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Chemistry

Biosynthesis of vitamin B_{12}: Concerning the origin of the methine protons of the corrin nucleus

(deuterium isotope effects/^{13}C NMR spectroscopy)

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ABSTRACT **^{13}C NMR spectroscopy has been used to locate six deuterium atoms incorporated biosynthetically on the periphery of the corrin nucleus of vitamin B_{12} (cyanocobalamin) derived from cells of *Propionibacterium shermanii* grown in a medium containing 50% ^2H_2O and ^{13}C-enriched δ-aminolevulinic acid. The implications of these results for the mechanism of vitamin B_{12} biosynthesis are discussed, and it is concluded that the same oxidation level of the intermediates is maintained throughout the biosynthetic pathway, from δ-aminolevulinic acid to corrin.**

Our knowledge of the carbon balance of the pathway leading to vitamin B_{12} (cyanocobalamin; **1**) is now complete, due to the application of ^{13}C NMR spectroscopy, which has defined the assembly process from δ-aminolevulinic acid (ALA; **2**) and methionine as depicted in Fig. 1 (1). However, the biochemical inventory of the protons at those positions on the corrin template that could be involved in oxidation–reduction or prototropic exchange with medium is still fragmentary. Early studies (2–4) showed that, during the insertion of the seven methionine-derived methyl groups, no exchange of methyl protons with the medium occurs, and it has been found that one proton is delivered stereospecifically to the C_{12} β-methyl group formed by the decarboxylation of the original acetate side chain at C_{12} (5). Furthermore, in a cell-free system that produces cobyrinic acid (**7a**) [but not cyanocobalamin (**1**)] it was observed that deuterium atoms at C-18 and C-19 are delivered from a medium containing ^2H_2O (6). Since the latter analysis of deuterium content depends on acid-catalyzed esterification of cobyrinic acid (**7a**) to cobester (**7b**), the cell-free approach is not applicable to the study of those protons (C-3, C-8, C-10, and C-13) capable of exchange during esterification. We now describe the complete methine proton inventory of cyanocobalamin (**1**) obtained by incubation of whole cells of *Propionibacterium shermanii* in the presence of ^2H_2O and two ^{13}C-enriched versions of ALA (^{13}C-3 and ^{13}C-4) followed by NMR measurement of the α- and β-deuterium isotope shifts on the ^{13}C-enriched nuclei (7, 8).

Cells of *P. shermanii* were grown anaerobically in normal production medium (1 liter; see ref. 18) containing 50% (vol/vol) ^2H_2O and [4-^{13}C]ALA (90 atom % ^{13}C; 100 mg) for 7 days. Isolation and purification of cyanocobalamin by HPLC afforded a specimen (14 mg) whose ^{13}C NMR spectrum is shown in Fig. 2. Incorporation of deuterium at C-3, C-8, and C-13 can be observed by the upfield α-deuterium shifts on the corresponding ^{13}C signals (see Table 1). Quantitative analysis of the relative intensities of the β-deuterium-shifted (7, 8) signals corresponding to C-1, C-17, and C-19 show that both C-18 and C-19 are deuterated, but unequally [(^{2}H at C-18)/(^{2}H at C-19) = 3/1 ± 10%] (see Fig. 2), a result in accord with the deuterium content observed at C-18 and C-19 in cobester as discussed above (6). It is also of interest to note that there is no deuterium at C-10, a position known to undergo prototropic exchange under acidic conditions (9).

Confirmation and extension of the assignments were made by analysis of the spectrum of cyanocobalamin (**1**) obtained by the same procedure but in the presence of [3-^{13}C]ALA, which labels a different set of carbon centers (see Fig. 3). Thus, in addition to an upfield α shift on C-18, β ^{2}H shifts are found at C-2, C-3a, C-7, C-8a, C_{12}, C-13a, and C-18 (Table 2). In addition, the C_{12} β-methyl group has a single deuteron substituent as discerned in the double shift on C_{12} (from ^{2}H at C-13 and C_{12} $C^1H_2{}^2H$).

