Biosynthesis of Porphyrins and Related Macrocycles. Part 42.[1] Pulse Labelling Experiments Concerning the Timing of Cobalt Insertion During Vitamin B_{12} Biosynthesis

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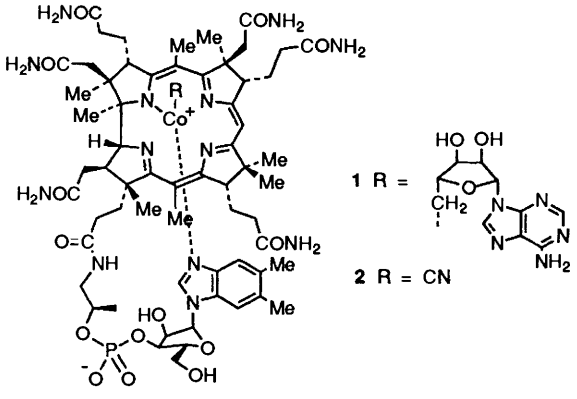
Pulse labelling experiments using $^{60}Co^{2+}$ and [*methyl*-^{13}C,^{14}C]-*S*-adenosyl-L-methionine have established that in the anaerobic organism *Propionibacterium shermanii* cobalt is inserted into the macrocycle after the second and before the fourth *C*-methylation step.

Cobalt plays a central role in the enzyme-catalysed chemistry carried out by coenzyme B_{12} **1** (the adenosylated derivative of vitamin B_{12} **2**) and it is of great interest to know at which point in the biosynthesis of this complex molecule the cobalt ion is inserted. For *Pseudomonas denitrificans*, an aerobic bacterium used for the commercial production of vitamin B_{12}, the biosynthesis of the corrin macrocycle, which involves, among many other steps, a characteristic ring-contraction process, is achieved without the formation of cobalt-complexed intermediates. In this organism the established[2] substrate for the cobalt-insertion process is hydrogenobyrinic acid a,c-diamide **11** (derived from **10**) and the complex cobaltochelatase enzyme



system responsible for catalysing this step has been isolated and characterised.[2] However, in a second B_{12}-producing organism, *Propionibacterium shermanii*, there were early signs that there is a different timing for cobalt insertion. When this bacterium is grown anaerobically in a strictly cobalt-free medium the normal cobalt-containing precursor of vitamin B_{12}, cobyrinic acid **9**, is not formed. Instead, pigments **7** and **8** are isolated[3] arising by aerial oxidation (during work-up) of the true biosynthetic intermediates precorrin-2 **4**† and precorrin-3A **5**.† A reasonable explanation for these observations is that normal cobalt insertion occurs at some point close to these stages (**4** and **5**) and the absence of cobalt causes the early cessation of corrin biosynthesis leading to the isolation of pigments **7** and **8**. This early insertion of cobalt in *P. shermanii* has also been recently supported by isolation and incorporation experiments.[4] Using a cell-free enzyme preparation the cobalt complexes of **7** and **8**, which are the dehydro forms of precorrin-2 **4** and precorrin-3A **5**, were found to be incorporated equally well (*ca.* 4%) into cobyrinic acid **9**.

