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Carbon-13 Nuclear Magnetic Resonance Studies of Cobalamins†

Gerald T. Bratt and Harry P. C. Hogenkamp*

ABSTRACT: The carbon-13 nuclear magnetic resonance spectra of aquocobalamin, adenosylcobalamin, methylcobalamin, and (carboxymethyl)cobalamin have been interpreted. The assignments were made by a comparison of the spectra with that of cyanocobalamin, by a study of the pH dependence of the chemical shifts, by an analysis of the effect of the axial ligands on the carbon atoms of the corrin ring, and by a study of the specific line broadening effect of the paramagnetic ions Mn^{2+} and Gd^{3+}. The chemical shift changes that accompany the "base-on" → "base-off" conversion of the organocobalamins demonstrate that the conformation of the "western" half of the corrin ring and the conformations of the a, b, c, d, f, and g side chains are relatively constant. In contrast, the conformations of the "eastern" half of the corrin ring and the e propionamide side chain are highly variable.

In earlier publications we (Anton et al., 1982; Bratt & Hogenkamp, 1982) presented the complete interpretation of the ^{13}C NMR spectrum of cyanocobalamin. The assignments of the 65 resonances were based on earlier biosynthetic studies (Scott et al., 1974, 1976; Battersby et al., 1976), on our systematic analysis of the ^{13}C NMR spectra of cyanocobalamin and a number of its analogues (Anton et al., 1982), and on a study of the effect of the paramagnetic ions Mn^{2+} and Gd^{3+} on the ^{13}C NMR spectra of three cyanocobalaminmonocarboxylic acids. The complete interpretation of ^{13}C NMR spectrum of heptamethyldicyanocobyrinate has been reported by Ernst (1981) and by Battersby et al. (1982). We have now extended these studies to the interpretation of the ^{13}C NMR spectra of the naturally occurring cobalamins: aquocobalamin, methylcobalamin, adenosylcobalamin, and (carboxymethyl)-cobalamin.

Thus far, the structures of only two cobalamins, cyanocobalamin and adenosylcobalamin, have been established by crystallographic analysis. Furthermore, all the cobinamides and the "base-off" forms of the cobalamins have not been obtained as crystalline preparations. Thus, alternate methods for the determination of their structures have to be explored.

Several years ago Doddrell & Allerhand (1971) determined the ^{13}C NMR spectra of cyanocobalamin, dicyanocobalamin, adenosylcobalamin, and dicyanocobinamide at 15.08 MHz. Several assignments were made by comparing these spectra with those of model compounds, by an analysis of the ^{13}C–H and ^{13}C–^{31}P coupling, and by the determination of the relative T_1 values of selected resonances. Unfortunately, the resolution at 15.08 MHz was not sufficient to resolve all the resonances, and many of the resonances could only be assigned in groups.

At 62.9 MHz all but a few of the resonances of the cobalamins are resolved, and therefore, the complete interpretation of their ^{13}C NMR spectra is now feasible. In the present study we have used several approaches to make the assignments. First, we have compared the spectra of the cobalamins with that of cyanocobalamin. Second, we have analyzed the pH dependence of the chemical shifts of the cobalamins. Third, we have studied the effect of the axial ligands on the chemical shift of the carbon atoms of the corrin ring (cis effect), and finally, we have monitored the specific line broadening effect of the paramagnetic ions Mn^{2+} and Gd^{3+}.

The carbon–cobalt bond of the organocobalamins is quite stable under physiological conditions in the dark, and thus, in the active holoenzymes, the enzyme must promote the cleavage of this bond. It has been generally accepted that this interaction between the cobalamin coenzymes and their re-

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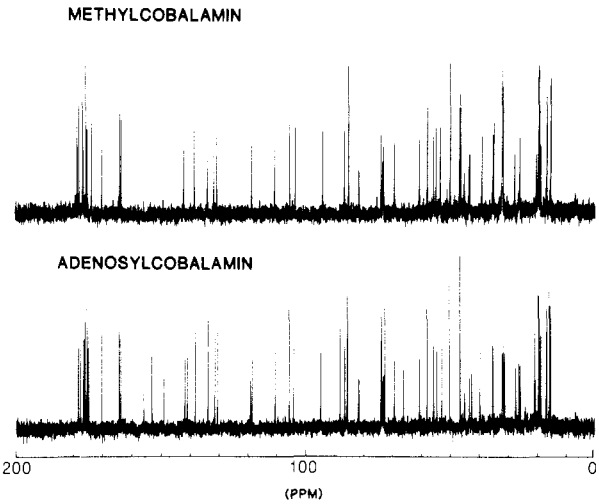

FIGURE 1: Proton noise decoupled ^{13}C NMR spectra (62.9 MHz) of aqueous solutions of methylcobalamin and adenosylcobalamin (10% D_2O).

spective apoenzymes induces conformational changes in the corrin ring that lead to the labilization of the carbon–cobalt bond. The complete interpretation of the ^{13}C NMR spectra of the three organocobalamins, methylcobalamin, adenosylcobalamin, and (carboxymethyl)cobalamin, in their "base-on" and "base-off" forms will enable us to assess the effect of modifications in the axial ligands on the corrin ring and on the acetamide and propionamide side chains.

