Studies on the Biosynthesis of Corrinoids and Porphyrinoids. II. The Origin of Nitrogen of Vitamin B_{12}

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To clarify the origin of nitrogen of vitamin B_{12}, ^{15}N-labeled aminolevulinic acid (ALA) was prepared and administered to *Propionibacterium shermanii*. Vitamin B_{12} thus isolated showed four signals in the nitrogen-15 nuclear magnetic resonance (^{15}N-NMR) spectrum. The nitrogen of [5-^{15}N]riboflavine was incorporated into the benzimidazole part of vitamin B_{12}. Hydroxycobalamin was transformed into cyanocobalamin by treatment with [^{15}N]potassium cyanide, and the ^{15}N-NMR spectrum was measured. The results of these experiments revealed the origin of the nitrogen atoms of vitamin B_{12}, and allowed the ^{15}N-NMR signals to be assigned.

Keywords vitamin B_{12}; biosynthesis; nitrogen origin; nitrogen-15-labeling; ^{15}N-NMR; ^{15}N-aminolevulinic acid; ^{15}N-riboflavine; ^{15}N-KCN

We have investigated the biosynthetic pathway of vitamin B_{12},[1-3] especially the origin of the heteroatoms. As regards hydrogen, most of the ring hydrogens were reported to be derived from the medium.[4,5] The origin of oxygen was reported in the previous paper.[6] In this paper, we deal with the origin of nitrogen of vitamin B_{12}.

Vitamin B_{12} has fourteen nitrogen atoms in its molecule (Fig. 1). It has been reported[7] that natural tetrapyrrole derivatives (vitamin B_{12}, chlorophyll, protoheme, *etc.*) are derived from porphobilinogen (PBG), which is itself derived from 5-aminolevulinic acid (ALA), which is in turn derived from glycine and succinyl CoA.[8] In the case of the biosynthesis of vitamin B_{12}, this theory is based on feeding exporiments with ^{13}C-labeled precursors, except for the case of the formation of ALA. ^{15}N-Labeled compounds were utilized to investigate the intermediate in the transformation of PBG to uroporphyrinogen III.[9,10] However, there is no report about the origin of nitrogen of vitamin B_{12}. We therefore planned to incorporate ^{15}N-labeled ALA into vitamin B_{12} produced by *Propionibacterium shermanii* ATCC 9614. According to the above pathway, ^{15}N of ALA should be incorporated into the corrin ring nitrogens of vitamin B_{12}.

