differs from ours in particular by not involving the corphin (**14**).

Whatman DE52 and XAD resin was Amberlyst XAD-2 the latter being purified before use by Soxhlet extraction (24 h) with methanol followed by batch-wise washing with acetone, ethanol, methanol, and aqueous hydrochloric acid (pH 2.5). Dowex-50W resin was 8% cross linked and 100—200 mesh; it was pre-washed with concentrated hydrochloric acid and then equilibrated with M-hydrochloric acid. Amberlite IRA-400 was analytical grade and was washed with saturated sodium hydrogen carbonate and then extensively with glass-distilled water. Sephadex DEAE A-25 was allowed to swell in 0.05M phosphate buffer, pH 7.7 and then equilibrated for 18 h with the same buffer.

Preparation of Cobester Uniformly ^{13}C-*Labelled at the Methyl Groups.*—[*methyl*-^{13}C]-L-Methionine[19] (90 atom % ^{13}C; 250 mg) was administered with δ-aminolaevulinic acid (250 mg) as earlier[19] to *Propionibacterium shermanii* growing in Kreb's yeast-lactate medium (4 l) containing Co^{2+}. Growth was continued for 3 days and the cells were then harvested before the aerobic growth stage used previously.[19] The cells were suspended in sodium acetate buffer (200 ml) pH 4.5, potassium cyanide (600 mg) was added and the suspension was autoclaved at 15 p.s.i. for 15 min. The cooled mixture was adjusted to pH 2.5 with concentrated hydrochloric acid, centrifuged (18 000 r.p.m., 30 min, 5 °C) and the supernatant was applied in portions (50 ml) to an XAD-column (4 × 15 cm). After running through each portion, the column was washed with degassed aqueous hydrochloric acid, pH 2.5.

The pellets from the above centrifugation were ground with solid sodium chloride, concentrated hydrochloric acid (2 ml) was added and the resultant paste was extracted with acetone (2 × 200 ml). The extracts were evaporated, the residue was redissolved in water (50 ml), and the solution was adjusted to pH 2.5 with saturated aqueous sodium hydrogen carbonate. This solution was applied to the same XAD-column used above which was then washed with degassed aqueous hydrochloric acid pH 2.5 (1.5 l) before the pigments were eluted with methanol.

The residue from the methanol was thoroughly dried at high vacuum, dissolved in degassed methanol–concentrated sulphuric acid (95:5, v/v; 75 ml) and after addition of trimethyl orthoformate (4 ml), the mixture was refluxed under argon in the dark for 65 h. Then saturated aqueous sodium hydrogen carbonate (150 ml) and potassium cyanide (100 mg) were added to the cooled solution which was extracted thrice each with carbon tetrachloride and then with dichloromethane. The material from the latter was recycled through the above esterification procedure.

The products from both carbon tetrachloride extractions was fractionated first by p.l.c. on 2 mm plates using chloroform–methanol (95:5) and the cobester band was fractionated again on 0.25 mm plates using toluene–methanol (9:1). The silica carrying the cobester band was washed with dichloromethane and then the cobester (3.65 mg) was eluted with methyl acetate–methanol (9:1). The ^{1}H n.m.r. spectrum of this product showed by ^{13}C-^{1}H coupling that the ^{13}C-enrichment was *ca.* 50 atom %; it was also clear that this sample contained a small quantity of 13-epicobester. Accordingly, part (0.73 mg) was diluted with unlabelled highly pure cobester (2.7 mg) and repeatedly crystallised from methyl acetate–hexane to give the standard sample (1.6 mg) at *ca.* 10 atom % ^{13}C; this sample contained only a trace of 13-epicobester.

[methyl-^{13}C]-S-*Adenosyl*-L-*methionine*.[cf.8]—Dried yeast (20 g) was suspended in water (2.67 l) containing the following nutrients: $MnSO_4 \cdot H_2O$ (220 mg), $ZnSO_4 \cdot 7H_2O$ (267 mg), $CaCl_2$ (267 mg), $MgCl_2 \cdot 6H_2O$ (1.33 g), trisodium citrate (2.67 g), ammonium sulphate (5.33 g), K_2HPO_4 (13.3 g), KH_2PO_4 (26.7 g), glucose (40 g), and [*methyl*-^{13}C]-L-methionine (2 g) (85.5 atom % ^{13}C). The culture was incubated with stirring and continuous aeration in a 5-l conical flask at 30 °C. Marble chips (2 g) were added after 6 h to prevent the pH from dropping below 3;[21] these were removed after 72 h and the mixture was centrifuged (2 600 r.p.m.; 15 min; 4 °C). The yeast pellets were resuspended in phosphate buffer (2 g K_2HPO_4 + 4 g KH_2PO_4 in 400 ml water), recentrifuged, resuspended in water (400 ml), and centrifuged again.

