Acknowledgments

We thank Dr. Akihiro Kusumi for providing us with spectral data for Figure 2 and for his advice, Professor Philippe Devaux for his discussions, and S. B. Sato for his help during this study. We also thank Dr. H. Saji for the supply of red blood cells.

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Carbon-13 Nuclear Magnetic Resonance Studies of Cyanocobalamin and Several of Its Analogues†

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ABSTRACT: The carbon-13 nuclear magnetic resonance spectrum of cyanocobalamin in aqueous solution has been interpreted. The assignments are based on the earlier biosynthetic studies with carbon-13-enriched precursors and on the present systematic analysis of the spectra of cyanocobalamin, cyanocobalamin lactone, cyanocobalamin lactam, cyanoepicobalamin, and several cyanocobalaminmonocarboxylic acids. The interpretation of the spectrum of cyanocobalamin greatly simplifies the structure determination of new corrinoids and should prove very helpful in future studies of these compounds. The structures of two cyanocobalamindicarboxylic acids and a cyanocobalaminmonocarboxylic acid lactone have been determined by comparing their carbon-13 magnetic resonance spectra with that of cyanocobalamin.

Vitamin B_{12} (cyanocobalamin) (Figure 1) is one of the most complex nonpolymeric molecules found in nature. The final elucidation of its structure in the 1950s by Hodgkin and coworkers (Hodgkin, 1965) required X-ray crystallographic studies because chemical degradative procedures were not sufficient for the determination of a structure as complex and as chemically inert as the corrin ring.

At present, nuclear magnetic resonance spectroscopy (NMR), and in particular ^{13}C NMR, is undoubtedly the most powerful technique for the elucidation of the structure of complex molecules. Doddrel & Allerhand (1971) determined the ^{13}C NMR spectrum of cyanocobalamin and made several assignments. Subsequent assignments were based on the la-

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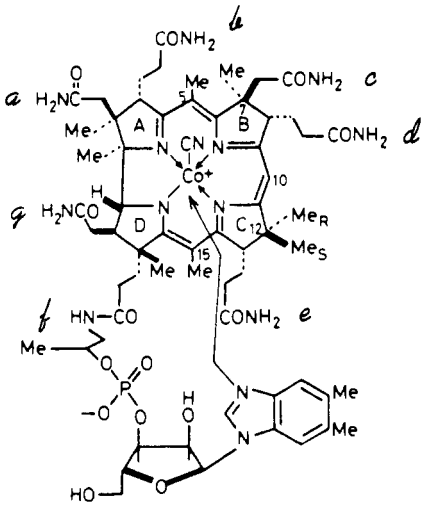

FIGURE 1: Structure of cyanocobalamin.

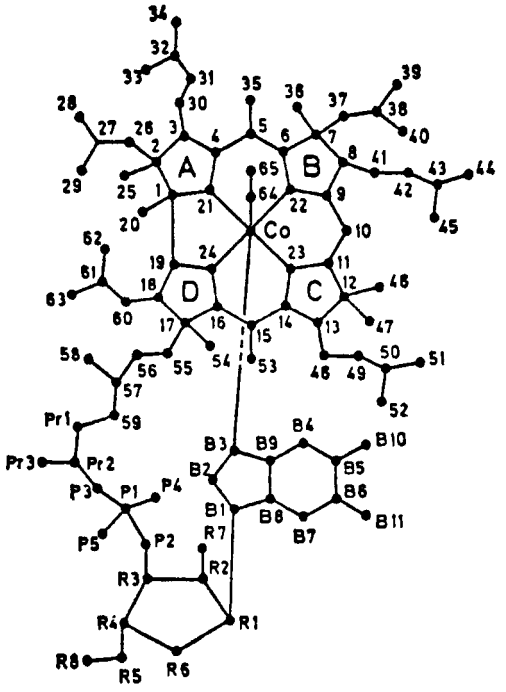

FIGURE 2: Numbering of atoms in cyanocobalamin.

beling pattern in cyanocobalamin derived from specifically labeled precursors. Thus, seven methyl groups, those at C-1, C-2, C-5, C-7, C-12 (*pro-R*), C-15, and C-17, are derived from the methyl group of methionine, while the α-methylene carbons of the propionamide side chains (C-31, C-42, C-49, and C-56), the methylene carbons of the acetamide side chains (C-26, C-37, and C-60), and the *pro-S* methyl group at C-12 are derived from C-2 of δ-aminolevulinic acid. Seven carbon atoms of the corrin ring (C-4, C-5, C-9, C-10, C-14, C-15, and C-16) are derived from C-5 of δ-aminolevulinic acid, while C-8 of porphobilinogen is the precursor of the β-methylene carbons of the propionamide side chains (C-30, C-41, C-48, and C-55) (Figure 2) (Scott et al., 1974, 1976; Imfeld et al., 1976; Battersby et al., 1976). In these studies most of the resonances were assigned in groups, and not many individual resonances were identified. Recently, Schlingmann et al. (1980) reported the assignments for more than half of the carbon resonances of several dicyanocobyrinic acid methyl ester amides, prepared by partial methanolysis of cyanocobalamin. Ernst (1981) has extended this work and has presented the complete interpretation of the carbon spectrum of dicyanocobyrinic acid hep-

