Biosynthesis of Vitamin B_{12}: the Site of Reduction of Precorrin-6x

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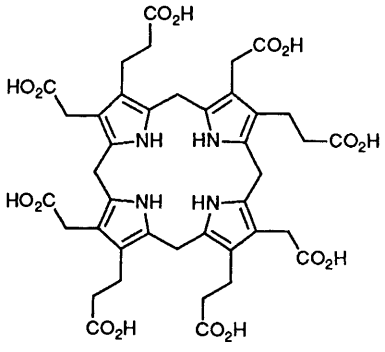
It is proved by deuterium labelling and NMR spectroscopy that a hydride equivalent from NADPH is transferred to C-19 of precorrin-6x as this intermediate is converted enzymically into hydrogenobyrinic acid.

The direction of research on the biosynthesis of vitamin B_{12} has recently been dramatically changed by the isolation and key properties[1] of precorrin-6x, a biosynthetic precursor of hydrogenobyrinic acid **2a**. The precursor is called precorrin-6x† because it was shown[1] to be formed by the introduction of six methyl groups from *S*-adenosyl-methionine into uro'gen III **1**. Hydrogenobyrinic acid **2a** is simply the cobalt-free form of cobyrinic acid **3a**, the latter being elaborated further[2] to produce vitamin B_{12} itself. The cobalt-free macrocycle **2a** is produced by a strain of *Pseudomonas denitrificans*[3] on which substantial genetic engineering has been carried out with the genes known[4] to be involved in the biosynthesis of vitamin B_{12}. It follows that precorrin-6x is of decisive importance for both the metal-free and cobalt-containing corrins **2a** and **3a**.

† The letter x will eventually be replaced by a capital letter B, C or D when it is known whether 1, 2 or 3 intermediates, also at the hexamethylated level, precede precorrin-6x on the pathway.



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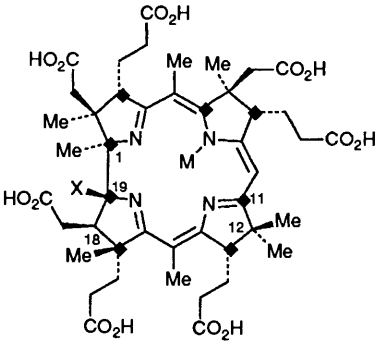
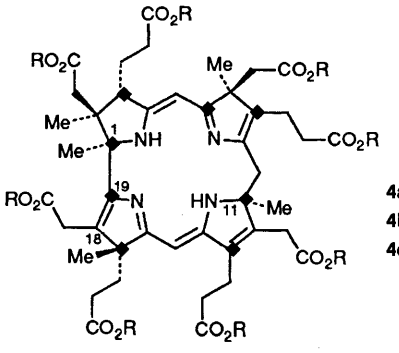
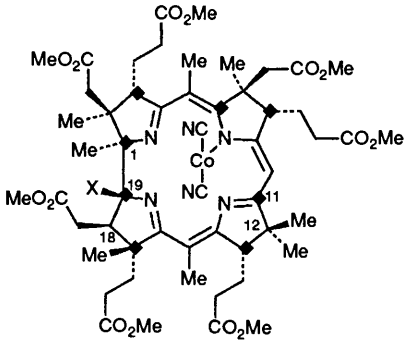


2a; M = X = H, ◆=^{12}C
2b; M = X = H, ◆=^{13}C
2c; M = H, X = ^{2}H, ◆=^{13}C
3a; M = Co^{III}, X = H, ◆=^{12}C
3b; M = Co^{III}, X = H, ◆=^{13}C
3c; M = Co^{III}, X = ^{2}H, ◆=^{13}C

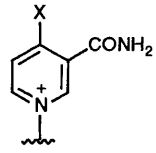


4a; R = Me, ◆=^{12}C
4b; R = H, ◆=^{12}C
4c; R = H, ◆=^{13}C

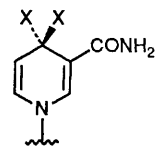


5a; X = H, ◆=^{13}C
5b; X = ^{2}H, ◆=^{13}C
5c; X = H, ◆=^{12}C
5d; X = ^{2}H, ◆=^{12}C