Results and Discussion

^{15}N-ALA was prepared by the method of Neuberger and

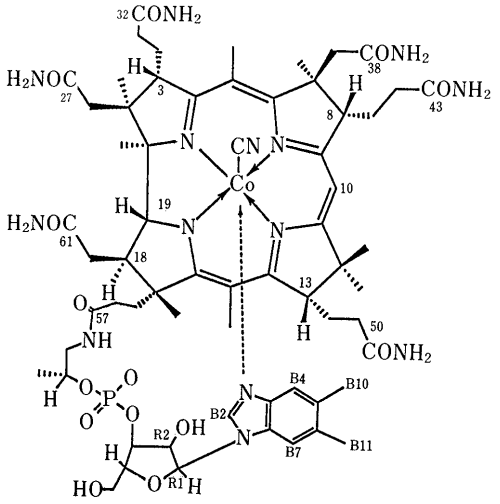

Fig. 1. Structure of Vitamin B_{12}

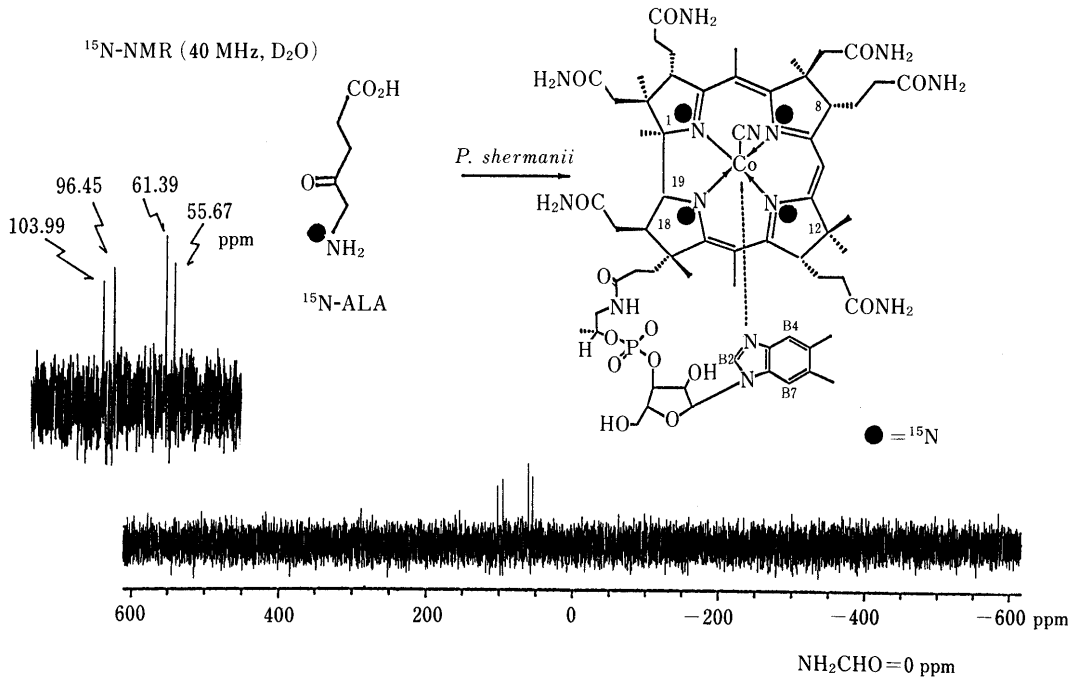

Fig. 2. ^{15}N-NMR Spectrum of ^{15}N-ALA-Incorporated Vitamin B_{12}

Scott.[11] In the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum, the C-5 signal of ALA appeared as a doublet owing to the coupling with neighboring ^{15}N (J= 7.3 Hz). This ^{15}N-ALA was fed to *P. shermanii*, and vitamin B_{12} was isolated. Figure 2 shows the nitrogen-15 nuclear magnetic resonance (^{15}N-NMR) spectrum of the isolated vitamin B_{12}. Four distinct signals were observed at 56, 61, 96, and 104 ppm (formamide = 0 ppm as an external reference). The chemical shifts of these peaks are in the range of pyrrole nitrogen (50—140 ppm), rather than amide nitrogen (−10—10 ppm).[12] As no ^{15}N-NMR signals were obtained on measurement of unlabeled vitamin B_{12}, these four peaks are due to incorporated ^{15}N.

To clarify the origin of nitrogen of the benzimidazole ring of vitamin B_{12}, [5-^{15}N]riboflavine was administered to *P. shermanii*. Horig and Renz reported that benzimidazole of vitamin B_{12} is derived from riboflavine, based on a feeding experiment with ^{13}C-labeled riboflavine, and a ^{13}C-NMR study.[13] Figure 3 illustrates the ^{15}N-NMR spectrum of vitamin B_{12}, isolated after incorporation of [5-^{15}N]-riboflavine. One signal was observed at 49 ppm, a chemical shift corresponding to imidazole nitrogen.[14] Figure