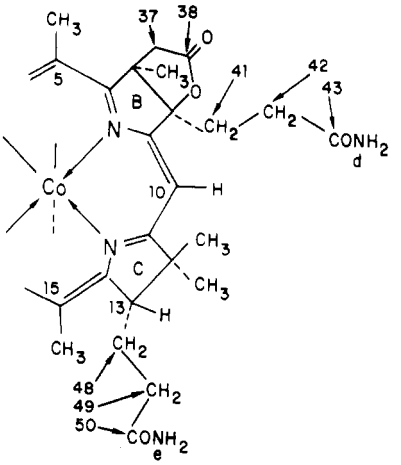

FIGURE 3: Partial structure of cyanocobalamin lactone. In cyanocobalamin lactam, the lactone ring oxygen is replaced by a nitrogen (N–H) while in cyanoepicobalamin the propionamide side chain at C-13 is projecting up from the corrin ring.

tamethyl ester. A comparison of this spectrum with those of several unknown partially amidated dicyanocobyrinic acid methyl esters allowed the identification of these corrinoids (Schlingman et al., 1979).

Our ^{13}C NMR studies of three monocarboxylic acids derived from vitamin B_{12} demonstrated that the earlier identification of these acids was incorrect (Anton et al., 1980a). The revision of the identity of these acids was based on an analysis of the NMR spectra of cyanocobalamin, cyanoepicobalamin, cyanocobalamin lactone, cyanocobalamin lactam, and the cyanocobalaminmonocarboxylic acids. We have now prepared a larger number of derivatives of cyanocobalamin and are thus in the position to extend this earlier work and assign the resonances of virtually all of the 63 carbon atoms of cyanocobalamin.

The complete interpretation of the ^{13}C NMR spectrum of cyanocobalamin should greatly simplify the identification of several new forms of vitamin B_{12} that have recently been recognized. For instance, Katada et al. (1979) have reported that pharmaceutical preparations of vitamin B_{12} inevitably contain an isomeric form of cyanocobalamin as an impurity. Kolhouse and co-workers have shown that human plasma and animal tissues contain relatively large amounts of analogues of a cobalamin of unknown structure (Kolhouse et al., 1978; Kondo et al., 1980).

Experimental Procedures

Methods. Pulse Fourier transform ^{13}C (62.9-MHz) nuclear magnetic resonance spectra were obtained at 29 °C with a Bruker WM 250 spectrometer. The transients resulting from the application of 90° pulses in a spectral width of 15 000 Hz were accumulated as 32K points in the time domain and transformed into a 16K point spectrum. The data acquisition time was 1.081 s with a 2-s pulse delay, and the spectra were obtained under conditions of simultaneous broad-band (2500-Hz) proton noise decoupling. Chemical shifts were measured with respect to a neat tetramethylsilane external standard. The spectra were obtained on solutions containing 20 mg/mL 10% D_2O in H_2O in 10-mm tubes. The purity of the corrinoids was established by spectral analysis with a Cary Model 15 spectrophotometer and by descending paper chromatography in three solvent systems (Dolphin, 1971).

Synthesis of Analogues of Cyanocobalamin. The cyanocobalamin-*b*-, -*d*-, and -*e*-monocarboxylic acids were prepared as previously described (Anton et al., 1980a). Cyanocobalamin lactone (Figure 3) was synthesized by a modification of the

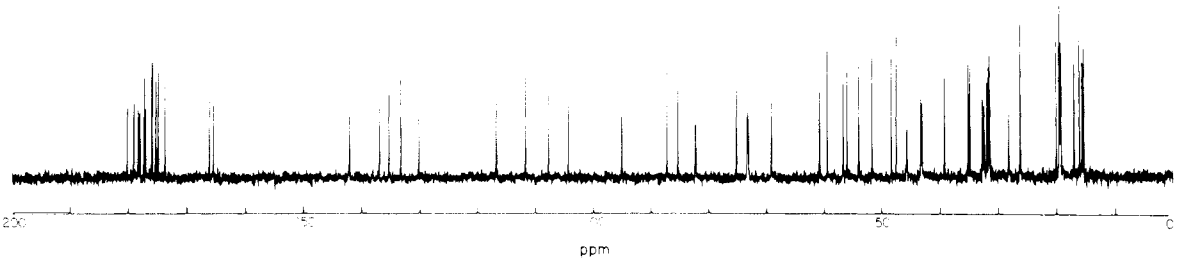

FIGURE 4: Proton noise decoupled ^{13}C NMR spectrum (62.9 MHz) of an aqueous solution of cyanocobalamin (10% D_2O).

procedure of Bonnet et al. (1957). To a solution of cyanocobalamin (2.0 g, 1.39 mmol) in 300 mL of water was added 400 mg of Chloramine T in 200 mL of water and 20 mL of 1.0 M HCl. The reaction mixture was stirred at room temperature for 2 h and then desalted by phenol extraction. The resulting aqueous solution was adjusted to pH 10.0 with sodium hydroxide and incubated at room temperature for 1 h to hydrolyze the lactone. The solution was then applied to a column of AG1 X2, 200–400 mesh (acetate form), 2.5 × 25 cm (equilibrated in water). The column was washed with water and eluted with 0.05 M sodium acetate buffer, pH 6.0. The fractions containing the desired corrinoids were pooled, desalted by phenol extraction, and applied to a second identical column of AG1 X2. Cyanocobalamin lactone was eluted with water, and a second corrinoid was eluted with 0.1 M sodium acetate buffer, pH 5.3. The second corrinoid was identified as a cyanocobalaminmonocarboxylic acid lactone on the basis of its ionophoretic mobility at several pH values. Both corrinoids were crystallized from aqueous acetone: yield, cyanocobalamin lactone 570 mg (28%) and cyanocobalaminmonocarboxylic acid lactone 420 mg (21%).

