The Biosynthesis of Vitamin B₁₂: A Study by ¹³C Magnetic Resonance Spectroscopy

(Corin/cyanocobalamin/porphyrins/porphobilinogen/δ-aminolevulinic acid)

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ABSTRACT The origin of the methyl group on C-1 of Ring A of the corrin ring of vitamin B₁₂ was investigated by ¹³C magnetic resonance spectroscopy. The proton-decoupled ¹³C spectra of vitamin B₁₂ synthesized from [5-¹⁴C]-δ-aminolevulinic acid by Propionibacteria were obtained by Fourier-transform nuclear magnetic resonance of high resolution, and spectra of high-resolution proton magnetic resonance of the ¹³C-labeled B₁₂ were also taken. The δ-carbon atom of δ-aminolevulinic acid is the source of seven or eight known positions of vitamin B₁₂, depending on whether the C-1 methyl group is also derived from the labeled substrate. We have found seven resonances whose chemical shifts enable us to identify the position of the ¹³C atoms in the molecule from the assignment of Dodderell and Allerhand. We observed no ¹¹C resonance corresponding to the C-1 methyl group of Ring A. Furthermore, the proton magnetic resonance spectrum showed no spin-spin splitting of the proton peak at the γ values assigned for the H atoms in this methyl group. It would thus appear that the methyl group at C-1, which has been considered to have the same origin as the δ-methylene bridge of porphyrins, does not originate from the δ-carbon atom of δ-aminolevulinic acid. This finding may aid in elucidation of the mechanism of synthesis of the functional isomer of vitamin B₁₂ and porphyrins. The ¹³C-labeled vitamin B₁₂ enabled us to make further assignments to the ¹³C magnetic resonance of the vitamin.

The corrin moiety of vitamin B₁₂ is structurally (1-3) and biosynthetically (4-6) similar to the porphyrin ring system although it is more highly reduced, lacks a bridge carbon atom between Rings A and D, and contains an additional seven "extra" methyl groups. These seven methyl groups include the methyl group at C-1, and six methyl groups at C-2, -5, -7, -12, -15, and -17 (one of the methyl groups at C-12 arises by decarboxylation of the original acetic acid side chain), which Bonnet et al. (3) suggested may arise by a C-alkylation of a tetracyclic intermediate (Fig. 1). It has been demonstrated that C-alkylation does indeed occur biologically and that at least six of the methyl groups in B₁₂ do arise from the methyl group of methionine (5, 6).

It has been suggested that the methyl group at C-1 does not arise by C-alkylation but that its origin is the same as that of the δ-methylene bridge carbon atom of porphyrins (6). This suggestion arose from structural considerations and from an experiment in which B₁₂ was biosynthesized from [5-¹⁴C]-δ-aminolevulinic acid. It has been demonstrated that [5-¹⁴C]-δ-aminolevulinic acid used for B₁₂ or porphyrin synthesis only labels carbon atoms 4, 9, 14, and 16 and the bridge carbon atoms; therefore, the labeled B₁₂ degraded by a Kuhn-Roth oxidation cannot yield ¹⁴C-labeled acetic acid unless a methyl group of the B₁₂ contained ¹⁴C. In this experiment the methyl group of the acetic acid formed contained radioactivity (8). The radioactivity was only 8-9% of the calculated value, and it was considered possible that it was present in the methyl group at C-1. This low yield of radioactivity could be rationalized because the yield of acetic acid was low, and the contribution of the methyl group at C-1 to the general pool of the total acetic acid yield could not be ascertained. On the other hand, the finding of methyl-labeled acetic acid in this degradation could have arisen if the labeled carbon atom of the δ-aminolevulinic acid was converted to the methyl group of methionine in the biological system, and thus indirectly gave rise to labeled methyl groups of B₁₂.

It is of considerable interest to establish the origin of the methyl group at C-1, for it would permit differentiation between the two possible biosynthetic pathways that may be responsible for the synthesis of the corrin ring, and furthermore eventually aid in the understanding of the mechanism of the formation of the functional isomer of the tetracyclic structure of porphyrins and corrins. The corrin ring system of B₁₂ has been considered to arise directly from porphobilinogen or from a related porphyrinogen.

\[ \text{δ-aminolevulinic acid} \rightarrow \text{Porphobilinogen} \rightarrow \text{Porphyrinogen} \]

The purpose of this investigation is to establish whether or not the C-5 of δ-aminolevulinic acid is the precursor of the methyl group at C-1 of B₁₂, as it is for the δ-bridge carbon atom of porphyrins. Since degradative procedures for B₁₂ are at present inadequate to uncover the origin of the methyl group at C-1, nuclear magnetic resonance studies were initiated to answer this particular question because this technique allows simultaneous observations on all the carbon atoms that arise from the ¹³C-labeled precursor.

