Proof that the Biosynthesis of Vitamin B_{12} involves a Reduction Step in an Anaerobic as well as an Aerobic Organism

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Labelling experiments with ^2H and ^3H prove that as cobyrinic acid is biosynthesised by the anaerobic bacterium *Propionibacterium shermanii*, its H-19 is derived from H_R (and not H_S) at C-4 of a reduced nicotinamide cofactor, so demonstrating that the biosynthetic pathway involves a reduction step.

Recently, knowledge of the biosynthetic pathway to corrins represented by hydrogenobyrinic acid **1**, a precursor[1] of vitamin B_{12} in the aerobic bacterium *Pseudomonas denitrificans*, has increased rapidly. This forward surge was initiated by the isolation[2] of precorrin-6x and proof[2–4] of its structure **4**. Precorrin-6x is an efficient biosynthetic precursor[2] of **1**. The structure of **4** implied several steps in the biosynthesis of **1**, which caused great surprise, one of these being the reduction step,[5] which converts precorrin-6x into the next intermediate precorrin-6y[5] **5**.[5,6] This was unexpected because consideration of the oxidation states of early precursors, *e.g.* precorrin-2 **6a**, and hydrogenobyrinic acid **1** together with the knowledge that the ring-contraction process in another B_{12}-producing organism (*Propionibacterium shermanii*) had been shown to release acetic acid,[7,8] had led to the view that external redox reagents would not be required. In fact the pathway in *P. denitrificans* involves an oxidative step at some stage prior to precorrin-6x **4** and the reduction to **5** brings the oxidation level back down to match that of **1**. The NADPH-specific reductase involved has been isolated[5] and it has been shown to transfer the H_R from C-4 of NADPH,[9] see part structure **8a**, to C-19 of precorrin-6x **4**.[10]

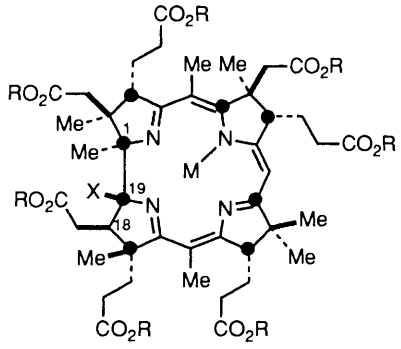
The foregoing facts relate to an *aerobic* B_{12}-producing bacterium but may not necessarily be true for *anaerobic* bacteria (such as *P. shermanii*), which are known to follow a slightly different biosynthetic pathways in that they produce cobyrinic acid **2** rather than the cobalt-free **1**. In particular it is important to know whether the redox changes occur in the anaerobe since these could have evolved only after oxygen became available in the atmosphere. We now report results that provide the answer for the anaerobe *P. shermanii*. The experimental design reflected the greater difficulties com-

pared with the experiments above; there, overproduced and substantially enriched preparations of the reductase were used. These are not available at present for *P. shermanii*; only a mixture of enzymes at the natural, unenhanced level is available, though this preparation is capable of converting precursors such as precorrin-2 **6a** into cobyrinic acid **2**.

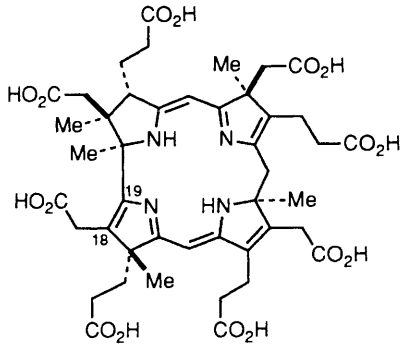
If a reductase is required for the biosynthesis of vitamin B_{12} in *P. shermanii*, its cofactor could be NADPH or NADH. Accordingly, a mixture of [4-$^2H_2$]NADH, part structure **7b**, and [4-$^2H_2$]NADPH, part structure **8b**, was incubated with the broken-cell enzyme preparation from *P. shermanii* and ^{13}C-labelled precorrin-2 **6b** (as the aromatised compound, sirohydrochlorin, which is known to be enzymically reduced *in situ* to precorrin-2). This precursor (*ca.* 40 atom% ^{13}C at each labelled site) had been biosynthesised from 90 atom% [4-^{13}C] 5-amino laevulinic acid. The cobyrinic acid produced was isolated with minimal unlabelled **2** added as carrier and then esterified to yield crystalline cobester **3b**. The signal from C-1 of **3b** was a doublet (coupling to C-19) superimposed on a larger singlet from ^{13}C at C-1 bonded to ^{12}C at C-19 and from natural abundance ^{13}C in the unlabelled carrier. Both the singlet and the doublet showed small additional signals 0.07 ppm upfield from the unshifted signals; this shift is in the range expected for a β-^{2}H isotopic shift,[11] which places ^{2}H at C-19 since this is the only skeletal position β to C-1 that carries a hydrogen atom. However, the level of ^{2}H-labelling was low (*ca.* 10–15%) and more sensitive experiments were needed for rigorous proof of reductive hydrogen transfer.