Cyanoepicobalamin-b- and -e-carboxylic Acids. Cyanocobalamin-*b*- and -*e*-carboxylic acids were epimerized by treatment with either anhydrous trifluoroacetic acid or trifluoromethanesulfonic acid as described before (Hogenkamp et al., 1975; Anton et al., 1980b). The epicobalamin acids were separated from the cobalamin acids by chromatography on AG1 X2. A desalted solution of the reaction mixture was applied to a 2.5 × 25 cm column of AG1 X2, 200–400 mesh (acetate form). The column was washed with water, and the monocarboxylic acids were eluted with 0.04 M sodium acetate buffer, pH 5.3, onto a second column (5 × 80 cm) of the same resin equilibrated with the eluting buffer. Cyanoepicobalamin-*e*-carboxylic acid was resolved from its normal isomer by elution with 0.04 M sodium acetate buffer, pH 5.3. Separation of the cyanoepicobalamin-*b*-carboxylic acid from its parent cobalamin required recycling twice through the same column. The two cyanoepicobalamincarboxylic acids were crystallized from aqueous acetone (yields: 12–15%).

Cyanocobalamin-d-carboxylic Acid Lactam. Cyanocobalamin-*d*-carboxylic acid (1.1 g, 730 μmol) dissolved in 200 mL of 0.1 M sodium hydroxide was heated at 100 °C for 10 min. The reaction mixture was cooled, neutralized, desalted by phenol extraction, and applied to a 2.5 × 25 cm column of AG1 X2, 200–400 mesh (acetate form). The column was washed with water and the desired cyanocobalamin-*d*-carboxylic acid lactam eluted with 0.04 M sodium acetate buffer, pH 5.3. Cyanocobalamin-*d*-carboxylic acid lactam was crystallized from aqueous acetone (yield: 0.62 g, 415 μmol, 57%).

1-α-D-Ribofuranosyl-5,6-dimethylbenzimidazole (α-Ribazole). Cyanocobalamin (3.0 g, 2.08 mmol) was treated with cerous hydroxide as described by Friedrich & Bernhauer (1956). The reaction mixture was then filtered through Celite, adjusted to pH 11, and applied to a 2.5 × 25 cm column of AG1 X2, 200–400 mesh (acetate form). The column was washed with water to remove the corrinoids, and the nucleoside was eluted with 0.1 M acetic acid. The fractions containing the nucleoside were pooled and evaporated to dryness. This preparation, which contained red contaminants, was further purified by chromatography on AG1 X2, 200–400 mesh (hydroxide form), as described by Dekker (1965). The nucleoside eluted with 60% methanol and was crystallized from hot water (235 mg, 41%).

Results

The ^{13}C NMR spectrum of cyanocobalamin at 62.9 MHz is shown in Figure 4. For ease of discussion the carbon resonances are grouped in five regions of the spectrum: (a) the carbonyl and imine carbon region, (b) the nucleotide region, (c) the corrin ring region, (d) the methylene carbon region, and (e) the methyl carbon region.

Carbonyl and Imine Carbon Region. In the downfield region (>160 ppm) 13 resonances are readily discerned; they correspond to the seven carbonyl carbons (C-27, C-32, C-38, C-43, C-50, C-57, and C-61) and the six nonprotonated pyrrolidine carbons (C-4, C-6, C-9, C-11, C-14, and C-16). Figure 5 contains a correlation diagram that summarizes the effect of modifications of the periphery of the corrin ring on these 13 resonances. The pyrrolidine carbon resonances as a group were assigned by Scott (1979) on the basis of the labeling studies with ^{13}C-enriched precursors. The correlation diagram unequivocally establishes the signal at 173.57 ppm as the resonance of C-9. Because the resonance at 165.32 ppm undergoes a large upfield shift (~3 ppm) in the spectra of cyanocobalamin lactone and cyanocobalamin lactam, it is identified as C-6. The other upfield resonance in this group (166.02 ppm) has been assigned to C-14 on the basis of its coupling of C-15 and C-16 (Scott, 1979). Indeed, this resonance shifts upfield (1.79 ppm) in cyanoepicobalamin, consistent with its assigment as C-14. Labeling studies are also the basis for the assignment of the two most downfield resonances (180.04 and 178.92 ppm) as C-4 and C-16. Although the incorporation of ^{13}C label in C-4 and C-16 does not differentiate between these two carbons, the resonance at 180.04 ppm can be assigned to C-4 because it undergoes a 0.47-ppm downfield shift when cyanocobalamin-*b*-carboxylic acid (C-32) is deprotonated. Similar downfield shifts are observed for C-9 (1.26 ppm) and for C-14 (0.59 ppm) when cyanocobalamin-*d*-carboxylic acid (C-43) and cyanocobalamin-*e*-carboxylic acid (C-50) are deprotonated. The carbonyl carbons C-32, C-43, and C-50 are readily assigned as the resonances at 177.87, 177.15, and 178.16 ppm, respectively, on the basis of the chemical shift changes that accompany the conversion of a propionamide residue to a protonated acid (~1 ppm) and the deprotonation of the latter (~4 ppm). The carboxamide carbon (C-38) of the *c*-acetamide side chain is assigned to the resonance at 175.13 ppm on the basis of its behavior in the cyanocobalamin lactone and lactam derivatives. The remaining pyrrolidine carbon, C-11, is assigned to the resonance