MATERIALS AND METHODS

Preparation of [5-¹³C]-δ-aminolevulinic acid

[2-¹⁴C]Malonic acid, 75% enriched in ¹⁴C, was purchased from Merck, Sharp, and Dohme of Canada Ltd., esterified (7), and used for the synthesis of δ-aminolevulinic acid essentially by the method of Shermin (8). The dimethylmalonic ester was condensed with β-carbomethoxypropionyl chloride in
anhydrous dioxane with the aid of sodium hydride, and the oximation reaction on the subsequent β-ketoacidipic acid was accomplished with isoamyl nitrite. The identification and purity of the [5-13C]β-aminolevulinic acid was verified by 1HMR (magnetic resonance) spectroscopy and by thin-layer chromatography.

**Synthesis of [13C]B12**

*Production of Vitamin B12.* Propionibacterium shermanii (ATCC 9615) maintained in stab cultures (Difco tomato-juice agar) were grown anaerobically for 3 days at 30°C in the medium described below. 25 ml of these starter cultures was used to inoculate 1 liter of medium containing 20 g of yeast extract, 46 g of glucose, 10 mg of Co(NO3)2·6H2O, 25 mg of dimethylbenzimidazole, 50 mg of L-methionine, and 1 mg each of riboflavin, calcium pantothenate, biotin, niacin, thiamine, and p-aminobenzoic acid and 5 mmol each of K2HPO4 and NaH2PO4. To each liter of the inoculated medium about 50 mg of the [5-13C]β-aminolevulinic acid was added. Usually 8 liters of medium were used for B12 production. The cultures were grown anaerobically for 3 days at 30°C, then aerated for 2 days at 37°C essentially by the method of Menon and Shemin (9). The pH was maintained at 6.85 ± 0.05 in a pH-stat by addition of concentrated NH4OH or 6 N HCl. About 50-60 g (wet weight) of cells per liter were obtained. The amount of B12 was determined by a bioassay (10) in Difco assay media.

**Isolation of vitamin B12**

Cells were collected by centrifugation and suspended in an equal volume of water, which was acidified to pH 2.2-2.5 with 6 N H2SO4 and broken by sonication. The suspension was adjusted to pH 5 with 5 N NaOH and centrifuged. Vitamin B12 was isolated from the supernatant solution by extraction with a solvent mixture of 55% o-cresol and 45% CCl4 (v/v), and back-extracted into water by the procedure of Bray and Shemin (6). The crude B12 was purified by ion-exchange chromatography on a Dowex-50 (Na+) pH 7 and a Dowex-2 (OH-) pH 9.5 column (11), and finally crystallized from an acetone-water mixture. The purity of the product was determined by thin-layer chromatography (12) and UV and visible spectroscopy. The yield of pure crystalline B12 was about 5 mg/liter.

**Spectra**

The nuclear magnetic resonance (NMR) studies on δ-aminolevulinic acid were performed on a Varian T-60 NMR spectrometer. The 13CMR spectra for the B12 were obtained on a Bruker HFX-90 MHz NMR spectrometer, adapted for Fourier pulse operation. Proton magnetic resonance spectra were recorded on Varian 100 MHz (37°C) and 220 MHz (21°C) NMR spectrometers.

The 13CMR spectra were recorded on a 12 mM solution of the 13C-labeled B12 at 25°C in D2O. The scan covered 5000 Hz, and C8 in a coaxial capillary was the external reference. 52,600 Pulses at a recycle time of 0.4 sec were collected. Natural abundance of 13C could not be observed on commercial B12 under these operating conditions.

![Fig. 1. Structure of Vitamin B12 (cyanocobalamin). The closed circles (●) represent the positions derived from the δ-carbon atom of β-aminolevulinic acid.](image)

![Fig. 2. Proton-decoupled 13CMR spectrum of 13C-labeled cyanocobalamin. The range of the spectrum is 0–206 ppm upfield from CS2. Conditions are given in text.](image)
RESULTS

The concentration of $^1$C in the isolated B$_{12}$ was determined by mass spectroscopy to be 5.7%. The labeled positions were 37 or 42% $^1$C depending on the number of positions arising from the $\delta$-carbon atom of $\delta$-aminolevulinic acid. This low-dilution factor was previously observed in experiments in which we added [4-$^1$C]$\delta$-aminolevulinic acid. It is likely that the added $\delta$-aminolevulinic acid either repressed or inhibited an enzyme or enzymes concerned with B$_{12}$ synthesis.

From the known mechanism of porphobilinogen and porphyrin synthesis (13, 14) and from a detailed analysis of the labeling pattern obtained in porphyrin synthesized from labeled precursors, it is possible to designate which of the carbon atoms of B$_{12}$ are derived from carbon atom-5 of $\delta$-aminolevulinic acid. The carbon atoms of B$_{12}$ derived from carbon atom-5 of $\delta$-aminolevulinic acid are C-4, -5, -9, -10, -14, -15, and -16, and possibly the C-1 methyl group, if this latter carbon atom is indeed the original $\alpha$-substituent of the porphobilinogen used for Ring A. It can readily be seen (Fig. 1) that all the $^1$C-labeled positions are adjacent to at least one other $^1$C atom, and C-15 is bonded to two other $^1$C-labeled positions. Since in each position about 40% of the carbon atoms are $^1$C, each resonance of the $^1$C in the position listed above should consist of a singlet corresponding to a $^1$C bonded to a $^1$C atom, and a doublet resulting from $^1$C-$^1$H spin-spin splitting. This doublet should be centered at the $^1$C-$^1$C singlet. Because C-15 can be bonded to two $^1$C atoms, one $^1$C atom, or two $^1$C atoms, a rather complex splitting pattern centered around the singlet results, with an intensity lower than the $^1$C-$^1$C singlets corresponding to the other labeled positions.