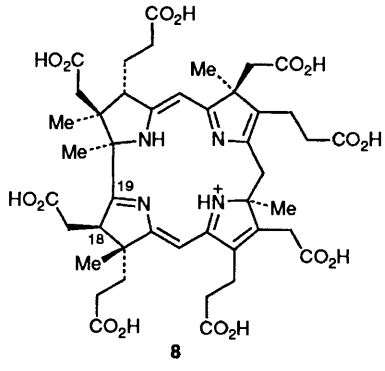
The structural properties[1] of precorrin-6x modified several earlier views about B_{12} biosynthesis (*e.g.* early decarboxylation of C-12 acetate) and the complete structure **4a** established[1,5] for its ester confirmed and added to these surprising discoveries. The crucial information which precorrin-6x‡ provides about the biosynthetic pathway can be summarised as follows. (*i*) Precorrin-6x **4b** is at the oxidation level of a dehydrocorrin and reduction is required[1] (specifically involving NADPH) for its eventual conversion into hydrogenobyrinic acid **2a**. When NADPH (reduced nicotinamide adenine dinucleotide phosphate) is excluded from the incubation, precorrin-6x accumulates. (*ii*) The ring-contraction step, which results in elimination as acetic acid[6,7] of C-20 and the attached methyl group, occurs before decarboxylation of the C-12 acetate residue. (*iii*) The methyl group which eventually appears at C-12 in **2a** and **3a** is inserted initially at C-11. (*iv*) The C-12 acetate residue undergoes decarboxylation late in the biosynthetic sequence, certainly beyond the sixth methylation step, and it is postulated[5] that decarboxylation allows methyl migration from C-11 to C-12. This information led to a pathway being proposed[5] for biosynthesis of the corrins **2a** and **3a** which differs from earlier ideas.



6a; X = H
6b; X = ^{2}H



7a; X = H
7b; X = ^{2}H



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We now describe experiments aimed at locating the site in precorrin-6x **4b** to which a hydride equivalent is transferred from NADPH, part structure **7a**, in the reduction step. The initial experiments were designed bearing in mind that the enzyme preparation for conversion of precorrin-6x **4b** into hydrogenobyrinic acid **2a** contained at that time some of the latter corrin as endogenous (and therefore unlabelled) material. Accordingly, ^{13}C-labelled precorrin-6x **4c** was used which was biosynthesised from 5-amino[4-^{13}C]laevulinic acid[5] and so carried diagnostic ^{13}C-centres at C-19 and C-1.

The hydrogen at C-4 of the pyridine ring of NADP, part structure **6a**, was exchanged for deuterium using cyanide ion catalysis,[8] this exchange being *ca.* 92% complete as shown by NMR analysis. The resultant [4-$^{2}H_1$]NADP, part structure **6b**, was reduced with sodium dithionite[9] in deuterium oxide (99.9% ^{2}H) to give [4-$^{2}H_2$]NADPH, part structure **7b** which was purified and desalted by ion-exchange on diethylaminoethyl cellulose in ammonium hydrogen carbonate buffer. In trial runs, the residual protium at C-4 of **7b** produced in this reduction was shown by NMR to be less than 5%.

The foregoing ^{13}C-labelled precorrin-6x **4c** and [4-$^{2}H_2$]-NADPH **7b** were incubated with the protein preparation from the aerobic organism *P. denitrificans*[1] which contains all the enzymes required to convert precorrin-6x into hydrogenobyrinic acid including the reductase dependent on NADPH. This reduction now involves transfer of deuterium to precorrin-6x and the site to which it is delivered was determined by allowing the biosynthesis to run through to hydrogenobyrinic acid. A second run was carried out in exactly the same way save that unlabelled NADPH was used to give a second sample of hydrogenobyrinic acid. Cobalt was inserted non-enzymically[10] into both products and the resultant samples of cobyrinic acid were esterified giving two preparations of

‡ Though the illustrated arrangement of double bonds **4a** is established[5] for precorrin-6x ester, there are obviously other prototropic tautomers available to precorrin-6x acid **4b** but these are interconvertible in the biological system.[1]

