Biosynthesis of Vitamin B_{12}: Origin of the Hydrogen Atoms at C-18 and C-19

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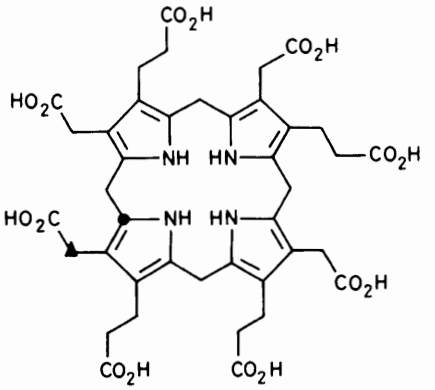
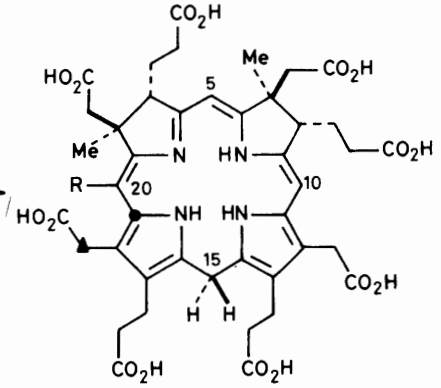
When cobyrinic acid is biosynthesised from uro'gen-III in deuterium oxide, deuterium is inserted at both C-18 and C-19 as shown by ^{13}C n.m.r.; this is a further result in keeping with a biosynthetic sequence for the corrin ring of vitamin B_{12} involving no external redox reagents.

Cobyrinic acid (**4**), a late precursor of vitamin B_{12}, is biosynthesised from uro'gen-III (**1**) by many steps of which the first three are *C*-methylations.[1] It is known that the dimethylated intermediate is the dihydroisobacteriochlorin[2] (**2**) and it is virtually certain that the trimethylated system is also produced and further transformed at the dihydro-level (**3**). Both uro'gen-III (**1**) and the macrocycles (**2**) and (**3**) have eight double-bonds so the conversion (**1**) into (**2**) and (**3**) involves no change of oxidation level.

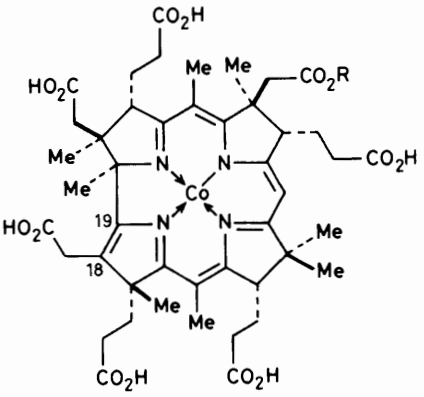
The next known intermediate on the pathway to vitamin B_{12}



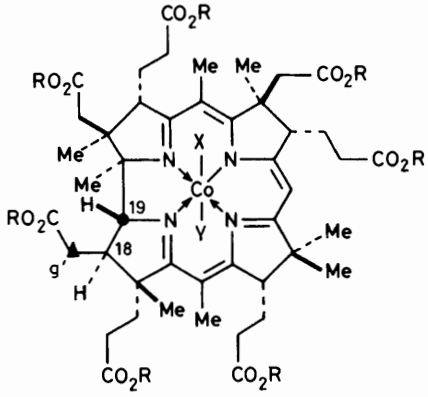
(**1**) ^{12}C at ● and ▲
(**1a**) ^{13}C at ▲, ^{12}C at ●
(**1b**) ^{13}C at ●, ^{12}C at ▲



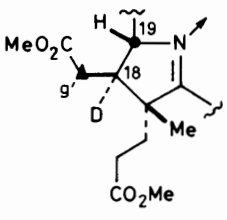
(**2**) R = H, ^{12}C at ● and ▲
(**3**) R = Me, ^{12}C at ● and ▲



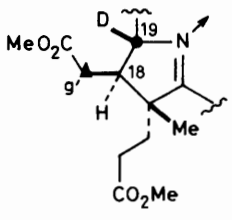
(**6a**) R = NH_2
(**6b**) R = OH



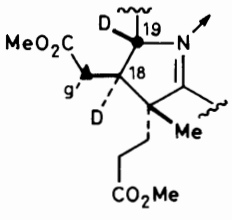
(**4**) R = H, ^{12}C at ● and ▲
(**4a**) R = H, ^{13}C at ▲, ^{12}C at ●
(**4b**) R = H, ^{13}C at ●, ^{12}C at ▲
(**5**) R = Me, ^{12}C at ● and ▲
(**5a**) R = Me, ^{13}C at ▲, ^{12}C at ●
(**5b**) R = Me, ^{13}C at ●, ^{12}C at ▲