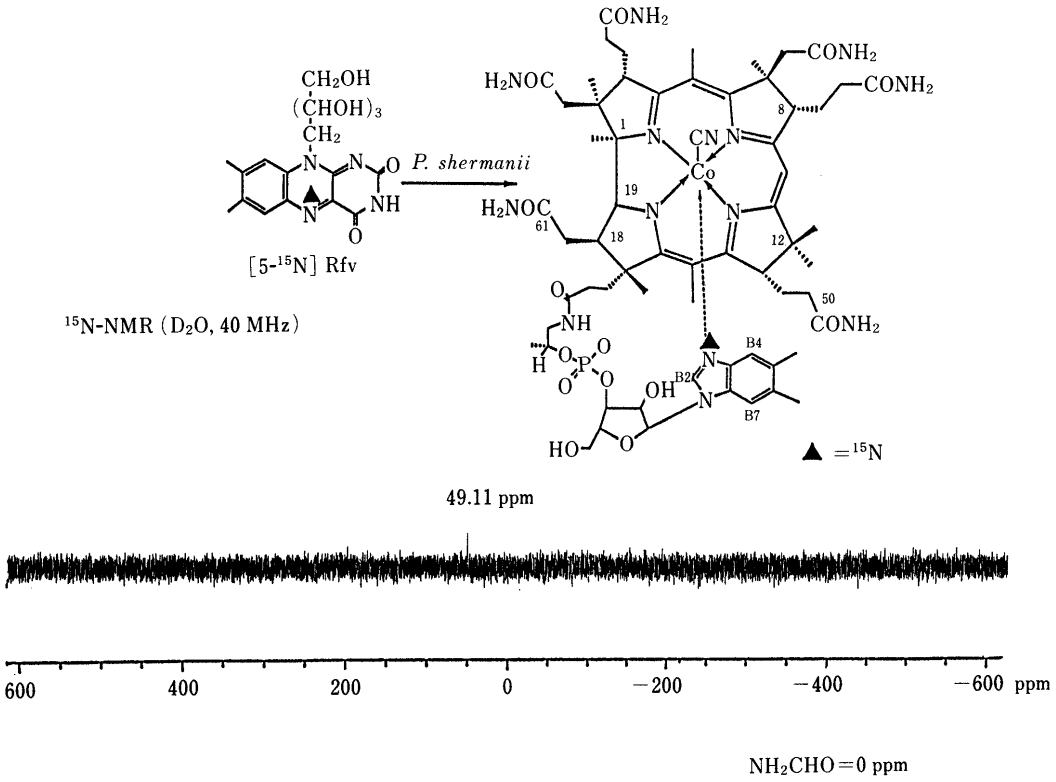

Fig. 3. ^{15}N-NMR Spectrum of ^{15}N-Riboflavine-Incorporated Vitamin B_{12}

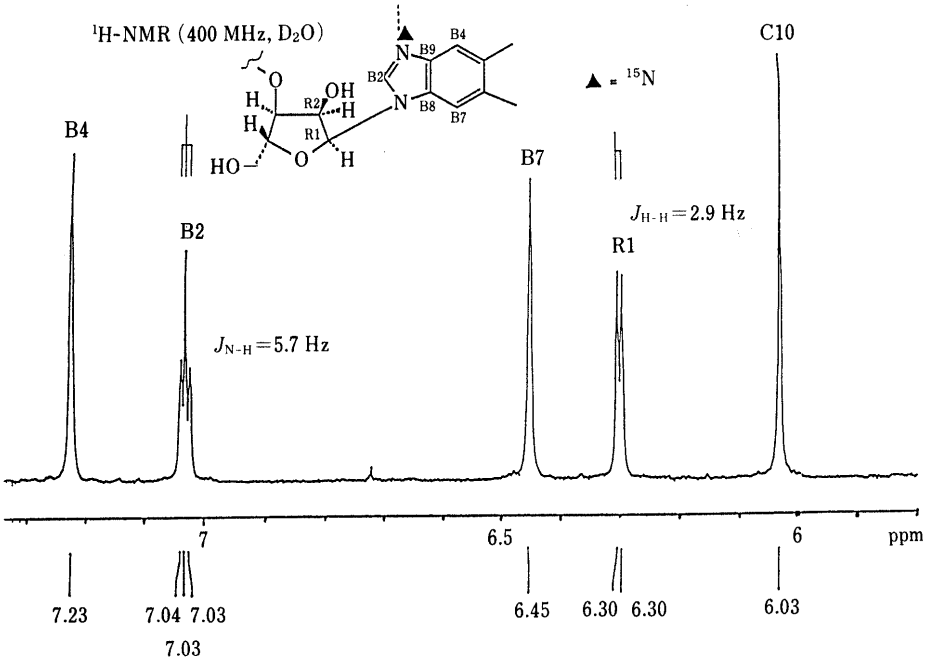

Fig. 4. Downfield Region of the ^{1}H-NMR Spectrum of ^{15}N-Riboflavine-Incorporated Vitamin B_{12}

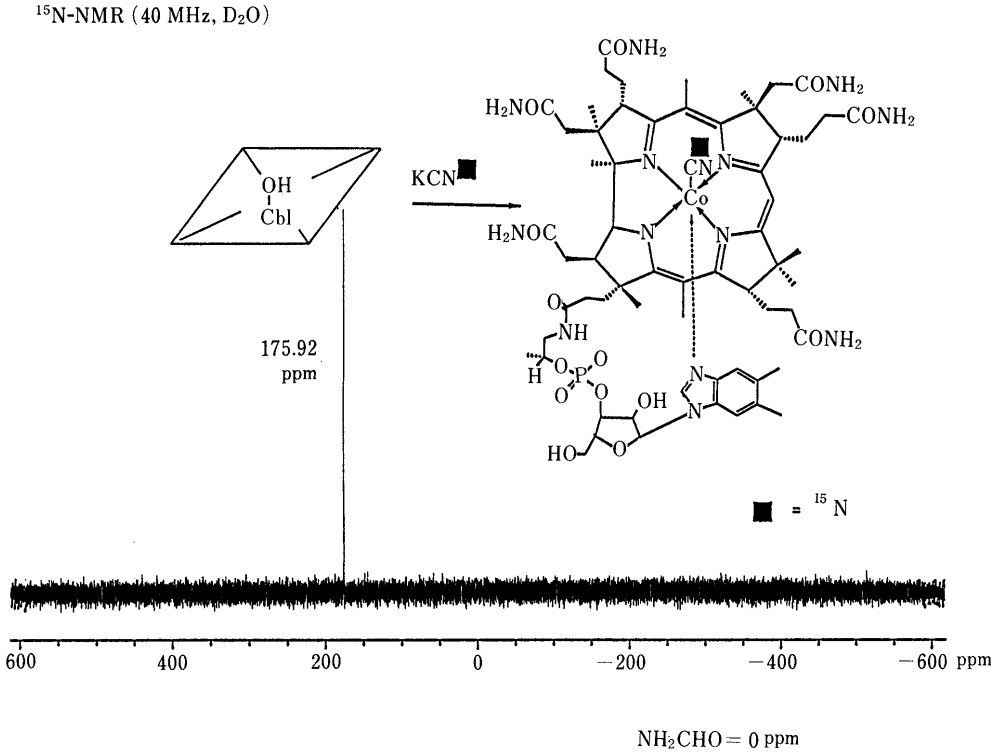

Fig. 5. ^{15}N-NMR Spectrum of [^{15}N-CN]Vitamin B_{12}
Cbl means cobalamin.

4 shows the downfield region of the ^{1}H-NMR spectrum of the above isolated vitamin B_{12}.[15] The B_2-H signal shows an apparent triplet, one peak being that of the unlabeled compound, and the other doublet being due to ^{15}N-labeled vitamin B_{12}. The coupling constant is 5.7 Hz. This ^{1}H-NMR spectrum also supports the incorporation of [5-^{15}N]ribofravine into vitamin B_{12}.

