Biosynthesis of Vitamin B_{12}: Pulse Labelling Experiments to Locate the Fourth Methylation Site

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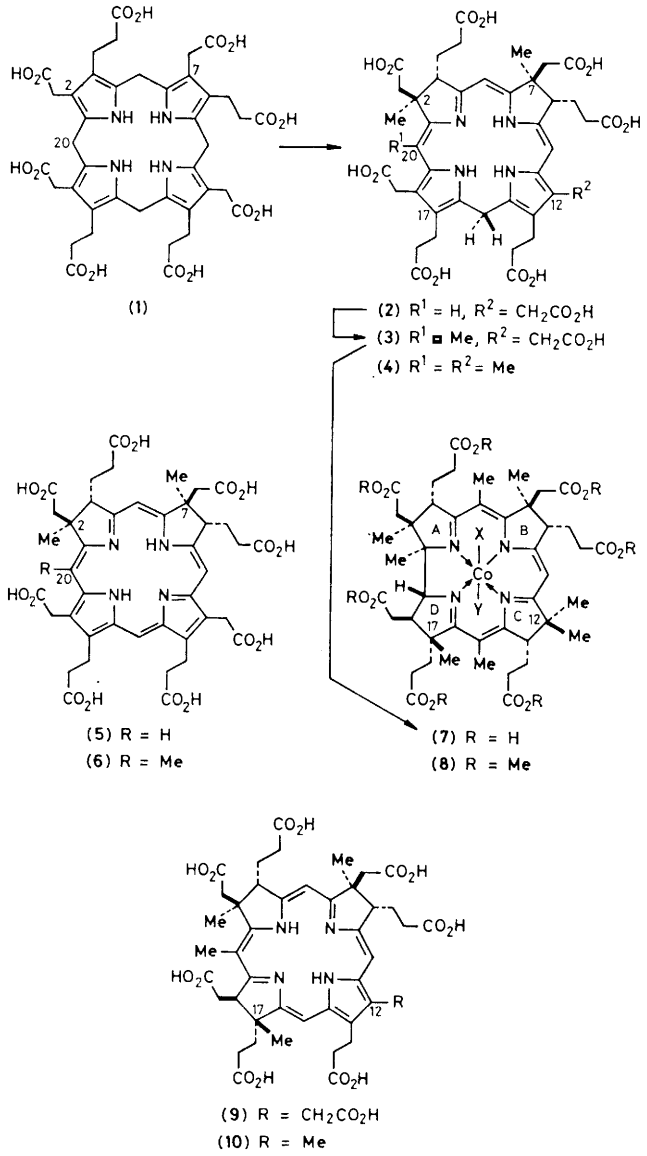
Pulse labelling experiments point to C-17 as the site where the fourth methyl group is introduced in the biosynthesis of vitamin B_{12}.

Cobyrinic acid (**7**), the precursor of vitamin B_{12}, is biosynthesised from uro'gen-III (**1**) by a multi-step sequence of reactions of which the first three are *C*-methylations. The first methyl group is introduced at C-2,[1] the second at C-7,[2] and the third at C-20.[3,4] It has also been recently shown[5] that the 2,7-dimethylated intermediate is the dihydroisobacteriochlorin (**2**) and it is highly probable that the trimethylated system is similarly formed and used in the dihydro-state, *i.e.* as structure (**3**). The dimethylated and trimethylated intermediates are normally isolated[2,3] as the aromatised macrocycles (**5**) [sirohydrochlorin] and (**6**) which are derived from the dihydro-systems by aerial oxidation. Enzymic reduction of the aromatic to dihydro-systems must occur when the former substances (**5**) and (**6**) are incorporated into cobyrinic acid (**7**) by enzymic preparations from B_{12}-producing organisms.[4]

The only knowledge of the pathway beyond the trimethyl system (**3**) is that the ring-contraction which generates the direct connection of ring-A to ring-D in cobyrinic acid (**7**) involves extrusion of C-20 and its attached methyl group as acetic acid;[6,7] this is mechanistically very important.