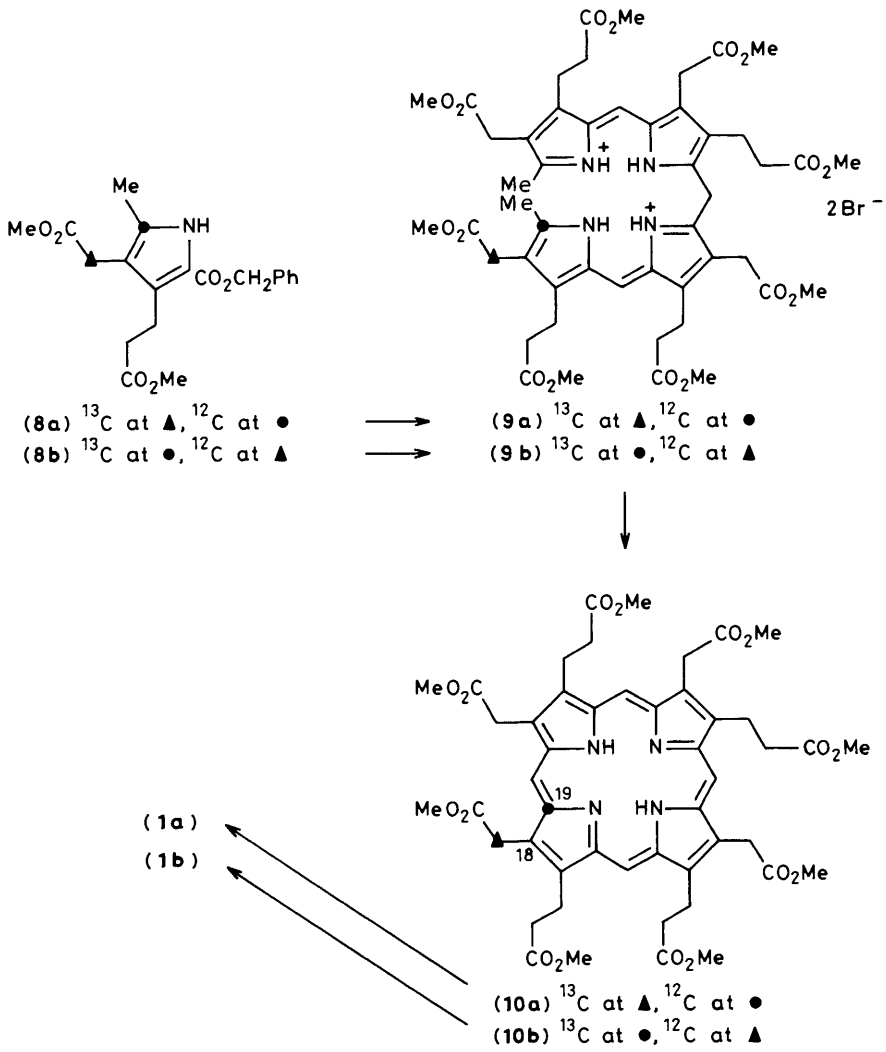
(**7a**)



(**7b**)



(**7c**)



is cobyrinic acid (**4**); also it is now established that during the corrin-forming ring-contraction, C-20 and its attached methyl group in structure (**3**) are extruded as acetic acid.[3,4] The change from (**3**) (8 double bonds) into cobyrinic acid (**4**) (6 double bonds) must involve hydration of one double bond at some stage; the second double bond is accounted for by the acetic acid lost. Thus, the two products together, *i.e.* (**4**) and acetic acid, correspond in oxidation level to the dihydroisobacteriochlorin stage (**3**). It remains to be discovered whether this constancy of oxidation level holds for each stage in the unknown sequence between the macrocycles (**3**) and (**4**).

The problem of oxidation level was further highlighted by the isolation[5] (*ca.* 4% yield) of the 18,19-dehydrocorrin (**6a**) from the mixture produced following Co^{II}-insertion into a crude sample of the metal-free hydrogenocobyrinic acid-*c*-amide [as (**6a**) but without Co^{II} and with the 18,19-double bond reduced] obtained from *Rhodopseudomonas spheroides*. It was suggested[5] that such a dehydrocorrin might be a biosynthetic intermediate *en route* to vitamin B_{12}. If this were so, then dehydrogenation would be required after formation of the dihydroisobacteriochlorins (**2**) and (**3**) followed by reduction at some later stage eventually to generate cobyrinic acid (**4**). Accordingly, it was important to determine by isotopic labelling whether the medium provides just one of the hydrogen atoms or both at C-18 and C-19 [see (**4**)]; the former result would point to a biological reducing agent [*e.g* NAD(P)H] as the source of the other hydrogen atom.