The low level of ^{2}H-incorporation from cofactors that were >95% ^{2}H-labelled is understandable from the known possibility of exchange with the medium *via* flavins of hydrogen atoms at C-4 of reduced nicotinamide cofactors. Loss of 60–70% of ^{2}H was experienced in the analogous experiments described above, even though substantially purified enzyme preparations were used.[9,10] Greater exchange was to be expected with the crude enzyme system from *P. shermanii* but importantly total exchange had been avoided.

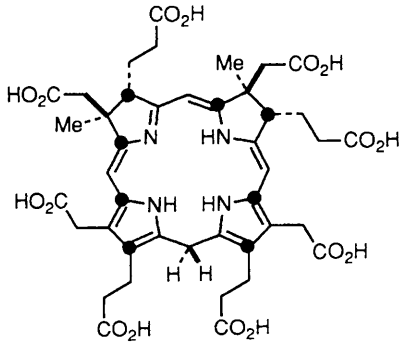
The interlocking study used ^{3}H-labelling and added a stereochemical probe. Synthesis of the required cofactors at high specific activities will be described in our full papers; they are [4*R*-^{3}H]NADH **7c**, [4*S*-^{3}H]NADH **7d**, [4*R*-^{3}H]NADPH **8c** and [4*S*-^{3}H]NADPH **8d**.† The enzyme system from *P. shermanii* was incubated with [^{14}C]precorrin-2 **6c** (again as the aromatised compound) as substrate and a mixture of approximately equal amounts of [4*R*-^{3}H]NADH **7c** and [4*R*-^{3}H]NADPH **8c**. A parallel run using the same enzyme preparation was identical apart from replacing the two [4*R*-^{3}H] cofactors with the two [4*S*-^{3}H] isomers, **7d** and **8d**. In order to have the right quantity (in mmol) of material, each ^{3}H-labelled cofactor was diluted with the corresponding [4-$^2H_2$]-labelled material, **7b** or **8b**, rather than with unlabelled samples. It was hoped that this would enhance the degree of ^{3}H-transfer by having ^{3}H competing against ^{2}H rather than against ^{1}H. The cobyrinic acid formed was isolated from each run after addition of unlabelled **2** as carrier, purified and then esterified. The cobester samples were extensively purified by chromatography and multiple recrystallisation until the ^{3}H and ^{14}C activities for the cobester sample in the



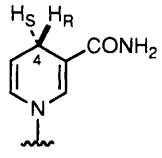
1 R = X = M = H, ● = ^{12}C
2 R = X = H, M = Co^{III}, ● = ^{12}C
3a R = Me, X = H, M = Co^{III}, ● = ^{12}C
b R = Me, X = ^{2}H, M = Co^{III}, ● = ^{13}C
c R = Me, X = ^{3}H, M = Co^{III}, ● = ^{14}C



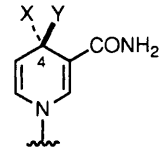
4
5 18,19-dihydro



6a ● = ^{12}C
b ● = ^{13}C
c ● = ^{14}C



7a
8a 3'-Phosphate of **7**



7b X = Y = ^{2}H
8b 3'-Phosphate of **7a**
7c X = H, Y = ^{3}H
8c 3'-Phosphate of **7b**
7d X = ^{3}H, Y = H
8d 3'-Phosphate of **7c**

† [4*R*-^{3}H] NADH and NADPH were synthesized by reduction of NAD^+ and $NADP^+$ using the alcohol dehydrogenases from horse liver and *Thermoanaerobium brockii* with [1-^{3}H]cyclohexanol and [1-^{3}H]isopropanol, respectively. [4-^{3}H]NAD^+ and $NADP^+$ were made by non-enzymic oxidation of the labelled NADH and NADPH using PQQ and O_2. [4*S*-^{3}H]NADH and NADPH were then generated using the same alcohol dehydrogenases as before but with unlabelled cyclohexanol and isopropanol.