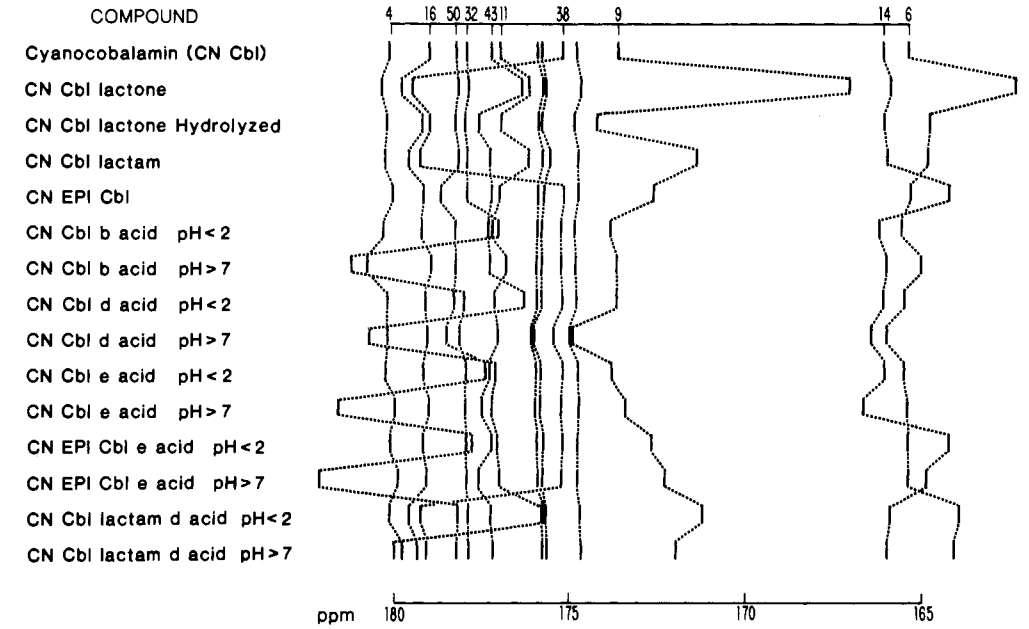

FIGURE 5: Correlation diagram of the carbon resonances in the carbonyl and imine carbon region for cyanocobalamin and its analogues.

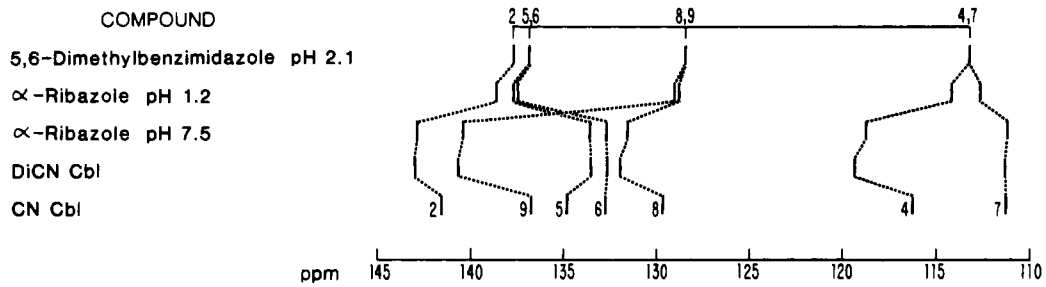

FIGURE 6: Correlation diagram for the benzimidazole resonances of 5,6-dimethylbenzimidazole, α-ribazole, dicyanocobalamin, and cyanocobalamin.

at 176.92 ppm because this resonance undergoes small shift changes in the cyanocobalamin-*e*-monocarboxylic acid. The other three resonances at 175.84, 175.72, and 174.73 ppm are amazingly constant in the cyanocobalamin derivatives and thus are assigned collectively to C-27, C-57, and C-61 (Table I).