The following procedures were performed at 4 °C. A suspension of the yeast in 1.5M perchloric acid (800 ml) was stirred for 1 h then centrifuged (14 000 r.p.m.; 20 min) and the clear supernatant was applied to an ion-exchange column (Dowex-50, 4 × 4.5 cm). The column was washed with 1M HCl (14 l) during 16 h and the product was eluted with 6M HCl (4 l) and precipitated by addition of a solution of ammonium reineckate (20 g) in water (400 ml) followed by concentrated hydrochloric acid (600 ml). After settling for 6 h, the crystals were collected, redissolved in water (250 ml), and the solution was extracted with butanol (4 × 50 ml) and ether (6 × 100 ml). The organic solvents were removed from the aqueous solution *in vacuo* or by purging with nitrogen and the pH was adjusted to 4.5 by addition of IRA-400 (HCO_3^--form); yield of SAM determined by u.v. spectroscopy[21] was 685 mg (12.9%), 84.9 atom % ^{13}C determined by ^{1}H n.m.r.-spectroscopy in D_2O. It was stored in the frozen state at −20 °C and was found spectroscopically to be stable over 16 months.

Cell-free Enzyme System from Clostridium tetanomorphum.—The cells were grown under standard anaerobic conditions for this organism by Mr. Ken Baker of the A.R.C. Unit of Nitrogen Fixation, Sussex. All operations below were carried out at 4 °C under argon.

The cell-paste (160 g) was partly thawed, chopped with a spatula, and suspended in 0.05 M-phosphate buffer pH 7.7 (160 ml). This was stirred until all lumps had broken and a few drops of polypropylene glycol were added if necessary to stop foaming. The cells were then broken by three passes through a French press and the resultant emulsion was centrifuged (18 000 r.p.m.; 90 min). The supernatant was applied to a column of DEAE-Sephadex (2.5 × 5 cm) and the filtrate was adjusted to pH 7.2 with saturated aqueous tripotassium phosphate. This enzyme preparation was held at 4 °C under argon and used immediately.

Determination of Endogenous S-*Adenosyl*-L-*methionine.*—To 30 ml of the foregoing enzyme system (corresponding to 20 g of cells) was added [*methyl*-^{13}C]-*S*-adenosyl-L-methionine (3.42 mg) (84.9 atom % ^{13}C) followed by 70% perchloric acid (3.7 ml) and the mixture was centrifuged (18 000 r.p.m.; 15 min). The supernatant was applied to Dowex-50 (1 × 5 cm), the column was washed with 1M HCl (1 l) and the SAM was eluted with 6M HCl (200 ml). It was precipitated by addition of ammonium reineckate (1 g) in water (20 ml). The crystals were collected after 8 h, redissolved in acetone–water (50 ml), and the solution was extracted with butanol (3 × 50 ml) and ether (3 × 100 ml). The dissolved organic solvents were removed from the aqueous solution *in vacuo* which was then freeze-dried.

This product was dissolved in saturated aqueous sodium hydrogen carbonate (1 ml) and kept at 20 °C for 5 days. The main degradation product (R_F 0.8) was isolated by t.l.c. using ethanol–acetic acid–water (93:5:2). It was dissolved in methanol, filtered twice through cotton-wool to remove silica, and evaporated. The ^{1}H n.m.r. spectrum of this product run in D_2O showed it to be *S*-methyl thioadenosine (**11**) carrying 82.1 atom % ^{13}C in the methyl group. The drop in enrichment from 84.9 to 82.1% corresponded to a dilution with 0.12 mg of natural SAM.