An attractive rationale for the isotopic discrimination between positions 18 and 19 can be found in the chemical analogy (10, 11) for the deacetylation of the model C-19 acetylcorrin (**3**), whereby the quenching of the stabilized C-19 carbanion (**4**) would take place under kinetic control (**4** → **5**) (Fig. 4). Although still only a hypothetical construct, the loss of acetic acid from C-20 and its attached methyl group (12–14) could be mediated via the 19-acetylcorrinoid (**6**) derived by the biosynthetic version of the model dihydrocorphinol → acetylcorrin rearrangement (10, 11) as shown in Fig. 5. Deacetylation in the presence of ^2H_2O would involve trapping of the corresponding carbanion by deuterium under kinetic control, leading to the observed ^{2}H ratio at C-18 and C-19. Thus C-18 (as well as C-3, C-8, and C-13, but not C-10) is protonated under equilibrium conditions during the conversion of uroporphyrinogen III to cobyrinic acid (**7a**) (Fig. 1), whereas proton delivery at C-19 is governed by a substantial kinetic isotope effect (Fig. 5).

These experiments render unlikely the possibility,* also considered earlier (6), that a Δ^{18}-dehydrocorrin undergoes reduction in the final stages (15, 16), mediated by a nicotinamide cofactor, and they are in accord with the observed (A.I.S., N. E. Georgopapadakou, and A. J. Irwin, unpublished data) lack of requirement for NADPH on NADH in the cell-free biosynthesis of cobyrinic acid, although the participation of flavin cofactors (e.g., FMNH) that could exchange with ^2H_2O or of valency changes in cobalt (17) that would lead to uptake of a proton at C-19 cannot yet be ruled out.

In summary, this work completes the proton inventory at C-3, C-8, C-10, C-13, C-18, and C-19 during vitamin B_{12} biosynthesis by a technique that avoids the possibility of proton exchange during isolation. The results strongly suggest that the original oxidation level is maintained throughout the biochemical pathway from glycine and succinate to the complete vitamin.†

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Abbreviation: ALA, δ-aminolevulinic acid.

*A Δ^{18}-dehydrocorrin has been isolated from *Rhodopseudomonas spheroides* (15). However, Δ^{18}-dehydrocobester has been shown to be a chemical artifact of the esterification procedure (16).

†It has been pointed out by Eschenmoser and colleagues (10, 11) that the chemical conversion of dihydrocorphinol to corrin provides a satisfactory rationale for maintaining the same oxidation level from the beginning to the end of vitamin B_{12} biosynthesis.