5,6-Dimethylbenzimidazole Nucleotide Region. The seven ring carbons of the 5,6-dimethylbenzimidazole moiety resonate between 110 and 150 ppm. These resonances (Table I) were assigned by comparison with the resonances of 5,6-dimethylbenzimidazole, 1-α-D-ribofuranosyl-5,6-dimethylbenzimidazole, and dicyanocobalamin (Figure 6). From the nuclear Overhauser effect carbons 2, 4, and 7 are readily identified as the three proton-bearing carbons, carbon 2 being most downfield at 141.85 ppm and carbons 4 and 7 at 116.50 and 111.47 ppm, respectively. Carbon 4 is assigned to the resonance at 116.50 ppm because it undergoes the larger chemical shift change when N-3 of the nucleoside is protonated and when the 5,6-dimethylbenzimidazole moiety in cyanocobalamin is displaced by cyanide ion as in dicyanocobalamin. The identity of the four nonprotonated carbons (B-5, B-6, B-8, and B-9) was established by line-broadening experiments with the paramagnetic ion Mn^{2+}. Kotowycz & Hayamizu (1973) have demonstrated that Mn^{2+} binds to N-7 of the adenine moiety of adenosine 5′-phosphate (AMP), and thus the corresponding interaction with α-ribazole should be at N-3. The addition of Mn^{2+} ions (2.5×10^{-5} M) to a neutral solution of α-ribazole causes the broadening of the resonances at 142.87 and 140.40 ppm. At higher Mn^{2+} concentrations (7.5×10^{-4} M) these resonances as well as the signal at 131.58 ppm are abolished, identifying the resonances at 140.40 and 131.58 ppm as B-9

Table I: Assignment of the Carbon-13 Nuclear Magnetic Resonance Spectrum of Cyanocobalamin[a]

carbon		carbon		carbon	
4	180.04	*5*	107.52	26[f]	42.84
16	178.92	*15*	104.10	18	39.06
50	178.16	*10*	94.86	31	35.02
32	177.87	*R-1*	87.03	49	34.69
43	177.15	1	85.12	60[f]	32.52
11	176.92	*R-4*[c]	82.05	56[f]	32.24
27, 57,	175.84	19	74.93	42	31.75
or 61	175.72	R-3[d]	73.12	*47*	31.50
38	175.13	R-2[e]	73.05	55[f]	31.31
27, 57,	174.73	Pr-2	68.92	48	27.99
or 61					
9[b]	173.57	*R-5*	60.53	30, 41	25.99
14	166.02	17[f]	59.17	B-10 or B-11	19.89
6	165.32	3	56.38	B-10 or B-11	19.37
B-2	141.85	8	55.72	20,[f] *46*	19.29
B-9	136.71	13	53.73	Pr-3[h]	19.12
B-5	135.11	7	51.43	25[f]	19.05
B-6	133.02	12	48.13	54[f]	16.80
B-8	129.95	2[f]	47.25	35	15.89
B-4	116.50	Pr-1[g]	45.45	36	15.43
B-7	111.47	37	43.05	53	15.16

[a] Chemical shifts downfield in parts per million with respect to external neat tetramethylsilane. Because of a different bulk magnetic susceptibility correction at the higher field, these shifts are 1-ppm upfield from those obtained at 25.2 MHz (Anton et al., 1980a). [b] The italicized carbons are those carbons assigned individually by Scott (1979) and Doddrell & Allerhand (1971). [c] J_{CCOP} = 7.4 Hz. [d] J_{COP} = 3.7 Hz. [e] J_{CCOP} = 6.5 Hz. [f] Tenative assignments. [g] J_{CCOP} = 5.0 Hz. [h] J_{CCOP} = 4.6 Hz.

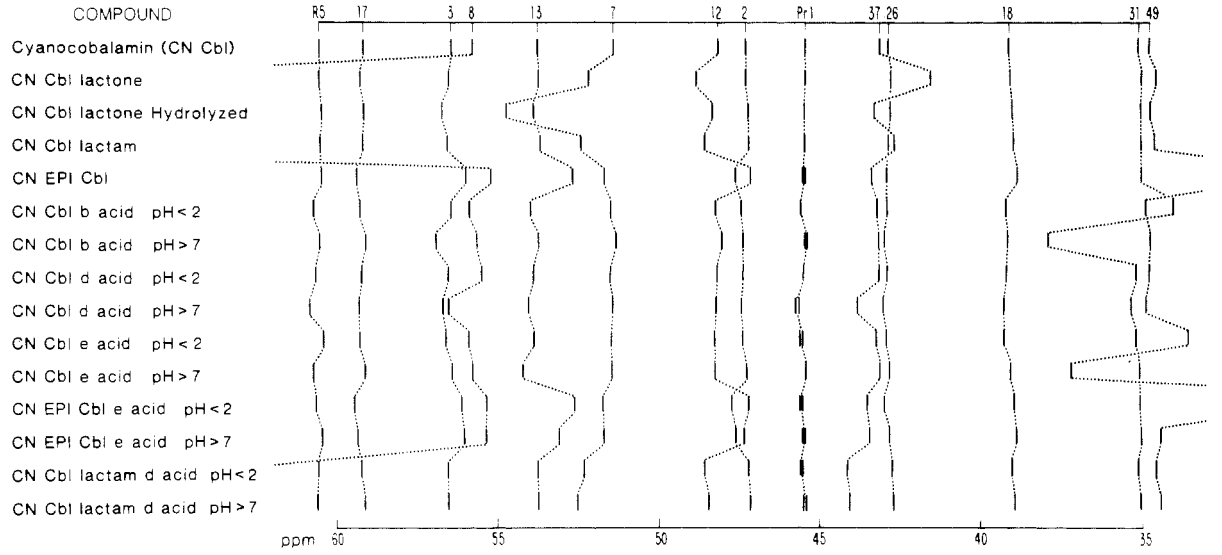

FIGURE 7: Correlation diagram of the carbon resonances in the corrin ring region for cyanocobalamin and its analogues.

and B-8, respectively. The corresponding carbons of cyanocobalamin resonate at 136.71 and 129.95 ppm. The resonance at 135.11 ppm is assigned to B-5 because it undergoes the larger chemical shift change when cyanocobalamin is converted to dicyanocobalamin.