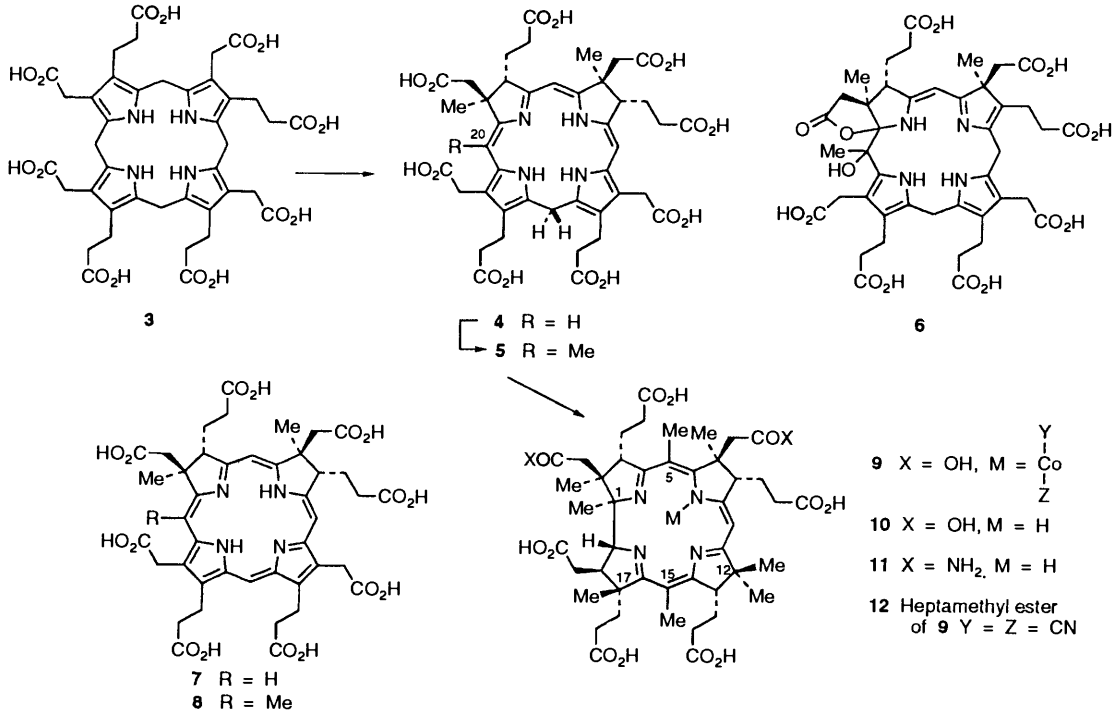
† Intermediates which follow uro'gen III **3** on the biosynthetic pathway to hydrogenobyrinic acid **10** and cobyrinic acid **9** are called precorrins and the accompanying number corresponds to the number of methyl groups derived from *S*-adenosyl-L-methionine which have been added to uro'gen III **3** to generate that intermediate. Letters, *e.g.* 3A, are used to distinguish different intermediates having the same number of such groups.

Results and Discussion

The precursors **4** and **5** form part of a series of methylated intermediates generated by the stepwise transfer of 8 methyl groups from *S*-adenosyl-L-methionine (SAM) and the first of these methyl groups is transferred onto uro'gen III† **3**. Our aim was to determine how many *C*-methyl groups had been added to the evolving macrocycle after the cobalt-insertion step by use of pulse labelling, a technique that provides information about the order of sequential events.[5]

The first experiments were with *P. shermanii* cells grown in the strict absence of cobalt ions; it was found that the derived cell-free enzyme preparation was able to biosynthesise cobyrinic acid **9** when cobalt ions were added. Accordingly, the experimental design was as follows: (*a*) to a cobalt-free enzyme preparation add the necessary cofactors for cobyrinic acid biosynthesis including unlabelled SAM, sirohydrochlorin **7** (which is known to be reduced *in situ* to precorrin-2 **4**) and a small quantity of $^{60}CoCl_2$ at known specific activity; (*b*) incubate the preparation for an appropriately short period (t_A) to allow the biosynthesis to progress a little beyond precorrin-2 **4**; (*c*) add [*methyl*-^{14}C]SAM of known specific activity together with sufficient unlabelled $CoCl_2$ greatly to reduce the specific activity of ^{60}Co in the medium; (*d*) incubate the preparation for a long period (t_B) in order to complete the conversion of intermediates present after t_A into cobyrinic acid **9**; (*e*) isolate the cobyrinic acid produced to measure the incorporation of ^{60}Co and ^{14}C. Clearly if the cobalt insertion step is entirely or largely completed during t_A then the resultant cobyrinic acid **9** will contain ^{60}Co at high specific activity. Conversely, if little or no cobalt has been incorporated during t_A **9** will carry ^{60}Co of low specific activity. Similarly, the ^{14}C-activity will give a measure of the approximate number of *C*-methyl groups added after the t_A period.

The appropriate period for t_A was selected after running a time-course for the incorporation of ^{14}C-labelled precorrin-2 **4** (derived *in situ* from ^{14}C-sirohydrochlorin **7**) into cobyrinic acid using the complete enzyme system in a standard incubation with unlabelled SAM and $CoCl_2$. The length of the incubation before taking the sample and resultant incorporation into **9** in each case were: 0.5 h, 0.9%; 1.5 h, 5.2%; 4 h, 7.9%; 8 h, 11.8%; 19 h, 16.1%. These results led to values of t_A being chosen in the range 15–45 min. Finally, the pulse experiments



require cobyrinic acid **9** to be isolated on a micro-scale without dilution with unlabelled carrier; the usual approach of esterification followed by purification of the heptamethyl ester **12** was not practical on the minute amounts involved. Direct chromatographic methods on ion-exchange or reverse-phase columns were therefore used.