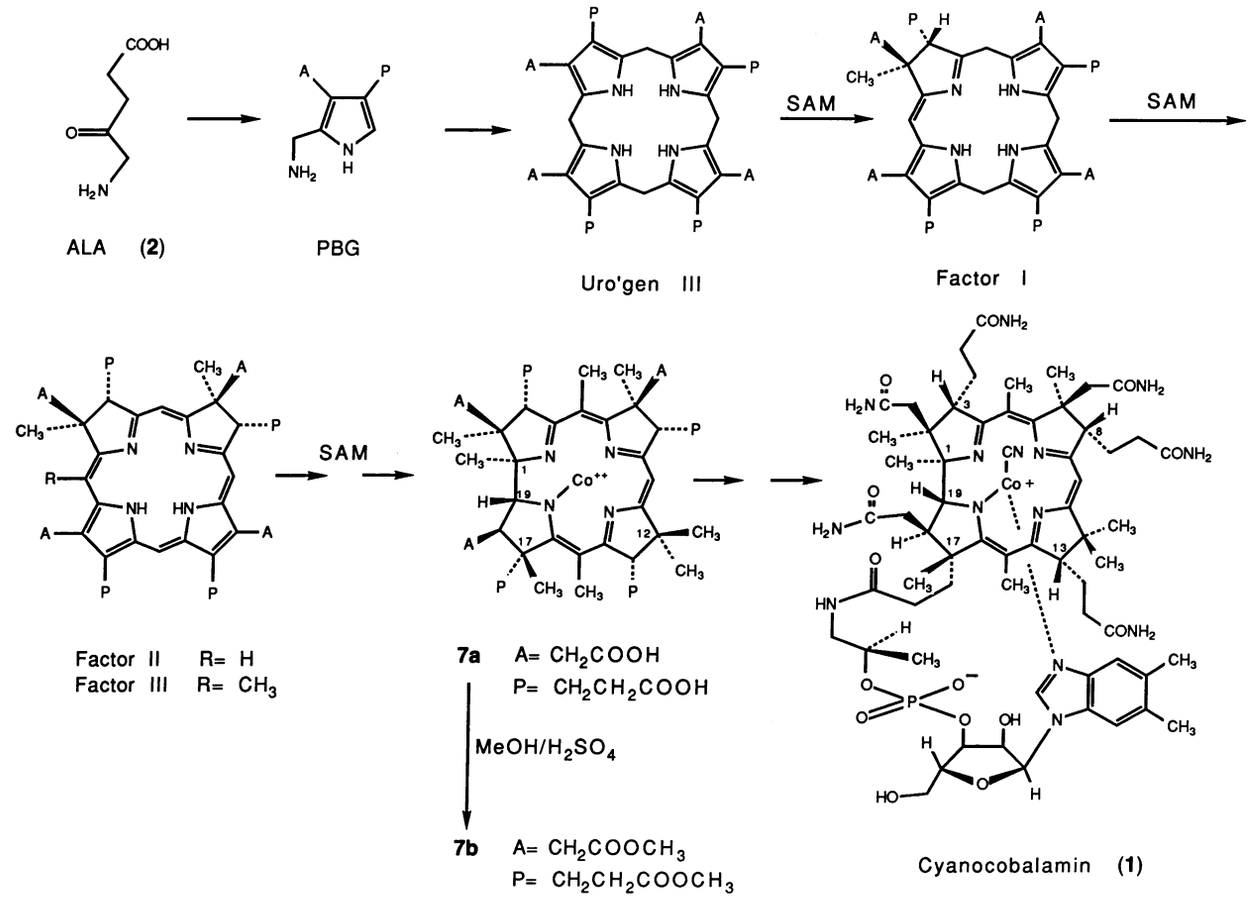


FIG. 1. Pathway for biosynthesis of vitamin B_{12}. PBG, porphobilinogen; A, acetyl (except for **7b**); P, propionyl (except for **7b**); Uro'gen III, uroporphyrinogen III; SAM, *S*-adenosylmethionine.

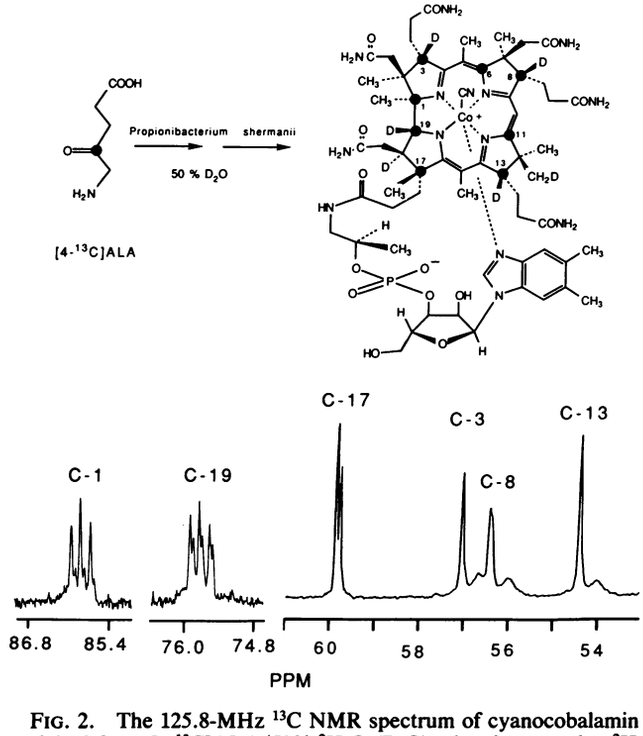


FIG. 2. The 125.8-MHz ^{13}C NMR spectrum of cyanocobalamin enriched from [4-^{13}C]ALA/50% ^2H_2O (D_2O), showing α and β ^2H shifts. In the structural formulas, ● = ^{13}C-enriched carbons. Solvent = ^2H_2O; waltz-decoupled (decoupler power = 13 H); number of scans = 37,300; acquisition time = 0.62 s; size of data table = 64,000 points; and pulse width = 64°. Shifts are relative to dioxane.