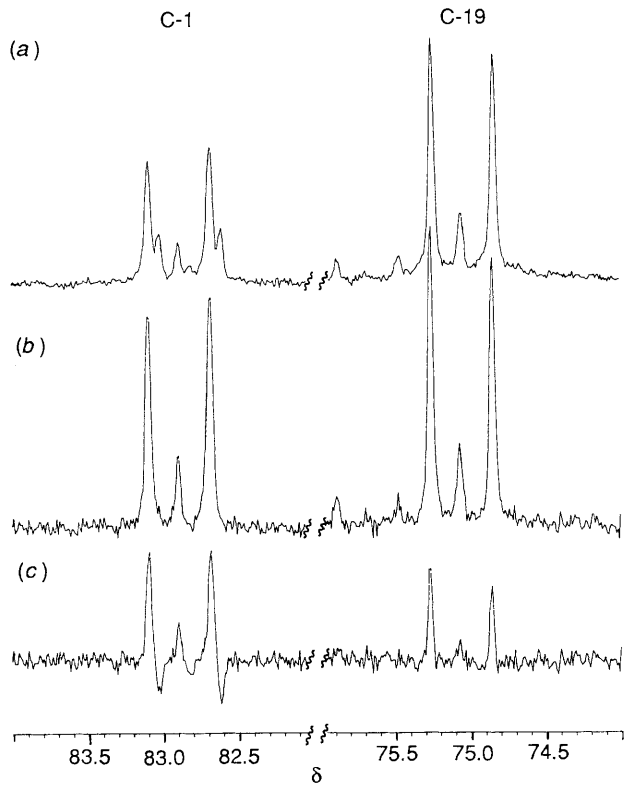

Fig. 1 Partial ^{13}C NMR spectra of cobester derived from 5-amino[4-^{13}C]laevulinic acid *via* precorrin-6x **4c**; (*a*) using [4-$^{2}H_2$]NADPH, (*b*) using unlabelled NADPH, (*c*) difference spectrum (*b*) − (*a*). For the difference spectrum the ratio for the subtraction was adjusted to give optimum nulling of the other methine signals (C-3, -8 and -13).

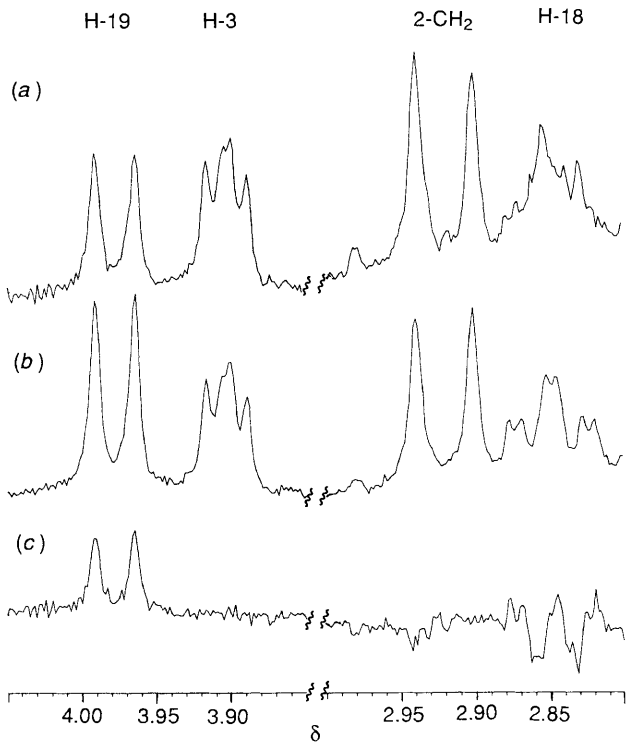

Fig. 2 Partial ^{1}H NMR spectra of (*a*) cobester derived from precorrin-6x **4b** using [4-$^{2}H_2$]NADPH, (*b*) unlabelled cobester, (*c*) difference spectrum (*b*) − (*a*). For the difference spectrum the ratio for the subtraction was adjusted to give optimum nulling of the signal for H-3.

cobester for NMR analysis. The doubly labelled sample from the [4-$^{2}H_2$]NADPH run is sample *A* and the standard one from NADPH is sample *B*. All the important ^{13}C-signals from cobester **5a** have been assigned.[11,12]