The resonances due to the ribose carbons are readily identified by comparison of their chemical shifts with those of α-ribazole. R-1 (the anomeric carbon) and R-5 are assigned to the resonances at 87.03 and 60.53 ppm, respectively, on the basis of their chemical shifts. Carbon 4 (R-4) is assigned on the basis of its chemical shift (82.05 ppm) and its three-bond coupling to phosphate, J_{CCOP} = 7.4 Hz. The doublets at 73.12 and 73.05 ppm are assigned to carbons R-2 and R-3, respectively, because as expected the three-bond coupling J_{CCOP} = 6.5 Hz for carbon 2 is larger than the two-bond coupling J_{COP} = 3.7 Hz for carbon 3. The broadened resonances at 68.92 and 45.45 ppm are assigned to carbon 2 (Pr-2) and carbon 1 (Pr-1) of the aminopropanol moiety, respectively. These assignments are based on their chemical shifts and the coupling to phosphate. In the spectra of cyanoepicobalamin and the cyanocobalaminmonocarboxylic acids the resonance for Pr-1 is clearly a doublet ($J_{CCOP} \sim 5$ Hz).

Corrin Ring Region. Nineteen carbon atoms constitute the corrin ring; of these the six nonprotonated pyrrolidine carbons (C-4, C-6, C-9, C-11, C-14, and C-16) resonate far downfield in the carbonyl region (vide supra). The three bridge carbons (C-5, C-10, and C-15) are derived from δ-amino[5-^{13}C]-levulinic acid. The resonance at 94.86 ppm was assigned to C-10 by Doddrell & Allerhand (1971), and C-5 and C-15 were assigned to the resonances at 107.52 and 104.10 ppm, respectively, by Scott (1979). The chemical shift changes (in parts per million) observed for these resonances in cyanoepicobalamin (C-10, 94.07; C-5, 108.58; C-15, 105.39) and in cyanocobalamin lactone and lactam (C-10, 91.72 and 91.34; C-5, 106.88 and 106.45; C-15, 105.75 and 104.97, respectively) are consistent with these assignments.

The nonaromatic pyrrolidine ring carbons have not been labeled biosynthetically. Seven of these distal pyrrolidine carbons (C-2, C-3, C-7, C-8, C-12, C-13, and C-17) resonate between 50 and 65 ppm. The three broad resonances in this region correspond to the protonated carbons C-3, C-8, and C-13; they are assigned to the resonances at 56.38, 55.72, and 53.73 ppm, respectively, on the basis of the titration shifts of the three cyanocobalaminmonocarboxylic acids (Figure 7). These three resonances all undergo small downfield shifts when

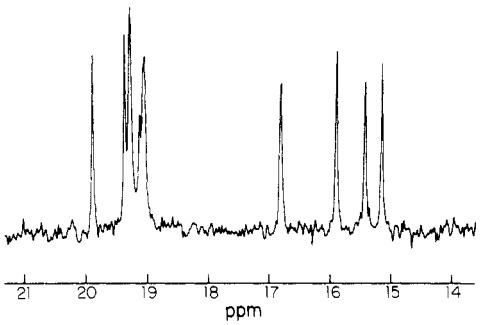

FIGURE 8: Expanded proton noise decoupled ^{13}C NMR spectrum of the upfield region of cyanocobalamin (10% D_2O).

their respective monocarboxylic acids are deprotonated. For instance, the resonance at 55.44 ppm in cyanocobalamin-*d*-carboxylic acid shifts 0.9 ppm downfield to 56.50 ppm when the acid is titrated from pH 1.7 to pH 9.0. Consistent with these assignments are the shifts observed for the C-8 resonance in cyanocobalamin lactam and cyanocobalamin lactone and for the C-13 resonance in cyanoepicobalamin (Figure 7). The assignment of the resonance at 51.43 ppm to C-7 and that of 48.13 ppm to C-12 are also based on the specific shifts seen for these resonances in the lactam and lactone and in cyanoepicobalamin. In the spectrum of cyanocobalamin lactam the resonance at 51.43 ppm is shifted to 52.40 ppm while in the spectrum of the lactone it is shifted to 52.14 ppm. Hydrolysis of the lactone ring results in an additional 2.55-ppm downfield shift for this resonance. Similar chemical shift changes are observed for 4-hydroxypentanoate, pentanoyl-γ-lactam, and pentanoyl-γ-lactone. Carbon 12 is adjacent to the inversion site in cyanoepicobalamin and thus can be readily assigned to the resonances at 48.13 ppm, which undergoes a 1.04-ppm upfield shift in cyanoepicobalamin.