To study this problem the medium must be labelled, so it was first established that the cell-free enzyme system,[6] prepared as usual from *Propionibacterium shermanii* and then freeze-dried, would function in essentially pure deuterium oxide for the biosynthesis of cobyrinic acid (**4**) with no serious loss of enzymic activity. Direct detection by n.m.r. of ^{2}H uptake into cobyrinic acid was not possible,[7] so the β-shift method,[8] where shifts in the ^{13}C n.m.r. spectra are used to detect ^{2}H at the β-position to a ^{13}C atom, was selected. Related experiments showed that such β-shifts were readily observed[9] in the ^{13}C spectra from standard deuteriated samples of the methyl ester of cobyrinic acid, cobester [as (**5**), X = Y = CN]. Uro'gen-III (**1**) was chosen as the biosynthetic precursor for cobyrinic acid (**4**) and the synthesis of (**1**) with ^{13}C-labels at C-19 and at the methylene group of the C-18 acetate residue† (as sensors for ^{2}H) was as follows.

The pyrrole (**8a**), prepared from 90 atoms % sodium [2-^{13}C]acetate, was built into the biladiene (**9a**) which by Cu^{II}-promoted cyclisation[10] gave [18-*methylene*-^{13}C]uropor-

† This is the C-18 g' position; see ref. 9 for notation.

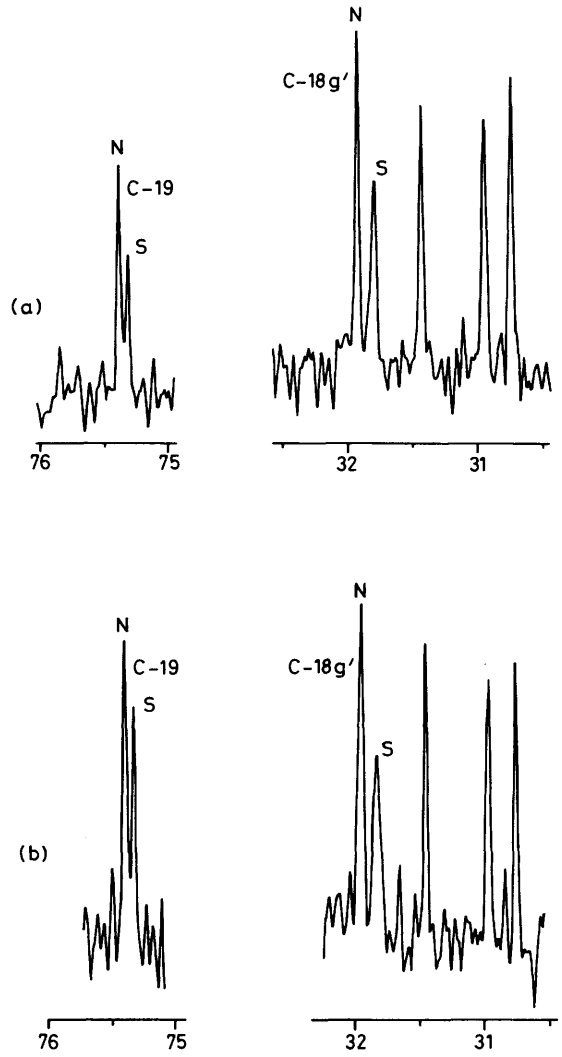


Figure 1. ^{13}C N.m.r. signals (δ in p.p.m.) at 100.6 MHz with ^{1}H noise decoupling from labelled cobester [see (**5a**, **b**) and (**7a**, **b**, **c**)] over regions round the unshifted signals from the C-18 g′ group (δ_C 31.9) and from C-19 (δ_C 75.3). Medium for enzymic runs was D_2O : H_2O (a) 94 : 6 and (b) 87 : 13; N = normal signal, S = shifted signal. Spectra run in C_6D_6 with the central line of the residual solvent peak as internal reference (δ_C 128.0); sweep width 8600 Hz, 16K data points, acquisition time 0.95 s, pulse angle 45°.