To clarify the ^{15}N-NMR signal of cyano nitrogen of vitamin B_{12}, hydroxycobalamin was transformed into cyano cobalamin with ^{15}N-KCN. Figure 5 shows the ^{15}N-NMR spectrum of the above vitamin B_{12}. It shows a ^{15}N signal at 175.9 ppm. Compared with ^{15}N-KCN (=159.1 ppm), it showed a downfield shift of 16.8 ppm. This shift is caused by coordination of the cyano group to the cobalt ion to form a Co–C bond.

The remaining nitrogens are the amide nitrogens of the corrin side chain. We consider that these nitrogens are derived from ammonia, available from the amino transfer of amino acids. We are planning to incorporate various ^{15}N-labeled amino acids into vitamin B_{12}, to identify the source of amide-nitrogens of vitamin B_{12}.

Experimental

Infrared spectra (IR) were recorded on a Jasco DS-701G spectrometer. ^{1}H-NMR spectra were taken on Hitachi R24B (60 MHz) and JEOL GSX-400 (400 MHz) spectrometer. ^{13}C- and ^{15}N-NMR spectra were taken on a JEOL GSX-400 spectrometer (100, 40 MHz). Chemical shifts are given downfield from DOH (=4.70 ppm) or sodium [2,2,3,3-$^2H_4$]-3-(trimethylsilyl) propionate (TSP) in the case of ^{1}H-NMR, from tetramethylsilane (TMS) or dioxane (=67.40 ppm) as an internal standard for ^{13}C-NMR, and from NH_4NO_3 (=0 ppm) or formamide (=0 ppm) for ^{15}N-NMR. Fast atom bombardment mass spectra (FAB-MS) were recorded on a JEOL DX-302 spectrometer equipped with a JMA-DA-5000 data system. Ultraviolet (UV) spectra were recorded on a Jasco UVIDEC 610C spectrometer.

Methyl [^{15}N]Phthalimidolevulinate (1) [^{15}N]Potassium phthalimide (99.7 atom% ^{15}N, purchased from Shoko Co. (0.80 g, 4.30 mmol), was dissolved in 6 ml of dry dimethylformamide (DMF). A solution of methyl 5-chlorolevulinate (prepared from succinic anhydride and diazomethane, 708 mg, 4.30 mmol) in 2 ml of DMF was added dropwise to the above solution of [^{15}N]potassium phthalimide under an argon atmosphere. The reaction mixture was stirred for 0.5 h at room temperature, warmed at 60—80 °C for 5 h, then diluted with water, and the product was extracted with chloroform. The organic layer was washed with saturated brine, dried over anhydrous sodium sulfate and evaporated. The residue was chromatographed on silica gel (hexane : ether = 4 : 1) to give **1** as colorless needles (431 mg, 36.3%). mp 89—94 °C. ^{1}H-NMR (60 MHz, $CDCl_3$) δ: 2.73 (4H, m, $COCH_2CH_2COO$), 3.61 (3H, s, $COOCH_3$), 4.48 (2H, s, $^{15}NCH_2CO$), 7.62 (4H, m, phenyl). IR (KBr): 1770, 1710 (C=O) cm^{-1}. MS *m/z*: 276 (M^+, 5.8%).

[^{15}N]Aminolevulinic Acid Hydrochloride (2) The above imide (**1**) was refluxed with 5 ml of 6 N hydrochloric acid at 110 °C for 14 h. After the solution had cooled, phthalic acid was removed by filtration. The mother liquor was concentrated and the residue was purified on a Dowex 50X8 column, freeze-dried, and then recrystallied from ethanol–ether to give **2** as colorless needles (227.0 mg, 87.2%). mp 144—147 °C. ^{1}H-NMR (400 MHz, D_2O) δ: 2.70 (2H, t, J=6.1 Hz, CH_2CH_2COO), 2.89 (2H, t, J=6.1 Hz, CH_2CH_2COO), 4.11 (2H, s, $^{15}NCH_2CO$). ^{13}C-NMR (100 MHz, D_2O) δ: 48.0 ($^{15}NCH_2CO$, d, $J_{^{15}N-^{13}C}$=7.3 Hz). ^{15}N-NMR (40 MHz, D_2O, NH_4NO_3=0 ppm) δ: −352.4. IR (KBr): 3425 (N-H), 1735, 1725 (C=O) cm^{-1}. MS (FAB-MS) *m/z*: 133 (M^++1−HCl).