Experimental Procedures

Materials. Cyanocobalamin was obtained from Rhone-Poulenc Industries, Paris, and hydroxycobalamin hydrochloride was obtained from Sigma Chemical Co. Adenosylcobalamin, methylcobalamin, (carboxymethyl)cobalamin, and the organocobalaminmonocarboxylic acids were prepared by published procedures (Hogenkamp, 1974; Hogenkamp et al., 1965; Anton et al., 1980).

Methods. The purity of the corrinoids was established by spectral analysis using a Cary Model 15 spectrophotometer and by descending paper chromatography in three solvent systems (Dolphin, 1971). Pulsed Fourier transform ^{13}C (62.9 MHz) nuclear magnetic resonance (NMR) 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 15000 Hz were accumulated as 32K data points in the time domain and transformed into a 16K point spectrum. The data acquisition time was 1.081 s with a 1-s pulse delay. 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 in solutions containing 20 mg of cobalamin/mL of 10% D_2O in H_2O in 10-mm tubes.

Results

The ^{13}C NMR spectra of methylcobalamin and adenosylcobalamin at 62.9 MHz are shown in Figure 1, and the assignments of the ^{13}C chemical shifts of cyanocobalamin, dicyanocobalamin, aquocobalamin, methylcobalamin, adenosylcobalamin, and (carboxymethyl)cobalamin are presented in Table I. In Table II are summarized the ^{13}C–^{31}P coupling constants of the ribose and 2-propanolamine moieties of these cobalamins. The numbering of the atoms of adenosylcobalamin is shown in Figure 2.

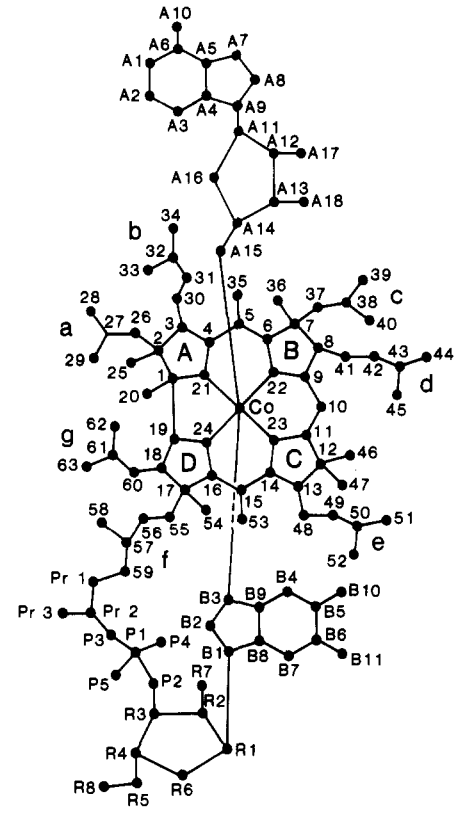

FIGURE 2: Numbering of atoms of adenosylcobalamin.

As before, we have divided the spectra in five regions: (a) the carbonyl and imine carbon region, (b) the nucleoside and nucleotide carbon region, (c) the corrin ring carbon region, (d) the methylene carbon region, and (e) the methyl carbon region.

Carbonyl and Imine Carbon Region. The ^{13}C NMR spectrum of cyanocobalamin shows 13 well-resolved resonances in this region (>160 ppm) (Anton et al., 1982). They were assigned to the six nonprotonated pyrrolidine carbons (C-4, C-6, C-9, C-11, C-14, and C-16) and the seven carbonyl carbons (C-27, C-32, C-38, C-43, C-50, C-57, and C-61). The correlation diagram presented in Figure 3 summarizes the effects of changes in the upper and lower ligands on these 13 resonances. It is clear that the substitution of a weak by a strong field ligand is accompanied by a upfield shift of the imine carbon resonances. For instance, the displacement of the lower 5,6-dimethylbenzimidazole ligand by cyanide in dicyanocobalamin causes large upfield shifts (1.2–2.6 ppm) of the C-4, C-6, C-9, and C-14 resonances and smaller upfield shifts (0.1 and 0.2 ppm) of the resonances of C-11 and C-16. Similar pronounced cis effects involving the resonances of C-4, C-9, C-11, and C-16 are observed when the cyanide ligand is displaced by either water or hydroxide ion as in aquocobalamin and hydroxocobalamin. The resonances of these four carbons are shifted downfield in aquocobalamin and upfield when the water ligand is deprotonated at high pH. As expected, the cis effect is most pronounced in the organocobalamins in their "base-on" forms. The resonances of C-4, C-10, C-11 and C-9 undergo large (~4 ppm) upfield shifts, so that in the spectra of the organocobalamins the most downfield resonances are those of the carbonyl carbons (C-50, C-32, and C-43) of the propionamide side chains. The base-on → base-off conversion of the organocobalamins at low pH is accompanied by a downfield shift of the imine carbon resonances except for the C-14 resonance, which shifts upfield. For adenosylcobalamin the pH dependence of the chemical