Time Course for Incorporation of ^{14}C-*Labelled Sirohydrochlorin* (**7**) *into Cobyrinic Acid.*—[2-*Methyl*,7-*methyl*-^{14}C]sirohydrochlorin octamethyl ester[22] (0.2 mg; 1.5 × 10^6 d.p.m.) was hydrolysed by stirring with aqueous 2M piperidine (5 ml) under argon in the dark for 18 h. The residue from evaporation was dried at high vacuum, dissolved in 0.2M phosphate buffer pH 8 (2 ml) and added to the foregoing enzyme preparation (70 ml corresponding to 40 g of frozen cells). 2-Mercaptoethanol (0.05 ml) and the following cofactors, dissolved in the minimum volume of the same phosphate buffer above were added: *S*-adenosyl-L-methionine (25 mg), ATP (50 mg), NADH (25 mg), glutathione (25 g), cysteine·HCl (8 mg), $CoCl_2 \cdot 6H_2O$ (6 mg), and disodium dihydrogen ethylenediaminetetra-acetic acid (50 mg). After these additions, the pH was readjusted to 7.2 and the flask was flushed with argon, wrapped in aluminium foil, and incubated at 37 °C. One fifth of the total incubate was withdrawn after 2 h, 4 h, 7.5 h, and 11 h. Cobyrinic acid (15 mg) and potassium cyanide (60 mg) were added to each of the samples, which were frozen in liquid nitrogen. After 18 h, the remaining fifth was treated in the same way.

All samples were worked up separately starting with the first one. The pH was brought to 2.5 with concentrated hydrochloric acid and the emulsion was centrifuged (15 min; 4 °C; 12 000 r.p.m.). The supernatant was applied to an XAD-column (2.5 × 10 cm) and the resin was washed with degassed, dilute hydrochloric acid, pH 2.5 (500 ml). The pigments were then eluted with methanol and recovered by evaporation, finally at high vacuum. A solution of the residue in degassed methanol–sulphuric acid (50 ml, 95:5; v/v) and trimethyl orthoformate (2 ml) was refluxed under argon in the dark for 24 h. Saturated aqueous sodium hydrogen carbonate (100 ml) and potassium cyanide (50 mg) were then added and the mixture was extracted with carbon tetrachloride (2 × 30 ml). The product from the organic layers was chromatographed on a 2 mm t.l.c.-plate using chloroform–methanol (95:5) as eluant. The cobester band was collected, washed with dichloromethane and eluted with methyl acetate–methanol (9:1). Multiple recrystallisation from methyl acetate–hexane was carried out to constant specific activity.

Pulse Labelling Experiments.—Both the Normal and Inverse pulse feedings were done in four separate runs and the general procedure for one of these runs is described. The Normal and Inverse pulses differed slightly in the amounts of substrates, cofactors, carrier, and times used, but the overall procedure was the same. Therefore letters are used for these quantities in the description below and the exact amounts for one run are given subsequently.

The foregoing enzyme preparation (220—240 ml corresponding to 160—170 g of cells) was diluted with 0.05M phosphate buffer pH 7.7 (160 ml). Then the following materials in the minimum volume of 0.2M phosphate buffer pH 8 were added: 2-mercaptoethanol (0.2 ml), ATP (200 mg), NADH (100 mg), NAD^+ (100 mg), glutathione (100 mg), cysteine·HCl (32 mg), $CoCl_2 \cdot 6H_2O$ (12 mg), and Na_2H_2EDTA (150 mg). The mixture was adjusted to pH 7.2 and maintained throughout the whole experiment at pH 7.0—7.2 by addition of saturated aqueous tripotassium phosphate. It was incubated with stirring in the dark under argon in a bath at 37 °C and when (after 30 min) the temperature of the incubate reached 37 °C, the following materials were added in the minimum volume of pH 8 0.2M phosphate buffer: sirohydrochlorin octapiperidinium salt (from *P* mg of the octamethyl ester, hydrolysed, evaporated, and dried as before) and *S*-adenosyl-L-methionine (*Q* mg).

After t_A, the following cofactors were dissolved in pH 8.0 0.2M phosphate buffer and added to the incubation flask: SAM (*R* mg), ATP (100 mg), NADH (40 mg), $CoCl_2 \cdot 6H_2O$ (12 mg), and Na_2H_2EDTA (50 mg). After t_B under the same conditions, the flask was cooled to 0 °C, cobyrinic acid (*S* mg) was added followed by DEAE-cellulose (*ca.* 3 g) which was collected after being stirred for 3—5 min. This treatment with DEAE-cellulose was repeated 3—4 times. The pigment-loaded resin was washed with water and stored at −20 °C (Resin *A*).

Potassium cyanide (100 mg) was added to the aqueous solution from the DEAE-cellulose treatment, the pH was adjusted to 2.5 with concentrated hydrochloric acid, and the resulting emulsion was combined for all four runs and centrifuged (30 min; 4 °C; 12 000 r.p.m.).