Initially, six experiments were carried out which followed the above protocol except for the substrate for the enzyme system being the precorrin-2 **4** and precorrin-3A **5** accumulated during the cobalt-free growth of the cells rather than from addition of sirohydrochlorin **7**. Radio-assay results from all these experiments left no doubt that cobalt insertion for B_{12} biosynthesis occurs early in *P. shermanii*, probably close to precorrin-3A **5** but candidates with 1 methyl group more or 1 methyl less also fitted the spread of ^{14}C and ^{60}Co labelling values. It is not necessary to give practical details for these experiments because their value lay in establishing the viability of the approach, in providing a sharper focus on what t_A should be, and in building the foundation for the definitive pulse studies now described with full experimental data.

The foregoing experience led to the following changes in approach: (*a*) *P. shermanii* cells grown normally on a cobalt-containing medium were used since the derived cell-free enzyme system gave higher incorporations into cobyrinic acid **9** than the enzyme preparation used for the earlier experiments; (*b*) the enzyme system was freed from possible endogenous B_{12} precursors by passage through DEAE-Sephadex A-25 and then sirohydrochlorin **7** was added as the source of precorrin-2 **4** so as to fix the starting point for the pulse study; (*c*) the possible presence of unlabelled Co^{2+} ions in the enzyme system was covered by carrying out an appropriate standardisation experiment (details below); (*d*) after the short incubation period (t_A), 99 atom% [*methyl*-^{13}C]SAM containing a tracer amount of [*methyl*-^{14}C]SAM was added together with the large amount of unlabelled $CoCl_2$. The use of [*methyl*-^{13}C]SAM at this point allowed those methyl groups incorporated after cobalt insertion to be examined individually by ^{13}C NMR.

The above new enzyme preparation was split into 1 part and 10 parts. To the first part, sirohydrochlorin **7** (to yield precorrin-2 **4**) and all the cofactors were added together with $^{60}CoCl_2$. After incubation for the long period t_B, the cobyrinic acid **9** formed was isolated by reverse-phase HPLC, the amount determined by absorption spectroscopy and the activity of the cobalt-60 it contained was measured. Using this analysis the specific molar activity of the cobyrinic acid could be calculated which corresponds to the specific activity of cobalt at the start of the incubation for any particular enzyme preparation. A standardisation experiment was carried out for each enzymic preparation as the final specific activity is dependent on the concentration of endogenous unlabelled Co^{2+} ions.

The larger part of the prepared enzyme system was used for the pulse labelling experiment and 10 times the amount of $^{60}CoCl_2$ was added at the outset so as to match the standardisation experiment above. Sirohydrochlorin **7** and all the cofactors (unlabelled SAM) were added as before. After a very short t_A (10 min) [*methyl*-^{13}C,^{14}C]SAM and the large amount of unlabelled $CoCl_2$ were added and incubation continued for t_B (19 h). One quarter of this incubation mixture was then used for the direct isolation of undiluted cobyrinic acid **9** and the pure material was radio-assayed. Remarkably, the specific activity of the cobalt-60 it contained was 93% of the value found in the standardisation experiment. This demonstrated that almost all of the cobalt finally present in cobyrinic acid is inserted very rapidly during the t_A period. This result also shows that the complexed cobalt remains securely held in the various macrocycles involved and little or no exchange occurs with the large excess of unlabelled Co^{2+} ions present throughout the remaining biosynthetic steps leading to cobyrinic acid **9**. In addition, the ^{14}C-specific activity of the cobyrinic acid **9** indicated that 5 ± 0.5 methyl groups had been transferred during t_B. Since the overall change **4**→**9** requires addition of 5 methyl groups, these results support the conclusion that the last 5 methyl groups for **9** are all added after t_A, *i.e.*, after cobalt insertion.