The $^1$C spectrum we observed (Fig. 2) was found to consist of resonances of the type described above, with singlets at the chemical shifts given below and doublets that are centered at the singlets. The singlets were found at 11.8 ppm, 12.8 ppm, two very closely spaced peaks at 26.6 ppm, 85.5 ppm, and 100.3 ppm upfield from CS$_2$, and the doublets to have a $J_{1^1C-^2H}$ of 60 to 70 Hz. A more complex splitting pattern was observed for the resonance centered at 88.0 ppm. Seven resonances were observed due to the incorporation of $^1$C into particular positions of B$_{12}$.

Doddrell and Allerhand (15) have assigned many of the $^1$C resonances in B$_{12}$. The chemical shifts we have observed are not only in good agreement with their assignments, but from our spectra we can make some additional assignments. The resonance at 100.3 ppm we assign to C-10, for this was the only carbon atom that appeared to exhibit enhancement of nuclear Overhauser effect due to bonding to hydrogen. Further, this assignment corresponds with the value reported (15). Although Doddrell and Allerhand could not differentiate between C-5 and C-15, they assigned the resonances at 85.9 ppm and 89.3 ppm to these carbon atoms. Since we observed resonances at 85.5 ppm and a complicated resonance centered at 88.0 ppm, we assign C-5 to 85.5 ppm and C-15 to 88.0 ppm. The remaining four downfield peaks are therefore assigned to carbon atoms-4, -9, -14, and -16, which are unsaturated and bonded to the nitrogen of the heterocyclic rings.

The seven carbon positions that were predicted to contain $^1$C indeed do, and the chemical shifts observed for these carbon atoms are in excellent agreement with those assigned (15). However, no additional $^1$C resonance was observed, and especially no resonance could be seen in the 154-162 ppm region, the range assigned by Doddrell and Allerhand to the resonance of the methyl group at C-1. Furthermore, although the proton magnetic resonances spectrum had a peak at the $r$ value assigned for the methyl group at C-1 (16-18), no $^1$C-$^1$H spin-spin splitting of the $^1$H resonance was observed in the proton spectrum. This finding is entirely consistent with the results of the $^1$CMR spectrum and with the observation that there is no excess $^1$C in this methyl group. These results demonstrate that the 5-carbon of $\delta$-aminolevulinic acid is not the precursor of the methyl group at C-1.

DISCUSSION

From an inspection of the structures of porphyrins and the corrin ring of B$_{12}$, it seemed reasonable to assume, since Rings A and D of the B$_{12}$ are fused and a methyl group is on the $\alpha$-position of Ring A, that this methyl group and the $\delta$-methine bridge carbon of porphyrins have the same biosynthetic origin. These carbon atoms could then both be presumed to come from the $\delta$-carbon atom of $\delta$-aminolevulinic acid (originally carbon atom-2 of glycine). Our finding that the methyl group at C-1 does not arise from the $\delta$-carbon atom of $\delta$-aminolevulinic acid implies that in the biosynthesis of B$_{12}$ the amino-methyl group of the porphobilinogen used for formation of Ring A is either lost to the medium or perhaps used to bridge Rings C and D, and that subsequently the methyl group at C-1 arises from another source.

In considering the mechanism of Ring A and Ring D fusion, investigators have proposed mechanisms that consider a porphyrinogen as an intermediate, with the implication that, in the conversion of the porphyrinogen to the corrin ring, the $\delta$-methine bridge carbon atom gives rise to the methyl group at C-1. Our negative finding rules out this mechanism and is consistent with the observation of Müller and Dieterle (19) who were unable to demonstrate the conversion of uroporphyrinogen to B$_{12}$. Therefore, in agreement with our observations and those of Müller and Dieterle, it would seem reasonable to assume that the corrin ring system is synthesized directly from porphobilinogen and that a porphyrin structure is not an intermediate according to the mechanism indicated above.

These experimental findings not only confirm several of the peak assignments of Doddrell and Allerhand (15), but also enable us to distinguish between the resonance due to carbon atoms 5 and 15, and make it possible to assign these. Furthermore, the findings in this investigation specifically assign the resonances between 11.8 and 26.6 ppm to the carbon atoms at positions 4, 9, 14, and 16, but more detailed assignments must await additional data. The range of these chemical shifts was previously indicated (15).

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