Incorporation of [^{15}N]ALA (2) into Vitamin B_{12} (3) *Propionibacterium shermanii* ATCC 9614 was incubated for 7 d in 12 l of casein I-B medium under a nitrogen atmosphere, with adjustment of the pH to 7.0 every day, and collected by centrifugation at 8000 rpm at 4 °C for 35 min. The cells were washed with brine, and divided into 6 batches, each of which was placed in a 500 ml sterilized flask containing a suitable medium.[6] They were incubated at room temperature for 3 d under the same conditions. The cells were gathered, washed with brine, and disrupted with an ultrasonicator (NIC US-300) at 0 °C for 15 min in 400 ml of 80% methanol solution containing 0.1% potassium cyanide, twice. The suspension was centrifuged at 8000 rpm at 4 °C for 30 min. The weight of wet cells was 138 g. The supernatant was concentrated to 150 ml, then extracted with 1 : 1 phenol–chloroform (40 ml × 2). The extract was washed with water (50 ml × 2), diluted with 1000 ml of ether, and re-extracted with water (50 ml × 3). The extract was washed with 50 ml of chloroform, then with 50 ml of ether. It was evaporated, and purified by column chromatography (SiO_2, methanol) (11 g, 1.5 i.d. × 13 cm). The red fraction (Rf=0.2) was collected and evaporated. The residue was recrystallized repeatedly from

water–acetone (1 : 8) to give 1.7 mg of **3** as needles. UV λ_{max}, nm: 550.0, 358.4. ^{15}N-NMR (40 MHz, D_2O, $\underline{N}H_2CHO$) δ: 55.7, 61.4, 96.5, 104.0.

Incorporation of [5-^{15}N]Riboflavin (4) into Vitamin B_{12} (5) *P. shermanii* was cultivated in 12 l of casein medium for 7 d under nitrogen, with adjustment of the pH to 7.0 every day. The cells were harvested, washed, and transferred into 6 flasks containing a suitable medium.[6] Then 125 mg of [5-^{15}N]riboflavine (**4**) (obtained from Dr. D. Alworth at E.T.H) was added and the cells were incubated for 3 d. The weight of wet cells was 161 g. After the same procedure as described above, 6.0 mg of **5** was obtained. UV λ_{max}, nm: 550.0, 358.4. ^{15}N-NMR (40 MHz, D_2O, $\underline{N}H_2CHO$) δ: 49.1. ^{1}H-NMR (400 MHz, D_2O, TSP) 7.88 (d, $J_{^{15}N-H}$= 5.7 Hz, B_2-H) 7.88 (s, B_2-H).

[^{15}N-CN]Vitamin B_{12} (5) A solution of [^{15}N]potassaium cyanide (99% atom ^{15}N, purchased from C.I.L. Co.) (1.2 mg) in 25 ml of water was added to a solution of hydroxycobalamin (25 mg) in 30 ml of water. The mixture soon turned dark red, and was extracted with phenol–chloroform (1 : 1) twice (40, 20 ml). The extract was washed with water (50 ml × 2), diluted with 600 ml of ether, and reextracted with water (50 ml × 4). This extract was washed with chloroform (50 ml) and evaporated. After recrystallization of the residue from water–acetone (1 : 5), 23 mg of **5** was obtained as red needles. UV λ_{max}, nm: 550.0, 358.4. ^{15}N-NMR (40 MHz, D_2O, $\underline{N}H_2CHO$) δ: 175.9.

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