Table I: Carbon-13 Chemical Shifts[a] for Cobalamins

assign-ments	CNCbl	diCNCbl	H_2OCbl	HOCbl	CH_3Cbl, pH 6.8	CH_3Cbl, pH 2.1	AdoCbl, pH 7.4	AdoCbl, pH 2.0	CMCbl, pH 8.5	CMCbl, pH 5.0	CMCbl, pH 2.0
4	180.0	178.8	181.5	179.1	175.3	176.0	175.9	176.6	176.3	177.4	177.6
16	178.9	178.7	181.3	178.4	174.9	175.4	175.9	176.2	176.0	177.1	177.6
50	178.2	177.9	178.1	178.3	178.4	178.5	178.3	178.3	178.5	178.4	178.4
32	177.9	177.6	177.8	178.0	178.2	178.3	178.3	178.3	178.3	178.2	178.2
43	177.2	177.4	177.0	177.3	177.6	178.3	177.5	178.2	177.6	177.5	178.0
11	176.9	176.8	179.0	177.0	173.3	175.2	174.7	176.2	174.7	175.6	176.5
27	175.8	176.1	176.0	176.4	176.4	176.4	176.5	176.3	177.1	176.6	176.5
61	175.7	176.0	175.7	176.0	176.2	176.4	176.2	176.3	176.5	176.2	176.2
38	175.1	175.4	175.7	175.4	175.2	175.4	175.3	175.4	176.0	175.6	175.4
57	174.7	175.2	174.7	175.0	175.1	175.0	175.1	174.7	175.5	175.3	175.3
9	173.6	172.1	174.7	173.0	169.7	171.1	170.2	172.2	169.9	171.2	172.6
14	166.0	163.4	166.0	165.2	163.6	163.3	164.2	163.4	163.7	164.6	164.0
6	165.3	163.1	164.3	164.0	163.1	163.3	163.7	163.8	163.5	164.1	164.7
A6							155.9	150.9			
A2							153.1	145.9			
A4							148.9	148.2			
B2	141.9	142.7	141.6	142.2	141.9	139.2	141.7	138.7	141.9	142.0	140.0
A8							140.9	142.6			
B9	136.7	140.5	136.2	136.8	138.2	130.8	138.2	129.7	138.1	137.7	133.3
B5	135.1	133.4	136.0	135.1	133.7	136.4	133.8	136.9	133.7	134.1	135.8
B6	133.0	132.5	133.9	132.9	131.5	136.2	131.5	136.8	131.5	131.8	135.0
B8	130.0	131.6	129.3	129.6	130.4	129.4	130.5	129.3	130.4	130.2	129.7
A5							119.0	118.7			
B4	116.5	119.1	115.6	116.8	118.3	114.9	118.5	114.4	118.6	118.1	115.9
B7	111.5	111.1	112.0	111.3	110.5	112.6	110.5	112.9	110.5	110.8	112.1
5	107.5	105.2	108.0	106.7	105.2	107.2	105.6	108.4	105.6	105.7	107.5
15	104.1	103.3	104.3	103.5	103.4	105.9	104.1	106.9	104.5	104.2	106.2
10	94.9	91.2	94.9	94.1	93.9	95.7	94.7	97.5	94.4	94.6	96.5
A-11							88.0	88.3			
R-1	87.0	85.9	87.5	87.1	86.4	87.2	86.4	87.3	86.5	86.7	87.1
A-14							85.6	86.3			
1	85.1	83.2	85.1	85.2	85.1	86.0	85.5	86.9	85.9	85.7	86.8
R-4	82.1	83.1	82.3	82.1	81.5	85.5	81.6	86.0	81.6	81.7	84.3
19	74.9	75.1	75.2	74.3	73.7	74.8	73.9	74.8	74.0	73.5	74.2