Now ^{13}C NMR could be used to show directly which *C*-methyl groups of cobyrinic acid **9** had been added during t_B. To this end, a small amount of unlabelled cobyrinic acid was added as carrier to the remaining three quarters of the incubation

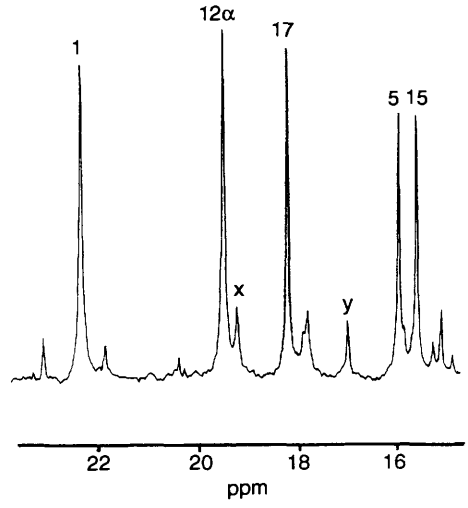


Fig. 1 ^{13}C NMR (100.6 MHz) spectrum run in C_6D_6 of ^{13}C, ^{14}C, ^{60}Co-labelled cobester **12** from the pulse experiment involving $^{60}CoCl_2$ and [^{13}C, ^{14}C-*methyl*]SAM. Data acquired over spectral width 21 739 Hz and 32K data points using CPD (Waltz-16) decoupling with 70K scans, acquisition time 0.754 s, line broadening factor 3 Hz and 1.327 Hz/data point. The large numbered signals are from the ^{13}C-labelled methyl groups and the small signals, marked x and y, are the natural abundance signals from the 7-Me and 2-Me groups, respectively.

mixture and the cobyrinic acid was isolated by the normal procedure[5] with esterification to yield cobester **12**. The ^{13}C NMR spectrum of this product (Fig. 1) was compared with the identically recorded ^{13}C NMR spectrum of a sample of cobester **12** uniformly ^{13}C-labelled in all its SAM-derived methyl groups; this standard sample was prepared biosynthetically[5] from δ-aminolaevulinic acid and 90 atom% [*methyl*-^{13}C]-L-methionine. Lorentzian line-fitting measurements, with corrections for the slightly differing peak areas from the uniformly labelled sample, gave the following values for the intensities of the signals from the methyl groups: 17-Me, 0.76; 12α-Me, 0.77; 1-Me, 0.93; 15-Me, 0.83; 5-Me, 1.0.

The key information provided by these values is that *all* the SAM-derived methyl groups added to precorrin-2 **4** and which remain* in cobyrinic acid **9**, are heavily ^{13}C-labelled. Importantly, this set includes the fourth methyl group to be added[8] at C-17 and, therefore, must largely be incorporated *after* the 10 min t_A period in agreement with the conclusions above. It is thus established by direct enzymic experiments based on normally grown cells that in *P. shermanii* cobalt is inserted into the macrocycle after the second but before the fourth *C*-methylation, *i.e.* into precorrin-2 **4**, precorrin-3A **5** or precorrin-3B[9] (**6** or isomer).

The intensities of the ^{13}C signals listed above from the pulse experiment fit the established order of *C*-methylation,[5,8,10,11] *viz.* 17, 12α, 1, 5/15, apart from the 15-Me signal. This signal would be expected to have a similar intensity to the signal from 5-Me but bearing in mind the precise repetitive measurements needed to ensure a small margin of error in our original studies on the methylation order,[5] a greater error margin for the single experiment described above is not surprising. This punctilio in no way affects the main conclusion above that in *P. shermanii* all five *C*-methylations (at 17, 12α, 1, 5/15) occur after cobalt insertion.

* The next methyl group to be added to precorrin-2 **4** is placed at C-20 to give precorrin-3A **5** but this methyl is lost as acetic acid[6,7] during the ring-contraction process.