Despite huge efforts, no traces of intermediates on the B_{12}-pathway carrying 4 methyls, 5 methyls, and so on up to 7 or 8 methyls have so far been found. Thus these intermediates must be (a) enzyme-bound (*cf.* polyketides), (b) present in minute amounts, or (c) unstable. Still worse, they



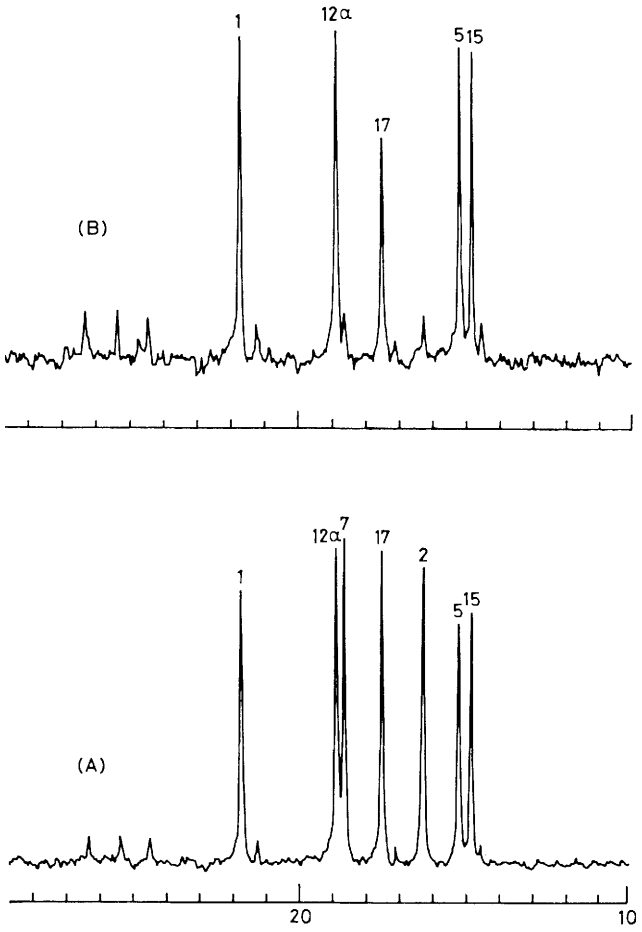


Figure 1. Proton-noise decoupled ^{13}C-spectra showing enhanced signals from (A) cobester [as (**8**)] uniformly ^{13}C-labelled at the SAM-derived methyl groups, and (B) cobester [as (**8**)] from the pulse labelling experiment with ^{13}C[SAM]. Both spectra recorded at 100.6 MHz in C_6D_6 with line-broadening (4 Hz); when line broadening was not used, the same overall pattern was obtained showing considerable reduction of the signal from C-17 in (B).

could combine some or all of these characteristics. These considerations guided our thinking for the pulse labelling experiments here described and the plans were as follows.

Use a cell-free enzyme system from *Clostridium tetanomorphum* which is known to convert added sirohydrochlorin (**5**) into cobyrinic acid (**7**); *S*-adenosylmethionine (SAM) is the source of methyl groups.[4] At the start of the experiment, add only a small amount of *unlabelled* SAM to the enzyme preparation containing added sirohydrochlorin (**5**). Allow a suitable incubation time t_A for methylated intermediates to be produced from the dimethyl precursor (**2**) and then add a large excess of [*methyl*-^{13}C]SAM. Continue the incubation for time t_B to produce cobyrinic acid (**7**). This product, isolated as cobester (**8**), 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 can be determined by ^{13}C-n.m.r. spectroscopy.