R-3	73.1	74.1	73.0	73.0	73.1	74.6	73.2	74.7	73.2	73.2	74.1
R-2	73.0	72.4	72.9	72.9	73.0	72.4	73.0	72.3	73.0	73.0	72.5
A-12							73.6	72.1			
A-13							72.6	73.1			
Pr-2	68.9	71.2	68.8	68.8	69.1	71.4	69.2	71.7	69.2	69.0	70.7
R-5	60.5	61.0	60.5	60.4	60.5	61.3	60.5	61.4	60.5	60.5	61.1
17	59.2	58.9	59.2	58.6	57.8	58.6	57.9	59.0	57.9	58.0	58.6
3	56.4	56.5	57.4	56.8	55.5	55.7	55.6	55.4	56.2	55.9	55.8
8	55.7	55.4	57.2	56.4	54.6	55.5	54.5	55.3	55.3	55.1	55.5
13	53.7	53.4	53.9	53.7	53.2	53.0	52.9	52.5	53.1	53.2	53.0
7	51.4	49.4	51.0	50.5	50.0	49.7	50.1	50.2	50.1	50.2	50.1
12	48.1	46.9	48.3	47.6	46.6	46.6	46.5	46.6	46.8	47.0	46.9
2	47.3	46.3	47.8	47.1	46.3	46.1	46.5	46.0	46.4	46.7	46.3
Pr-1	45.5	45.0	45.6	45.4	45.0	44.8	45.0	44.7	45.0	45.1	44.9
37	43.1	43.9	45.5	44.6	43.3	43.7	43.3	43.3	42.8	43.1	43.1
26	42.8	42.4	43.5	43.0	43.0	43.4	42.5	43.0	42.8	42.7	43.1
13	39.1	39.0	39.7	39.5	39.1	39.1	39.7	39.4	39.7	39.8	39.6
31	35.0	35.1	35.0	35.2	35.3	35.3	35.4	35.2	35.5	35.3	35.3
49	34.7	33.1	34.8	35.0	34.9	32.7	35.2	32.5	35.5	35.3	33.6
60	32.5	32.5	33.0	32.7	32.0	32.6	32.0	32.4	32.4	32.4	32.6
56	32.2	32.2	32.7	32.7	32.0	32.6	31.9	32.4	32.1	31.9	32.3
42	31.8	32.0	32.3	31.9	32.0	32.4	31.8	32.2	32.0	31.9	32.2
47	31.5	32.0	31.5	31.8	31.9	31.8	31.6	31.6	31.8	31.8	31.8
55	31.3	31.9	31.5	31.8	31.8	31.3	31.2	31.4	31.5	31.4	31.3
48	28.0	26.8	28.1	27.9	27.8	26.7	27.4	26.7	27.8	27.8	27.1
30	26.0	25.8	26.5	26.5	26.1	26.5	26.4	26.2	26.5	26.3	26.6
41	26.0	24.9	26.5	26.3	26.0	26.0	25.9	26.0	26.0	26.0	26.2
A-15							24.3				
B-10	20.0	21.7	21.2	20.3	20.3	23.0	21.0	23.9	20.9	20.7	22.3
B-11	19.4	20.0	20.0	20.1	19.7	19.8	20.6	19.8	20.6	20.4	20.2
20	19.3	19.5	19.9	19.9	19.6	19.8	19.6	19.7	19.6	19.7	19.7
46	19.3	18.9	19.7	19.9	19.3	19.7	19.3	19.7	19.4	19.3	19.6
Pr-3	19.1	18.9	19.2	19.1	18.8	18.7	18.8	18.6	18.9	18.9	18.7
25	19.0	18.7	19.3	19.3	19.1	19.3	18.9	18.9	19.2	19.2	19.4
35	16.8	17.4	17.8	17.6	16.8	17.8	16.9	17.9	17.3	17.0	17.5
54	16.0	16.5	16.7	16.8	16.7	16.7	16.7	16.5	17.1	16.8	16.7
36	15.4	15.3	15.5	15.6	15.5	15.7	15.8	15.5	15.9	15.6	15.7
53	15.2	14.8	15.4	15.5	15.4	15.3	15.4	15.5	15.6	15.5	15.6

[a] Chemical shifts downfield in parts per million with respect to external neat tetramethylsilane.