Experimental

General Directions.—Except where stated otherwise, the following procedures were adopted. Electronic spectra were recorded on a Kontron Uvikon spectrophotometer. All NMR spectra were recorded on a Bruker WH-400 instrument operating at 400 MHz (^1H) or 100.6 MHz (^{13}C) and the solvent signal was used as internal reference. The cells and cell-free systems were maintained at 0 °C throughout and protected against light and oxygen where possible; all water used in the biological work was glass-distilled. Centrifugations were performed on a Europa 24M centrifuge with an 8 × 50 cm^3 rotor at 4 °C. The bacterial cells were broken by three passes through a French pressure cell press (American Instruments Company) at a pressure of 10 000–16 000 p.s.i. under argon. Enzymic incorporation experiments were carried out in the dark under argon in a system equipped with a pH electrode and an automatic pH titration system consisting of a PH 82 standard pH meter, TTT 80 titrator and AUB80 autoburette (Radiometer, Copenhagen). HPLC purifications were carried out using a Waters 600E multisolvent delivery system and pigments were detected by a CE 272 linear readout UV spectrometer (Cecil Instruments) at 365 nm (sensitivity range 0.1 to 1.0) equipped with an RE 511.20 recorder (Venture, 10 mV range). The reverse-phase column was a Hichrom S 5 ODS 1 (4.6 × 250 mm) and, before injection, samples were filtered through Acrodisc 3 (0.45 mm, Gelman Sciences). Sep-Pak C-18 cartridges were from Waters. Preparative TLC was performed on plates coated to 2 mm with Kieselgel H60 and all solvent systems used for chromatography of cobester **12** were saturated with KCN. Radiochemical analyses were carried out on a liquid scintillation counter (United Technologies Packard, 2000 CA, Tricarb liquid scintillation analyser). ATP, NAD^+ and $NADP^+$ were from Boehringer Mannheim, glutathione, DL-cysteine, *S*-adenosyl-L-methionine, DL-dithiothreitol and DEAE-Sephadex A-25 from Sigma, and $CoCl_2 \cdot 6H_2O$, EDTA and $MgCl_2 \cdot 6H_2O$ from Fisons. Radioactive [^{60}Co]-$CoCl_2$ solution was purchased from Amersham (nominally 1.85 MBq, 50 μCi, 0.23 cm^3 containing 0.95 $\mu g/cm^{-3}$ cobalt). Radioactive [^{14}C-*methyl*]-*S*-adenosyl-L-methionine (60 μCi/mmol) was from Sigma. The [^{13}C-*methyl*]-SAM was prepared from [^{13}C-*methyl*]-L-methionine, 99 atom% ^{13}C, using a yeast cell-culture procedure.[5] Acetonitrile, 1,2-dichloroethane and tetramethylethylenediamine were distilled from calcium hydride. Wet organic solutions were dried over anhydrous Na_2SO_4. Solvents were evaporated on a Buchi rotavapor at reduced pressure.

Preparation of Cell-free Enzyme System and Precursors.—Two batches of sirohydrochlorin octamethyl ester (0.2 mg and 2.0 mg) were hydrolysed in a glove box (oxygen content < 6 ppm) with 2 mol dm^{-3} aqueous piperidine at room temperature for 48 h and then freeze dried.

Deep-frozen cells of *P. shermanii* (275 g, grown in medium containing cobalt chloride) were thawed on ice then transferred to a stoppered 500 cm^3 conical flask containing potassium phosphate buffer (50 mmol dm^{-3}, pH 7.7, 275 cm^3) and stirred to make a homogeneous suspension. The cells were broken in a French pressure cell press as above and the product centrifuged (40 000 × *g*, 1 h). The supernatant was passed through a column of DEAE-Sephadex A-25 (10 g; the resin had previously been kept under vacuum for 10 min and then swollen under argon in the pH 7.7 phosphate buffer overnight at 4 °C). The column was washed with pH 7.7 phosphate buffer and the eluent and washings were collected under argon. The cell-free preparation was divided, 1 part (30 cm^3, equivalent to 25 g of cells) was taken for the standardisation experiment and 10 parts (300 cm^3, equivalent to 250 g of cells) for the pulse experiment.