The supernatant was applied to an XAD column (15 × 4 cm) in portions (100 ml) followed by washing with degassed, dilute hydrochloric acid (pH 2.5, 500 ml). After 10 such portions had been applied, the pigments were eluted with acetone–water (6:4) and the same resin used again after equilibrating with degassed, dilute hydrochloric acid (pH 2.5). The combined orange-red eluate was neutralized with saturated sodium hydrogen carbonate and the acetone was evaporated with checks that the pH did not exceed 8 during this procedure. The resulting aqueous solution was treated with DEAE-cellulose (3 × 3 g) as before and the resin washed with water and stored at −20 °C (Resin B).

The pellets from the above centrifugation (*ca.* 160 g) were ground with the same amount of sodium chloride and transferred to a blender for stirring with acetone–water (6:4) (1.3 l) and then centrifuged again. The remaining pellets were extracted 3 times more in the same way after which they were essentially colourless. The combined extracts were evaporated to a residual volume of *ca.* 1 l with control of the pH between 7 and 8. The solution was acidified to pH 2.5 with concentrated hydrochloric acid and filtered through glass-wool. The pigments were then adsorbed onto DEAE-cellulose as described before (Resin C).

The combined DEAE-cellulose resins A, B, and C were washed with water, methanol, acetone, and ether and dried at high vacuum. It was then extracted with degassed methanol–sulphuric acid (150 ml 95 : 5, v/v), trimethyl orthoformate (3 ml) was added and the mixture was stored at 20 °C under argon in the dark for 40 h. It was then neutralised with saturated aqueous sodium hydrogen carbonate (300 ml), potassium cyanide (150 mg) was added, and the resulting mixture extracted with carbon tetrachloride (3 × 100 ml) and dichloromethane (3 × 50 ml). The product from the carbon tetrachloride was purified on a 2 mm plate using chloroform–methanol (95:5) as eluant to yield the main sample of cobester (**10**). A further part was obtained by reesterification of the material from the dichloromethane together with lower R_F compounds from t.l.c. of the main sample. The combined cobester was further purified on a 0.25 mm plate with toluene–methanol (9:1). The yield (*T*) was determined by u.v.-spectroscopy (ε_{593} = 12 000).

Normal Pulse Conditions.—Sirohydrochlorin octamethyl ester (*P*, 1.1 mg), *S*-adenosyl-L-methionine iodide (*Q*, 20 mg; Sigma; 85—90% pure; natural isotopic composition), [*methyl*-^{13}C]-*S*-adenosyl-L-methionine (*R*, 85.6 mg; 84.9 atom % ^{13}C), cobyrinic acid (*S*, 0.59 mg); heptamethyl dicyanocobyrinate (*T*, 1.77 mg; cobester); t_A 90 min; t_B 60 min.

Inverse Pulse Conditions.—Sirohydrochlorin octamethyl ester (*P*, 2.25 mg), [*methyl*- ^{13}C]-*S*-adenosyl-L-methionine (*Q*, 20.5 mg; 84.9 atom % ^{13}C), *S*-adenosyl-L-methionine chloride (*R*, 600 mg; Sigma; 70% pure; natural isotopic composition), cobyrinic acid (*S*, 0.55 mg), heptamethyl dicyanocobyrinate (*T*, 1.20 mg); t_A 135 min; t_B 30 min.

N.m.r. Spectra.—All three samples of cobester (*ca.* 1.5 mg) were dissolved in [$^2H_6$]benzene (0.25 ml) in a 5-mm n.m.r. tube and sealed under an inert atmosphere. The ^{13}C n.m.r. spectra

were recorded at 100.57 MHz on a Bruker WH400 with polarization transfer using the DEPT pulse sequence.[23] Typically the 90° ^{1}H pulse was 19 μs and the 90° ^{13}C pulse was 11 μs; the third ^{1}H pulse in the sequence was set at 36° to give optimum intensity for $^{13}CH_3$ groups and an optimum evolution time of 3.94 ms was used (1/2J for J = 127 Hz). Broad-band decoupling was accomplished by means of a carefully adjusted WALTZ-16 sequence[24] (γB_1 = 2.5 kHz) with a decoupler power of 0.5 W. In one set of experiments, acquisition was over a spectral width of 2.5 kHz (*ca.* 25 p.p.m.) and 8K data points; a relaxation delay of 0.5 s gave a recycle time of 2.1 s and 70 000 to 100 000 scans were acquired for each sample. In a second set of experiments, acquisition was over a spectral width of 3.0 kHz (*ca.* 30 p.p.m.) and 16K data points; a relaxation delay of 2.0 s gave a recycle time of 4.7 s and 27 000 to 30 000 scans were acquired for each sample.

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