Table II: ^{13}C–^{31}P Coupling Constants for Cobalamins

J (Hz)	CNCbl	diCNCbl	H_2OCbl	HOCbl	CH_3Cbl, pH 6.8	CH_3Cbl, pH 2.1	AdoCbl, pH 7.4	AdoCbl, pH 2.0	CMCbl, pH 8.5	CMCbl, pH 5.0	CMCbl, pH 2.0
R-4, J_{CCOP}	7.4	5.6	7.0	7.4	8.4	<0.9	8.9	3.6	8.7	8.4	4.9
R-3, J_{COP}	4.1	5.3	4.1	4.4	3.9	4.6	4.9	2.1	4.5	4.6	3.7
R-2, J_{CCOP}	6.3	5.4	6.3	8.3	6.1	6.0	5.9	6.2	6.1	6.1	4.2
Pr-2, J_{COP}	<0.9	3.1	<0.9	<0.9	<0.9	3.7	<0.9	4.6	<0.9	<0.9	5.2
Pr-1, J_{CCOP}	5.1	6.9	5.8	4.6	<0.9	3.1	<0.9	6.3	3.3	4.1	4.7
Pr-3, J_{CCOP}	4.7	<0.9	<0.9	2.6	4.4	3.8	1.7	3.8	4.6	4.3	4.1

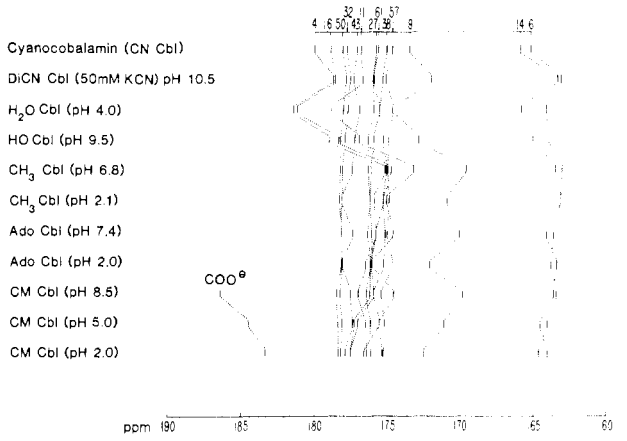

FIGURE 3: Correlation diagram of carbon resonances in the carbonyl and imine carbon region for cobalamins.

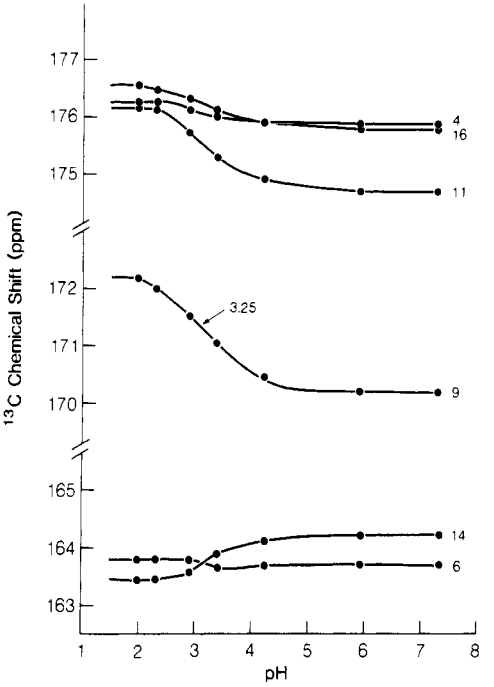

FIGURE 4: pH dependence of ^{13}C chemical shifts of the imine carbons of adenosylcobalamin.

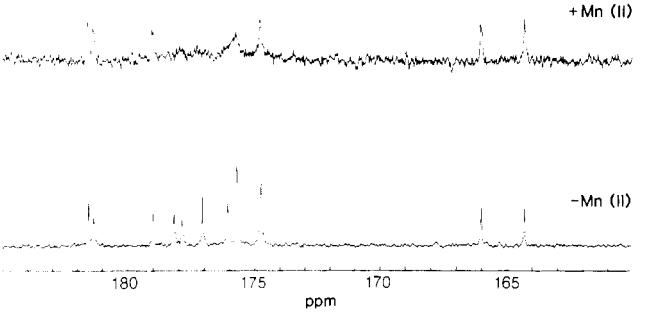

FIGURE 5: Effect of 5×10^{-4} M Mn^{2+} ions on resonances of aquocobalamin in the carbonyl and imine carbon region of the spectrum.

shifts of the imine carbons is presented in Figure 4. These titration curves show a pK_a = 3.2–3.3, which is very similar to the pK_a = 3.3–3.5 determined by UV–visible absorption spectroscopy, direct titration with acid, and electrophoresis (Ladd et al., 1961). Protonation of the upper carboxymethyl ligand of (carboxymethyl)cobalamin (pK_a = 7.0) causes an upfield shift of the carboxyl carbon resonance of the organoligand and downfield shifts of the imine carbon resonances. These downfield shifts reflect the weaker ligand donor strength of the protonated carboxymethyl ligand.

To confirm the assignments of the imine carbon resonances of the cobalamins, in particular where the chemical shift changes are very large, the specific line broadening of the carbonyl resonances by Mn^{2+} ion was investigated. The effect of the addition of 5×10^{-4} M Mn^{2+} on the downfield region of the NMR spectrum of aquocobalamin is shown in Figure 5. In the presence of the paramagnetic ion the carbonyl resonances are eliminated and thus the remaining six signals arise from the imine carbons. Similar experiments with the other cobalamins confirmed the assignments of their imine carbon resonances.