Standardisation Experiments.—The above cell-free extract

(30 cm^3) was maintained at 30 °C in a three-necked flask (100 cm^3) equipped with a pH electrode and automatic pH titrator and the cofactors ATP (38.2 mg), NAD^+ (18.6 mg), NADH (19.9 mg), glutathione (18.3 mg), DL-cysteine (4.3 mg), $MgCl_2 \cdot 6H_2O$ (14.3 mg) and SAM (20 mg) were added. These additions were followed by the addition of labelled cobalt $^{60}CoCl_2$ solution (2.41 × 10^5 dpm, specific activity, 4.0 × 10^{13} dpm/mmol) and the sirohydrochlorin (*ca.* 0.2 mg) prepared as above. The stirred mixture was incubated in the dark under argon for 19 h at pH 7.7 maintained by automatic titration with argon-saturated 1 mol dm^{-3} NaOH. To the final mixture was added two volumes of pyridinium acetate buffer (27 mmol dm^{-3}, pH 4.1), the pH was adjusted to 4.0 with 2 mol dm^{-3} HCl and the precipitated proteins were removed by centrifugation (40 000 × *g*, 10 min). The supernatant was passed through reverse-phase C-18 cartridges (pre-treated with 1% TFA in ethanol followed by 0.02 mol dm^{-3} aqueous HCl) and the adsorbed cobyrinic acid was eluted with 20% acetonitrile in pyridinium acetate buffer. The eluent was freeze dried and the residue was dissolved in phosphate buffer, filtered through an Acrodisc cartridge and injected onto a reverse-phase ODS, C-18 HPLC column eluting with a 0→20% acetonitrile gradient in potassium phosphate buffer (100 mmol dm^{-3}, pH 5.0 containing 10 mmol dm^{-3} KCN) over 75 min. The fractions containing cobyrinic acid (eluting at *ca.* 28 min) were collected, desalted by adsorption on and elution from C-18 Sep-pak cartridges (as above) and freeze-dried to give pure cobyrinic acid for radiochemical analysis. Cobyrinic acid was measured by UV–visible spectroscopy (FW 991, extinction coefficient 1.018 × 10^4 dm^3 mol^{-1} at 577 nm) and 2.29 nmol was counted (^{60}Co, 1640 dpm, specific activity of ^{60}Co in cobyrinic acid = 7.16 × 10^8 dpm/mmol). This value corresponds to the specific activity of the ^{60}Co after dilution with unlabelled Co^{2+} present in the cell-free system at the outset and shown by these specific activity values to amount to *ca.* 0.04 mg.

Determination of ^{60}Co *and* ^{14}C *Counting Efficiencies.*—Although radioactive counting of samples labelled with both ^{14}C and ^{3}H is routine, we are not aware of cases where samples labelled with both ^{14}C and ^{60}Co have been counted. ^{60}Co emits both γ- and β-radiation with a half life of 5.27 years. Although counting the γ-emissions is possible, a much greater efficiency, approaching 100%, is obtained by counting the β-emissions in a liquid scintillation counter. The energy of the β-emissions from ^{60}Co is in the range 0–318 KeV, whereas for ^{14}C it is in the range 0–156 KeV. This difference in the ranges is sufficient for the two types of emission to be distinguished but clearly a considerable proportion of the ^{60}Co emissions will occur in the same energy range as the ^{14}C emissions and so the accuracy of counting both nuclei (especially ^{14}C) will be affected.

After various trials the following procedure was adopted for dual counting of ^{14}C and ^{60}Co. The scintillation counter was set to count in the ranges 0–110 KeV (region A) and 110–1000 KeV (region B) and quench calibration curves were generated by counting a series of ^{14}C and ^{60}Co standards. The counting efficiencies were for ^{14}C *ca.* 88% in region A and 5–7% in region B and for ^{60}Co *ca.* 55% in region A and *ca.* 38% in region B. However, despite this calibration, it was routinely found that a small proportion of the ^{60}Co counts spilled over into the computed value for the ^{14}C activity. As a result, the following method for correcting the computed ^{14}C counts was used. After counting each dual labelled sample, two separate aliquots carrying known activities of ^{14}C and ^{60}Co were added, each approximately equal to the activities computed after the first count, and the samples were then recounted. If the activities computed after the first count for ^{14}C and ^{60}Co were a_1 and b_1, the computed activities after the second count were a_2 and b_2, and the actual activities added between these counts were a_0 and b_0, then the spill-over of the added ^{60}Co into the computed ^{14}C activity is $a_2 - a_1 - a_0$ and represents a fraction $(a_2 - a_1 - a_0)/b_0$ of the added ^{60}Co counts. Since the same fraction of the ^{60}Co counts spilled over into the ^{14}C counts during the first count, the ^{14}C count can be calculated as a_1-$(a_2 - a_1 - a_0)b_1/b_0$. The correction required was typically about 7% of the ^{60}Co activity.

The ^{14}C standards used for calibration of the regions A (0–110 KeV) and B (110–1000 KeV) employed for ^{14}C/^{60}Co dual counting were supplied by Packard. The ^{60}Co standards were made up by adding equal aliquots of a stock solution of $^{60}CoCl_2$ to each of six scintillation vials and adding varying amounts of cobester to each vial to vary the quenching. The ^{60}Co standards were counted in the energy range 0–2000 KeV and 100% counting efficiency was assumed for this range.