Success in this approach requires (i) assignment of the ^{13}C-n.m.r. spectrum of cobester (**8**); this has been completed,[8,9] (ii) determination by dilution analysis of the quantity of endogenous (unlabelled) SAM in the enzyme preparation, (iii) selection of appropriate times t_A and t_B for a particular enzyme preparation and set of conditions; this was achieved by measuring the time-course for incorporation of ^{14}C-labelled sirohydrochlorin (**5**) into cobyrinic acid (7—8 h for completion) and then optimising t_A and t_B by a series of trial labelling experiments, and (iv) the development of new work-up methods which allow isolation in good yield of the small quantity of biosynthesised cobacid (**7**) from the proteinaceous enzyme preparations without burying the information it contains by excessive dilution with either endogenous corrins or exogenous cobacid (**7**) added as carrier.

When these foundations had been built and with the preparation of [*methyl*-^{13}C]SAM enzymically from [*methyl*-^{13}C]-methionine,[10] the pulse labelling studies were possible. The enzyme system was prepared from *C. tetanomorphum* cells (160 g) using a French press, (*cf.* ref. 11), the set of cofactors[11] was added, and after the mixture at pH 7.0—7.2 had warmed to 37 °C, sirohydrochlorin piperidinium salt [as (**5**), 1.1 mg] and unlabelled SAM (20 mg) were added. The incubation was continued for t_A and then [*methyl*-^{13}C]SAM (85.6 mg) was added. After t_B under the same conditions, unlabelled cobyrinic acid (0.59 mg) was added as carrier and the mixture was worked up to isolate total cobyrinic acid (**7**). Four such experiments were run, the combined cobyrinic acid was esterified, and the resultant ^{13}C-labelled cobester (**8**) was rigorously purified for ^{13}C-n.m.r. spectroscopy.

Next, a standard sample of cobester was prepared having uniform ^{13}C-labelling at the SAM-derived methyl groups by feeding a large quantity of [*methyl*-^{13}C]methionine to growing *Propionibacterium shermanii* cells.[4] Figure 1(A) shows the proton-noise decoupled ^{13}C-signals from the ^{13}C-enriched methyl groups of this standard cobester [as (**8**)]. The ^{13}C-spectrum of the cobester from the pulse labelling experiment was then determined under *precisely* the same conditions (including all n.m.r. parameters, concentration of solute, *etc.*); this spectrum is shown in Figure 1(B). Because the precursor for the pulse feeding was unlabelled sirohydrochlorin (**5**), which already carries methyl groups at C-2 and C-7, the spectrum in Figure 1(B) has, as expected, only very small signals corresponding to these two methyl groups. However, the striking result is that the signal from the methyl group at C-17 of the pulse labelled cobester [as (**8**)] is of considerably lowered intensity (*ca.* 70% of standard).†

This result points to the fourth methyl group being introduced at C-17 for the biosynthesis of cobyrinic acid (**7**) and vitamin B_{12}.‡ At present there are two possibilities. If *C*-methylation at C-17 precedes decarboxylation of the acetate residue at C-12 of (**3**), then our results point to the pyrrocorphin§¶ (**9**) as the next intermediate beyond the trimethylated system (**3**). However, if decarboxylation occurs first, then the dihydroisobacteriochlorin (**4**) and the pyrrocorphin (**10**)¶ are picked out as candidates for the next two intermediates following the trimethylated macrocycle (**3**).

† The intensity differences for the other signals relative to standard were not significantly distinguishable from the experimental error.

‡ In principle, this result could also be explained if the last five methyl groups are introduced in a random order and the enzyme for methylation at C-17 binds its substrate most strongly. This possibility seems so remote as to be neglectable.

§ Pyrrocorphins carrying alkyl substituents have recently been synthesised, J. E. Johansen, V. Piermattie, Ch. Angst, E. Diener, Ch. Kratky, and A. Eschenmoser, *Angew. Chem., Int. Ed. Engl.*, 1981, **20**, 261; R. Schwesinger, R. Waditschatka, J. Rigby, R. Nordmann, W. B. Schweizer, E. Zass, and A. Eschenmoser, *Helv. Chim. Acta*, 1982, **65**, 600; C. J. R. Fookes, A. R. Battersby *et al.*, forthcoming paper.

¶ Or conceivably metal complexes thereof.

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