The seven carbonyl resonances undergo relatively small chemical shift changes in the various cobalamins. The three most downfield resonances in the spectra of methyl- and adenosylcobalamin were assigned unambiguously to C-50, C-32, and C-43, respectively, by a comparison of the spectra of the methyl- and adenosylcobalamin b, d, and e monocarboxylic acids with those of the corresponding cyanocobalaminmonocarboxylic acids (data not shown). We have demonstrated previously (Anton et al., 1982) that the conversion of a propionamide side chain to the protonated acid is accompanied by an ~1 ppm upfield shift of the carbonyl resonance, while the deprotonation of the acid causes an ~4 ppm downfield shift of this resonance. It is of interest to note that the C-43 resonance is more sensitive to the base-on → base-off conversion than the carbonyl resonances of the b and e propionamide side chains (C-32 and C-50). Inspection of molecular models based on crystal data (Lenhert, 1968) reveals steric interactions between the lower dimethylbenzimidazole ligand and the carbonyl group of the d propionamide side chain in the base-on form. Thus, the downfield shift of the C-43 resonance in the base-off forms reflects the reduction in this interaction.

Nucleoside and Nucleotide Carbon Region. The seven ring carbons of the 5,6-dimethylbenzimidazole ligand and the five adenine carbons of adenosylcobalamin resonate between 110 and 150 ppm. The adenine carbon resonances were assigned by a comparison of the spectrum of adenosylcobalamin with that of adenosine and by an analysis of the pH dependence of the chemical shifts of these resonances. In contrast to our earlier observations (Ladd et al., 1961), the titration curves of adenosylcobalamin (Figures 6 and 7) demonstrate that the pK_a of the 5′-deoxyadenosyl ligand is 2.8 rather than 3.3. The

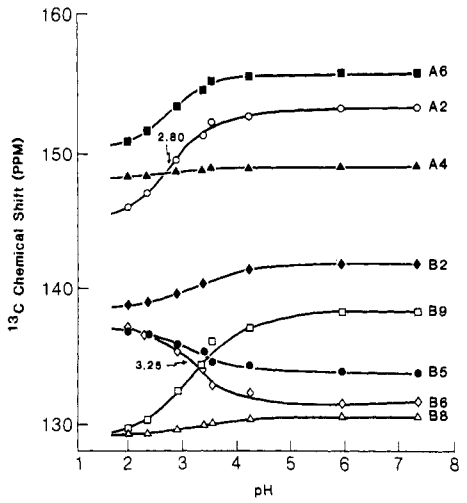

FIGURE 6: pH dependence of ^{13}C chemical shifts of selected carbons of the 5′-deoxyadenosyl ligand and 5,6-dimethylbenzimidazole moiety of adenosylcobalamin.

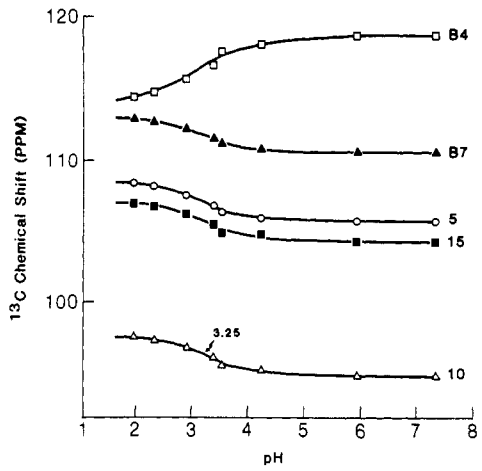

FIGURE 7: pH dependence of ^{13}C chemical shifts of methine bridge carbons (C-5, C-10, and C-15) of the corrin ring and of B-4 and B-7 of the 5,6-dimethylbenzimidazole ligand of adenosylcobalamin.

lower pK_a value readily distinguishes the adenine carbon resonances from those of the 5,6-dimethylbenzimidazole moiety. This titration behavior also confirms the assignment of the resonances at 155.9 and 153.1 ppm to carbons A-6 and A-2, respectively. These two resonances undergo the expected larger chemical shift changes when nitrogen A-1 is protonated. The titration curves presented in Figure 6 clearly demonstrate that the upper and lower ligands of adenosylcobalamin have distinct pK_a values.

It should be noted that the resonances assigned to B-2, B-4, B-8, and B-9 in the spectrum of cyanocobalamin undergo downfield shifts when the lower ligand is replaced by cyanide, as in dicyanocobalamin. These downfield shifts are consistent with the expected reduction in steric compression at those carbon atoms in this base-off form. In contrast, the base-on → base-off conversion in the organocobalamins in acid is accompanied by an upfield shift of the same resonances as a result of the protonation of nitrogen B-3.