phyrin-III ester (**10a**). Similarly, the [5-^{13}C]pyrrole[11] (**8b**) was converted by the same sequence *via* (**9b**) into [19-^{13}C]uroporphyrin-III ester (**10b**). To a 1 : 1 mixture of the labelled samples (**10a**) and (**10b**) was added a small quantity of ^{14}C-uroporphyrin-III ester for assay of the incorporation levels. Note that the mixture of ^{13}C species allowed information to be gained for both C-18 and C-19 in one run; also, for experiments dependent on ^{13}C shifts, it was essential to have the two ^{13}C atoms in different molecules. Alkaline hydrolysis of the mixture [(**10a**) and (**10b**)] gave labelled uroporphyrin-III which was reduced to [18-*methylene*-^{13}C,19-^{13}C]uro'gen-III [(**1a**) and (**1b**)] and this was incubated in two separate runs with the *P. shermanii* enzyme system (a) with D_2O : H_2O as 94 : 6 and (b) with D_2O : H_2O as 87 : 13. These produced, respectively, 160 μg and 125 μg of cobyrinic acid biosynthesised from the labelled uro'gen-III [(**1a**) and (**1b**)]. It was essential to isolate the products without overwhelming dilution with added carrier[12] and the resultant samples of labelled cobester [(**5a**) and (**5b**), X = Y = CN] were purified to homogeneity.

Figure 1(a) shows those parts of the ^{13}C n.m.r. spectrum of the sample from (a) of labelled cobester [(**5a**) and (**5b**)] close to the signals from the labelled sites at δ_C 75.3 and δ_C 31.9 p.p.m.; the analogous spectrum for the sample from (b) is in Figure 1(b). In each case, only the resonances from C-19 and C-18 g′ showed new shifted signals (S) immediately upfield of the normal signals (N); the other signals illustrated are from natural abundance ^{13}C at other sites of the carrier cobester.

The shifted signal from C-19 arises from species (**7a**), the upfield change (7.3 Hz at 100.6 MHz) being typical for a β-shift.[9] Also, for both experiments, the shifted C-19 signal was of substantially smaller size than the shifted signal from C-18 g′; this loss of C-19 signal shows that there is also ^{2}H at C-19. The shifted signal from C-18 g′ confirmed the presence of ^{2}H at both C-18 and C-19 because the size of the upfield movement (13.7 Hz) was far too large to be accounted for by a γ-shift (normally 0—7 Hz) involving the species (**7b**) with ^{2}H only at C-19; ^{2}H must also be present at C-18 causing a β-shift. The shifted resonance from C-18 g′ thus arises from two unresolved signals due to species with ^{2}H at C-18 and ^{1}H at C-19 (**7a**) and with ^{2}H at both sites (**7c**); the latter species causes a (β + γ) shift.‡

Thus the hydrogens at both C-18 and C-19 of cobyrinic acid (**4**) are derived from the medium, a result which does not support the involvement of 18,19-dehydrocorrins (**6**) in the biosynthesis of vitamin B_{12}.§ Furthermore, Nussbaumer, Kräutler, and Arigoni have found[13] that (i) the 18,19-dehydrocorrin system can be consistently formed as an artefact (*ca.* 5% yield) during manipulation of the corresponding corrin and (ii) when the product from enzymic conversion of an early labelled precursor into cobyrinic acid (**4**) was worked up by esterification, radioactive cobester (**5**) and its 18,19-dehydro analogue [(**6b**) ester], [*ca.* 5% of the quantity of (**5**)] were both isolated and they had exactly the same specific activity, thus confirming the artefactual relationship of (**6b**) to (**4**). All these findings interlock with other results[3,4,14] supporting the attractive possibility that the entire sequence for the biosynthesis of cobyrinic acid (**4**) from uro'gen-III (**1**) may occur without participation of external redox reagents.

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‡ Analysis of the peak areas in Figure 1, taking into account their size in a spectrum of natural abundance cobester run under exactly the same conditions, showed that the incorporation of ^{2}H at C-18 was substantially greater than at C-19. The mechanistic significance of these isotope effects will be discussed in our full paper.

§ The sum of evidence is extremely strong against reduction of an 18,19-dehydrocorrin being involved in B_{12}-biosynthesis but, strictly, the conceivable possibility of reduction by a flavin (which could undergo hydrogen-exchange with the medium) has not been excluded. Rigorous exclusion using the present complex enzyme preparation is well-nigh impossible; but we expect this remote possibility to be finally eliminated by future chemical and enzymic developments.

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