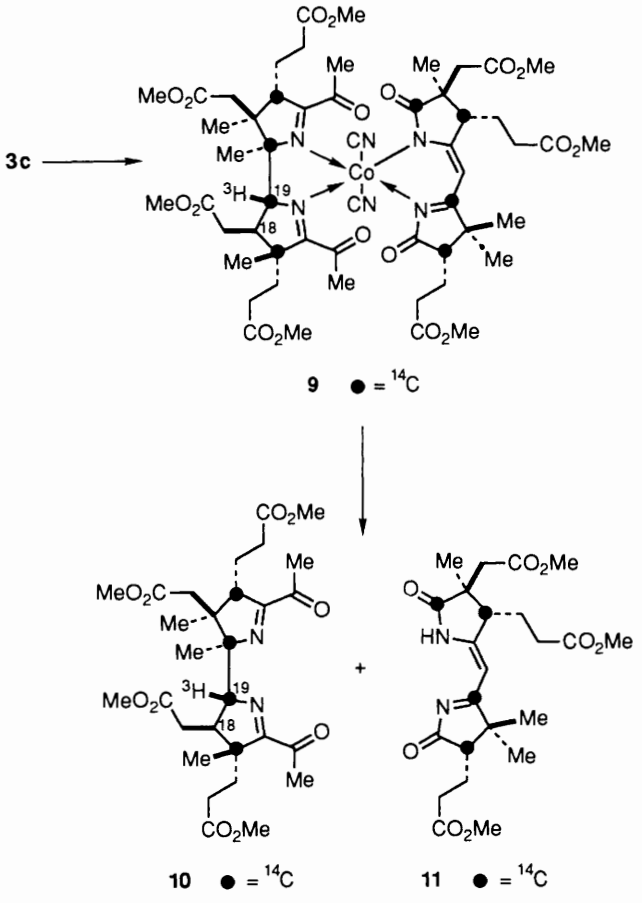


Table 1 Degradation of cobester **3c** derived from [4*R*-^{3}H]nicotinamide cofactors **7c** and **8c**

Substance	Specific activity (dpm/mmol × 10^{-5})	
	^{14}C	^{3}H
Cobester **3c**	47 ± 3	3.7 ± 0.3
Intermediate **9**	39 ± 2	2.5 ± 0.2
A/D fragment **10**	20 ± 2	2.9 ± 0.2
B/C fragment **11**	17.5 ± 2	0

4*R*-series **3c** were almost constant (incorporation of **6c** based on ^{14}C was 22%). At this same stage of purification, the ^{3}H-level for the cobester from the 4*S*-series had already fallen to a level that could not be accurately determined so this sample was not carried further; however, the ^{14}C-activity demonstrated that there had been an excellent incorporation of **6c** (30%). The decision not to seek perfect constancy for the 4*R*-derived sample at this point was based on the need to preserve material for degradation and on the fact that there would be opportunities to achieve the last small step to radiochemical purity during two further purifications, involving different substances, during degradation by Kräutler's photochemical method[12] to the separate A/D and B/C fragments, **10** and **11**. The three cobalt-containing intermediates in this degradation[12] (the two singly cleaved products are not shown but the doubly cleaved **9** is) were isolated and carefully purified to ensure radiochemical purity of **10** and **11**.

The results in Table 1 show that the [4*R*-^{3}H]cofactors led to the formation of ^{3}H-labelled cobyrinic acid isolated as cobester **3c** with the ^{3}H-label entirely in the A/D fragment **10**; interestingly, the reductase from *P. denitrificans* also is specific for H_R of its cofactor.[9] Apart from the methyl hydrogen atoms, which need not concern us, only H-18 and H-19 are introduced during the conversion of **6a** into **2** and since the former is derived from the medium (see below), the ^{3}H-label is at C-19 (see **3c**) in agreement with the ^{2}H-study above.‡ The combined results of the ^{2}H and ^{3}H experiments demonstrate that reduction is a step in B_{12} biosynthesis even in an anaerobic organism.

Early experiments[13] on the origin of H-18 and H-19 of **2** in *P. shermanii* were run in D_2O with 6–13% H_2O. These results showed that H-18 is derived from the medium but the ^{2}H-labelling at C-19 was significantly lower than expected for straightforward derivation from the medium. Both results were confirmed some years later.[14] It is clear from the present work that the earlier results for H-19 were due to substantial exchange of the reducing cofactor with D_2O of the medium.

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‡ The degree of dilution of the cobyrinic acid biosynthesised with unlabelled carrier can be calculated from ratio of the specific ^{14}C activities of the initial substrate **6c** and the final sample of cobester **3c**. Using this dilution factor, the specific activity of ^{3}H in the biosynthesised cobyrinic acid before dilution can be calculated. That value is more than 500 times greater than the *maximum* specific activity possible by exchange of all the ^{3}H from the cofactors into the medium and derivation of H-19 from that source.