As pointed out before (Anton et al., 1982), the resonances due to the ribose carbon atoms can be readily assigned by a comparison of their chemical shifts with those of ribazole and adenosine. R-1, A-11, A-12, A-13, and R-5 are assigned on the basis of their chemical shifts, and the assignments of R-2, R-3, and R-4 are based on their chemical shifts and the coupling to phosphate. The most upfield of these last three doublets is assigned to R-2 because it shows the larger three-bond coupling. In several of the spectra the resonances due to Pr-1 and Pr-2 appear as doublets, and thus they can be readily assigned.

A comparison of the spectra of the organocobalamins in the base-on and the base-off form demonstrates that the ribose moiety of the lower ligand and the f propionamide side chain undergo substantial conformational changes when the 5,6-dimethylbenzimidazole ligand is protonated and no longer coordinated to the cobalt atom. For instance, the spectrum of adenosylcobalamin at pH 2.0 shows downfield shifts for R-1 (0.9 ppm), R-4 (4.6 ppm), R-3 (1.5 ppm), R-5 (0.9 ppm), and Pr-2 (2.5 ppm) and upfield shifts for R-2 (0.7 ppm), Pr-1 (0.3 ppm), and Pr-3 (0.2 ppm) relative to the corresponding chemical shifts of adenosylcobalamin in the base-on form. An examination of the ^{13}C–^{31}P coupling constants (J_{COP} and J_{CCOP}) presented in Table II also points to conformational changes in the f propionamide side chain in the base-off forms of all the cobalamins. In the base-on forms the coupling constant for the Pr-2 doublet (J_{COP}) is small (<0.9 Hz) while in the base-off forms this coupling constant varies between 3.1 and 5.2 Hz. Less dramatic and less consistent changes are also seen for the other doublets. As reported before (Walker et al., 1974), the resonance of the 5′-methylene carbon of the upper 5′-deoxyadenosyl ligand (A-15) and the other Co–alkyl ligands (–CH_3 and –CH_2COOH) undergo large upfield shifts in the base-off forms.

Corrin Ring Carbon Region. As shown above, the six nonprotonated pyrrolidine carbons resonate downfield in the carbonyl region. The remaining 13 corrin ring carbon resonances can be readily assigned by a comparison of the ^{13}C NMR spectra of the cobalamins with that of cyanocobalamin. Inspection of Table I reveals that the displacement of a weak ligand by a stronger one is accompanied by an upfield shift of most, but not all, of the 19 corrin carbon resonances. However, there is not a linear correlation between the chemical shifts of these carbon atoms and the β-band of the visible spectrum. This nonlinearity suggests that the cis effect is not just electronic but also steric in nature. The steric interaction between the corrin ring and the lower 5,6-dimethylbenzimidazole ligand has been well documented. Lenhert (1968) has pointed out that the length of the axial Co–N bond correlates with the pK_a of the base-on → base-off conversion. The Co–N bond length in cyanocobalamin (pK_a = 0.1) is 1.97–2.06 Å and in adenosylcobalamin (pK_a = 3.4) 2.24 Å, and thus the steric interaction between the corrin ring and the lower ligand is more severe in cyanocobalamin. Although the structure of the other cobalamins has not yet been established by X-ray crystallography, the very low pK_a (–2.4) of aquocobalamin predicts a very short Co–N bond length and, consequently, severe steric hindrance between the corrin ring and the 5,6-dimethylbenzimidazole ligand. Such a steric interaction should cause upfield shifts of the corrin ring carbon resonances of aquocobalamin compared to those of cyanocobalamin. Moreover, the substitution of a strong field ligand (cyanide ion) by a weak one (water) should be accompanied by a downfield shift of the ring carbon resonances. The observed chemical shift differences between cyanocobalamin and aquocobalamin are the sum of these opposing effects, and thus, it is not at all surprising that the correlation between the chemical shifts of the corrin ring carbons and the β-band of the visible spectrum is not linear. An examination of the chemical shifts of the corrin carbon atoms of the organocobalamins in the base-on and the base-off forms reveals that the effect of the substitution of the 5,6-dimethylbenzimidazole ligand by water is communicated primarily to the methine

carbons (C-5, C-10, and C-15) and to selected carbons of the pyrrolidine rings (C-1, C-4, C-8, C-9, C-11, and C-19) (Figure 7 and Table I).