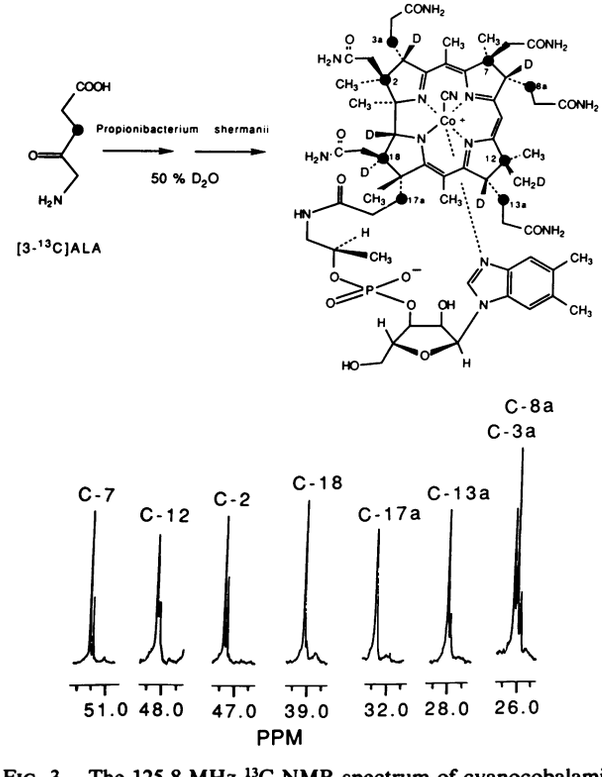


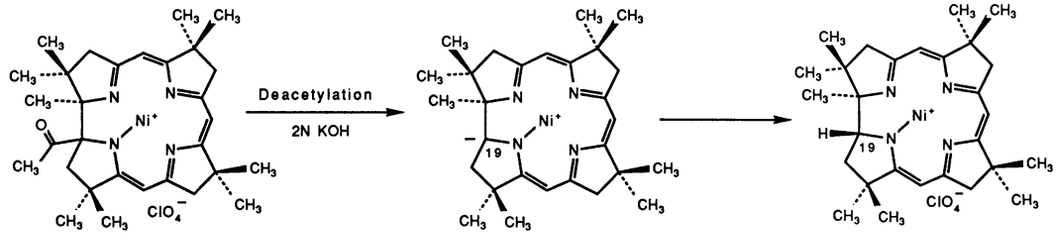
FIG. 3. The 125.8-MHz ^{13}C NMR spectrum of cyanocobalamin enriched from [3-^{13}C]ALA/50% ^2H_2O (D_2O), showing α and β ^2H shifts. Solvent = ^2H_2O; broad-band-decoupled (decoupler power = 6 H); number of scans = 53,800; acquisition time = 1.18 s; size of data table = 64,000 points; and pulse width = 41°.

Table 1. ^{13}C NMR data of cyanocobalamin derived from [4-^{13}C]ALA

Carbon	Nonshifted signal, ppm	α shift, Hz	β shift, Hz
C-11	176.61		
C-6	166.00		
C-1	85.88		8.84
C-19	75.69		6.42
C-17	59.99		11.24
C-3	57.04	48.99	
C-8	56.50	57.02	
C-13	54.42	50.60	

Table 2. ^{13}C NMR data of cyanocobalamin derived from [3-^{13}C]ALA

Carbon	Nonshifted signal, ppm	α shift, Hz	β shift, Hz
C-7	51.43		11.02
C-12	48.17		10.18 (2.55)
C-2	47.29		9.32
C-18	39.10	42.39	10.18
C-17a	32.33		
C-13a	28.00		10.51
C-3a	26.11		11.87
C-8a	26.01	(48.32)	13.56



3 4 5

FIG. 4. Deacetylation of the model C-19 acetylcorrin (3).

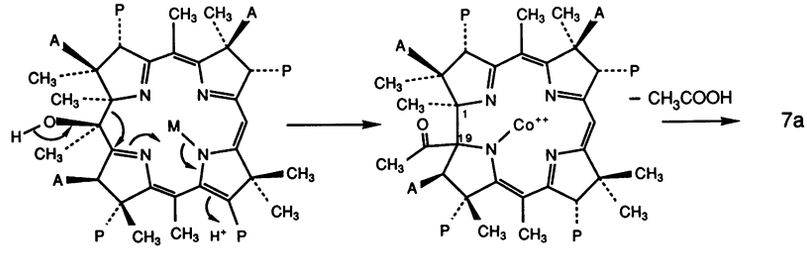


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FIG. 5. Proposed biosynthetic dihydrocorphinol → acetylcorrin rearrangement. A = acetyl; P = propionyl.

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