Fig. 1 shows the ^{13}C-signals from C-1 and C-19 of samples *A* and *B*. Both signals are doublets (*J* 41 Hz) in both spectra due to coupling with each other and they are centred on a small singlet arising from material having ^{12}C at the adjacent position. (In some runs the doublets due to C-1 show a very small further coupling of *ca.* 2 Hz due to long-range coupling to C-3 but this splitting, which is at the limit of resolution of these spectra, was not always clearly visible). In the spectrum of the doubly-labelled sample *A*, Fig. 1(*a*), the doublet for C-1 shows in addition another 41 Hz doublet shifted slightly to higher field due to the β-deuterium isotopic shift.[13] This β-shift must be from C-19 since this is the only centre carrying hydrogen in the β-position to C-1. The upfield shift of 0.07 ppm is within the normal range for β-shifts. The presence of ^{2}H at C-19 (as shown in structure **5b**) was confirmed by the diminished ^{13}C-signal from C-19 (due to the α-effect of deuterium) clearly shown by the difference spectrum [sample *B* − sample *A*], Fig. 1(*c*).

The fact that the level of deuterium incorporation is only *ca.* 25–30%, despite there being initially a much higher level of deuteriation at C-4 of [4-$^{2}H_2$]NADPH, probably indicates that the cofactor (used in a large excess) undergoes some exchange with the medium (*via* flavins?) and then experiences a $^{1}H/^{2}H$ isotope effect, which will favour transfer of ^{1}H.

During the course of the above experiments, an enzyme preparation was developed containing the key reductase enzyme but free from endogenous hydrogenobyrinic acid **2a**. Confirmation of the foregoing results was thus possible by direct ^{1}H NMR spectroscopy. Unlabelled precorrin-6x **4b** was incubated as before with [4-$^{2}H_2$]NADPH, part structure **7b**, but now with the new enzyme preparation, finally to give cobester, sample *C* **5d**.

Fig. 2(*a*) shows the ^{1}H-signals from H-19, H-3, one of the 2-acetate protons and H-18 of sample *C* **5d** produced in the [4-$^{2}H_2$]NADPH run. Fig. 2(*b*) shows the same signals from unlabelled cobester **5c** as a standard. The signal from H-19 is clearly diminished in Fig. 2(*a*), as is made obvious by the difference spectrum shown as Fig. 2(*c*). The signal for H-18 in Fig. 2(*a*) has a different appearance from that in Fig. 2(*b*) consistent with deuteriation at H-19. The difference spectrum shows, however, that there is no significant net diminution of the signal for H-18. The integration of these spectra revealed that in this experiment the level of deuteriation at H-19 is also *ca.* 25–30%. The fast atom bombardment mass spectrum of sample *C*, when compared with that of unlabelled cobester, again showed about the same level of monodeuteriation, indicating that there are no other sites of deuteriation in the molecule.

These experiments rigorously establish that the NADPH-dependent reductase transfers a hydride equivalent to C-19 of precorrin-6x and so presumably it is the C-18 protonated form of precorrin-6x **8** to which the transfer of hydride occurs. The structure of the reduction product, precorrin-6y, is currently being studied.

It is interesting that early experiments[14] based on the anaerobic bacterium, *Propionibacterium shermanii*, in which the biosynthesis of cobyrinic acid (as **3a**) was carried out in D_2O (containing, in two separate experiments, 6 and 13% of H_2O) showed clearly that its hydrogen atom at C-18 is derived from the medium. However, the amount of deuterium which appeared at C-19 was significantly lower than expected.[14] Both these findings were confirmed some years later.[15] Two explanations were considered for the result at C-19: (*i*) that the C-19 hydrogen is derived from the medium but that isotope effects discriminate against deuterium uptake, or (*ii*) that hydrogen at C-19 arises from a reducing cofactor which undergoes flavin-mediated partial exchange with the medium.

There was no way to discriminate between (*i*) and (*ii*) at that time but explanation (*i*) was preferred.[14,15] The results outlined in this Communication using an aerobic organism show that explanation (*ii*) should be directly tested in the anaerobic system.

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