The pyrrolidine carbons C-2 and C-17 must be responsible for the resonances at 47.25 and 59.17 ppm. Doddrell & Allerhand (1971) have assigned the resonance at 59.17 ppm to C-2 because this carbon should be deshielded due to the steric interaction with the methyl group at C-1 (C-20). However, this assignment then identifies the resonance at 47.25 ppm as C-17, which is not consistent with our previous assignments. Ring carbons C-3, C-8, and C-13 are substituted with propionamide residues and resonate at higher frequencies (downfield) from C-7, which carries an acetamide residue. C-17 is also substituted with a propionamide side chain and

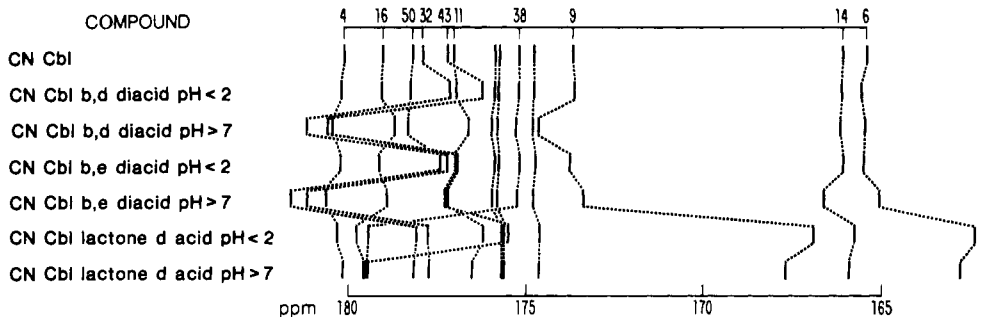

FIGURE 9: Correlation diagram of the carbon resonances in the carbonyl and imine carbon region of cyanocobalamin and three analogues whose structures were determined with ^{13}C NMR spectroscopy.

in addition is deshielded by a methyl group (C-54). Thus C-17 should resonate downfield, not upfield from C-3, C-8, and C-13. Furthermore, since C-2 and C-7 are structurally very similar, they should resonate in the same region of the spectrum. On the basis of these considerations, we assign the resonance at 47.25 ppm to C-2 and the resonance at 59.17 ppm to C-17. The last pyrrolidine carbon, C-18, has been assigned to the resonance at 39.1 ppm on the basis of its chemical shift. In contrast with the other ring carbons substituted with an acetamide group (C-2 and C-7), C-18 is protonated and thus the nuclear Overhauser enhancement of this resonance is consistent with its assignment.

Methylene Carbon Region. The four β-methylene carbons (C-30, C-41, C-48, and C-55) of the propionamide side chain are labeled biosynthetically with [8-^{13}C]porphobilinogen, while the α-methylene carbons of the propionamide side chains (C-31, C-42, C-49, and C-56) as well as the three methylene carbons of the acetamide substituents are labeled with δ-amino[2-^{13}C]levulinic acid (Scott, 1979). Using this information and the chemical shifts changes in several derivatives of cyanocobalamin, we earlier (Anton et al., 1980a) assigned the following resonances: C-30, 26.99 ppm; C-31, 35.02 ppm; C-41, 25.99 ppm; C-42, 31.75 ppm; C-48, 27.99 ppm; C-49, 34.69 ppm. The resonance at 43.05 ppm is identified as C-37 (methylene carbon of the *c*-acetamide side chain) on the basis of its upfield shift in cyanocobalamin lactone and its downfield shift in cyanocobalamin-*d*-carboxylic acid (Figure 7). The resonance at 42.84 ppm is assigned to C-26 (the methylene carbon of the *a*-acetamide side chain) on the basis of its chemical similarity to C-37. The resonances of the α- and β-methylene carbons of the *f*-propionamide side chain (C-56 and C-55) and the methylene carbon of the *g*-acetamide side chain (C-60) do not undergo specific chemical shift changes in the analogues of cyanocobalamin. However, since the methylene carbons of the other acetamide side chains (C-37 and C-26) resonate downfield from the other methylene carbons, we tentatively assign the resonance at 32.52 ppm to C-60. Furthermore the β-methylene carbons of the propionamide side chains resonate upfield from the α-methylene carbons, and thus the resonances at 31.31 and 32.24 ppm can be reasonably attributed to C-55 and C-56, respectively.

Methyl Carbon Region. Cyanocobalamin contains 11 methyl groups, seven of which are labeled with [methyl-^{13}C]methionine. These are the methyl groups at C-1 (C-20), C-2 (C-25), C-5 (C-35), C-7 (C-36), C-12 *pro-R* (C-46), C-15 (C-53), and C-17 (C-54). The *pro-S* methyl group (C-47) attached to C-12 is derived from δ-amino[2-^{13}C]levulinic acid; this carbon resonates in the methylene carbon region at 31.5 ppm. The other three methyl groups are B-10 and B-11 of 5,6-dimethylbenzimidazole and Pr-3 of the 2-propanol moiety. The expanded spectrum between 14 and 21 ppm (Figure 8) shows nine well-resolved resonances. Six of these correspond to single carbon atoms, while the peaks at 19.29, 19.12, and 19.05 ppm integrate to 2, 0.5, and 1.5 carbon atoms, respectively. The resonances at 19.89 and 19.37 ppm are assigned to the methyl groups of the 5,6-dimethylbenzimidazole moiety (B-10 and B-11) on the basis of a comparison with the corresponding methyl groups of α-ribazole. The resonance at 19.12 ppm is identified as the downfield part of the Pr-3 doublet, the upfield part coinciding with the line at 19.05 ppm (J_{CCOP} = 4.6 Hz). On the basis of the assignment of the corresponding carbon atoms of dicyanocobyrinic acid heptamethyl ester by Ernst (1981), we tentatively assign the resonance at 19.29 ppm to both C-20 and C-46 and the resonance at 19.05 ppm to C-25.