Pulse Experiments.—The pulse experiment was the same as the standardisation run as far as the addition of sirohydrochlorin but on 10 times the scale *except* that 32 mg of unlabelled SAM were added not 10 × 20 mg. Addition of substrate (*ca.* 2 mg) marked the starting time. After incubation for 10 min the following were added: (i) unlabelled cobalt chloride (101.4 mg $CoCl_2 \cdot 6H_2O$) and (ii) [*methyl*-^{13}C,^{14}C]SAM (135 mg; 99 atom% ^{13}C; ^{14}C 9.34 × 10^6 dpm, specific activity 2.87 × 10^7 dpm/mmol). The incubation was continued for 19 h and the mixture was divided into 1 part (60 cm^3) and 4 parts (240 cm^3).

Work up of the 1 *part.* To the mixture was added twice its volume of pyridinium acetate buffer (27 mmol dm^{-3}, pH 4.1) and, after adjustment of the mixture to pH 4.0 with 2 mol dm^{-3} HCl, the procedure described for the standardisation experiment was followed. Pure cobyrinic acid so isolated was assayed as above using liquid scintillation with the instrument programmed to discriminate ^{60}Co and ^{14}C decompositions. Following correction for ^{60}Co spill over, the values found for cobyrinic acid were: specific activity of ^{60}Co, 6.7 × 10^8 dpm $mmol^{-1}$ (93% of that in the standard experiment) and of ^{14}C, 1.28 × 10^8 dpm $mmol^{-1}$, which on the basis of the specific activity (2.87 × 10^7 dpm $mmol^{-1}$) of the added labelled SAM corresponds to the transfer of 5.0 ± 0.5 methyl groups during t_B. The error margins have been set to contain the true value bearing in mind (*a*) that the specific activity of the labelled SAM at the start of t_B is probably somewhat lower than that of the added SAM because of dilution with an unknown quantity of unlabelled SAM remaining after t_A which lies in the range 0–32 mg and (*b*) the extensive experience in Cambridge of the errors in double-labelling experiments.

Work up of 4 *parts* (*for* ^{13}C *NMR spectroscopy*). To the 4 parts of the foregoing incubation mixture (240 cm^3) were added unlabelled cobyrinic acid (4 mg) as carrier material and KCN (50 mg) and the pH was adjusted to 3.5 with 3 mol dm^{-3} HCl. The suspension was centrifuged (18 000 × *g*, 15 min), the supernatant stored at 4 °C and the pellets ground with an equal amount of sodium chloride then with acetone–water (6:4; about 100 cm^3). After centrifugation, the supernatant was stored at 4 °C and treatment of the pellets with sodium chloride and acetone–water repeated once to give colourless pellets. The combined supernatants were evaporated to remove acetone, adjusted to pH 2.5 with 3 mol dm^{-3} HCl and passed through a column of Amberlite XAD-4 resin; the resin had been prewashed with methanol, water and finally with pH 2.5 degassed water. The adsorbed pigments were washed with degassed acidic water (pH 2.5), eluted with degassed methanol and the eluent was concentrated to a small volume then freeze-dried. To the residue was added methanol saturated with HCN and the filtered solution was evaporated at high vacuum to remove all moisture. The residue was treated with dry methanol (12 cm^3), conc. H_2SO_4 (0.15 cm^3) and freshly distilled trimethyl orthoformate (0.6 cm^3) and the mixture was heated at 60 °C

under argon in the dark for 20 h. The cooled mixture (0 °C) was treated with saturated aqueous sodium hydrogen carbonate (50 cm^3) and extracted into carbon tetrachloride (4 × 30 cm^3), the extracts being washed with degassed water, dried and evaporated to dryness. The cobyrinic acid heptamethyl ester (cobester) so obtained was purified by PLC using benzene–methanol (9:1) saturated with KCN. The band corresponding to cobester was eluted with methyl acetate and this material in C_6D_6 was used for ^{13}C NMR spectroscopy at 100.6 MHz (Fig. 1). The ^{13}C NMR spectrum of uniformly [^{13}C]-labelled cobester[5] was also recorded using identical NMR parameters for comparison with labelled cobester from the pulse experiment.

Acknowledgements

Grateful acknowledgement is made to Tim Dickerson for growth of the cells, to Dr. S. K. Basu (Director, National Institute of Immunology, New Delhi) for providing leave of absence for R. A. V., to the Deutsche Forschungs Gemeinschaft and The Royal Society for the award of a postdoctoral fellowship (to A. P.) and to Zeneca, Hoffmann-La Roche, Roche Products, SERC and the Leverhulme Trust for financial support.

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Paper 3/06807B
Received 15*th November* 1993
Accepted 30*th November* 1993