The most upfield resonance (15.16 ppm) is identified as C-53 (methyl at C-15) because it undergoes an upfield shift in cyanoepicobalamin. The next resonance (15.43 ppm) is assigned to C-36 (methyl at C-7) on the basis of the large downfield shifts of this peak in cyanocobalamin lactone and cyanocobalamin lactam. Ernst (1981) identifies the penultimate resonance in the spectrum of dicyanocobyrinic acid heptamethyl ester as C-35; however, this carbon, attached to C-5, should be less affected than C-36 by lactone or lactam formation. The resonance at 15.89 ppm is assigned to C-35 because it undergoes a small downfield shift in the lactone and lactam. Finally C-54, the methyl group at C-17 , is assigned to the resonance at 16.80 ppm. This resonance is remarkably constant in all the derivatives of cyanocobalamin, and indeed, all the modifications in these derivatives are far removed from this carbon.

Discussion

The complete interpretation of the carbon-13 nuclear magnetic resonance spectrum of cyanocobalamin allows the identification of unknown cyanocorrinoids by ^{13}C NMR on a routine basis. The resonances in the downfield region of the spectrum (>165 ppm) are particularly sensitive to modifications of the acetamide or propionamide side chains on the periphery of the corrin ring. Thus the hydrolysis of an amide to a protonated acid is accompanied by an upfield shift of approximately 1 ppm, while the deprotonation of an acid causes a larger downfield shift (~4 ppm) of the carbonyl carbon. The resonances at 173.57 and 175.13 ppm, corresponding to carbons 9 and 38, undergo large chemical shift changes upon lactam and lactone formation at the B ring. Furthermore the chemical shift changes in the analogues with more than one modification, e.g., cyanoepicobalamin-*b*-carboxylic acid (not shown), cyanoepicobalamin-*e*-carboxylic acid, and cyanocobalamin-*d*-carboxylic acid lactam (Figures 5 and 7), clearly reflect each individual modification. These unique chemical shift changes were used to identify two unknown cyanocobalamindicarboxylic acids isolated from a mild acid hydrolysate of cyanocobalamin (Bernhauer et al., 1966). The correlation diagram presented in Figure 9 clearly identifies

one as cyanocobalamin-*b*,*e*-dicarboxylic acid and the other as cyanocobalamin-*b*,*d*-dicarboxylic acid. The ^{13}C NMR spectrum (>165 ppm) of the first dicarboxylic acid at low pH shows significant chemical shift changes for only two resonances, which in turn undergo large downfield shifts when the solution is neutralized. These chemical shift changes pinpoint the modifications at C-32 and C-50 and unambiguously identify this corrinoid as cyanocobolamin-*b*,*e*-dicarboxylic acid. Similarly the spectra of the second dicarboxylic acid locate the modifications at C-32 and C-43, identifying this corrinoid as cyanocobalamin-*b*,*d*-dicarboxylic acid.

Our preparations of cyanocobalamin lactone synthesized by the procedure of Bonnet et al. (1957) invariably contain appreciable amounts of a cyanocobalaminmonocarboxylic acid lactone, which can be separated from cyanocobalamin lactone by chromatography on AG1 X2. A comparison of the downfield region of the spectrum of this monocarboxylic acid with that of cyanocobalamin unambiguously identifies the acid as cyanocobalamin-*d*-carboxylic acid lactone (Figure 9). In addition to chemical shift changes of the resonances of C-38 and C-9 associated with lactone formation, the resonance assigned to C-43 undergoes the typical shifts at low and high pH expected for the *d*-carboxylic acid.

The spectral region between 110 and 160 ppm is a window that allows the identification of the lower ligand. Indeed, the spectra of dicyanocobinamide and dicyanocobyrinic acid heptamethyl ester, which lack the nucleotide ligand, do not show any resonances in this region.

Modifications of the corrin ring are most readily discerned in the spectral region between 30 and 70 ppm. For instance, the spectrum of cyanoepicobalamin, prepared in deuterated trifluoroacetic acid, shows a greatly diminished resonance at 52.71 ppm but normal peaks at 55.25 and 56.01 ppm, demonstrating that deuteration occurs only at C-13 and not at C-8 or C-3. Also evident in this region of the spectrum are the isotope effects of the deuterium at C-13 on C-12 and C-47. In a similar experiment Ernst (1981) has shown that deuterium substitution at C-13 of dicyanocobyrinic acid heptamethyl ester abolishes the resonance of C-13 and causes a shift or broadening of all the resonances adjacent to the substitution site. He noted a significant effect even on C-53. These few examples clearly demonstrate that with the complete interpretation of the ^{13}C NMR spectrum of cyanocobalamin, ^{13}C NMR spectroscopy can be used to elucidate the structure of any corrinoid on a routine basis.

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