Methylene Carbon Region. The methylene carbons of the acetamide side chains (C-26, C-37, and C-60) and those of the propionamide side chains (C-30, C-31, C-41, C-42, C-48, C-49, C-55, and C-56) of the cobalamins were assigned by a comparison of their chemical shifts with the corresponding chemical shifts of cyanocobalamin. For the cluster of resonances assigned to C-60, C-56, C-42, C-55, and C-47, the assignments are only tentative because crossovers may have occurred in this region that are not readily detected. The assignments of the methylene carbons of the b, d, and e propionamide side chains were confirmed by a comparison of the spectra of the three adenosylcobalaminmonocarboxylic acids at neutral and acidic pH and by a study of the specific line broadening of these resonances by Gd^{3+} (Bratt & Hogenkamp, 1983) (data not shown).

Glusker (1982) has reported that in adenosylcobalamin the methylene groups C-41, C-48, C-55, and C-56 of the propionamide side chains and the methyl group C-20 prevent the rotation of the 5,6-dimethylbenzimidazole ligand. An even more severe steric hindrance would be expected in cyanocobalamin because the length of the Co–N bond to the dimethylbenzimidazole ligand in cyanocobalamin is considerably shorter. The spectrum of dicyanocobalamin shows that displacement of the dimethylbenzimidazole ligand by cyanide causes large upfield shifts of C-41, C-48, and C-49 and only small downfield shifts of C-42 and C-55. The basis of these upfield shifts is not clear at present because they seem to reflect stronger rather than weaker steric interaction in the base-off form. Similarly, the protonation of the dimethylbenzimidazole ligand and its replacement by water in the organocobalamins are accompanied by large upfield shifts of the C-48 and C-49 resonances while the other methylene resonances undergo only small chemical shift changes.

Methyl Carbon Region. All cobalamins contain eleven methyl groups; seven of these (C-20, C-25, C-35, C-36, C-46, C-47, and C-54) are attached to the corrin ring, two (B-10 and B-11) are part of the dimethylbenzimidazole ligand, and Pr-3 is from the 2-propanolamine moiety. The *pro-S* methyl group (C-47) resonates downfield in the methylene region. The other resonances are assigned by a comparison of their chemical shifts with those of the corresponding resonances of cyanocobalamin. The most downfield resonance in this region is assigned to B-10. This methyl group should experience severe steric compression in the base-on forms, and in accord with this assignment, this resonance shifts downfield in the base-off forms. The most upfield doublet in this cluster of resonances is assigned to Pr-3 because of the coupling to phosphate. In cyanocobalamin, this doublet is partially obscured by the C-25 resonance. However, in the other cobalamins this doublet is well resolved from the other resonances and can be unambiguously assigned to Pr-3. As before (Anton et al., 1982), the assignments of B-10, C-20, C-46, and C-25 are only tentative. In several of the spectra some of these resonances overlap, and thus crossovers may have occurred. The four most upfield methyl resonances are assigned to C-35, C-54, C-36, and C-53 by a comparison of their chemical shifts with those of cyanocobalamin. In the organocobalamins only the resonances assigned to C-35 undergo substantial downfield shifts in the base-off form. In contrast, the other three methyl resonances are quite constant, suggesting that in the base-on form of the organocobalamin these three methyl groups do not experience severe steric compression.

Discussion

The chemical shift data of the various cobalamins, presented in Table I, demonstrate that the effect of the axial ligand (cis effect) is transmitted only to selected carbon atoms of the corrin ring. For instance, the deprotonation of aquocobalamin to hydroxocobalamin, the substitution of a weak field ligand by a strong one, is accompanied by large upfield shifts of the resonances of C-4 (2.4 ppm), C-5 (1.3 ppm), C-9 (1.7 ppm), C-11 (2.0 ppm), and C-16 (2.9 ppm). Smaller upfield shifts are observed for some of the other ring carbon resonances. In contrast, the resonances of C-1, C-6, C-13, and C-18 are virtually constant in the spectra of these cobalamins. Similarly, the substitution of the lower 5,6-dimethylbenzimidazole ligand by cyanide as in base-off dicyanocobalamin brings about very large upfield shifts of only 11 of the 19 corrin ring carbon resonances. The resonances of the four carbons of the D pyrrolidine ring (C-16, C-17, C-18, and C-19) are not affected by these rather drastic electronic and steric modifications. The chemical shift changes that accompany the base-on → base-off conversion of the organocobalamins in acid are also limited to only half of the corrin ring carbon resonances. For example, the spectrum of adenosylcobalamin in the base-off form shows that only the resonances for C-1 (1.4 ppm), C-5 (2.8 ppm), C-9 (2.0 ppm), C-10 (1.8 ppm), C-11 (1.5 ppm), C-15 (2.8 ppm), C-17 (1.1 ppm), and C-19 (0.9 ppm) have undergone substantial downfield shifts.

An even more striking specificity of the cis effect is evident in the methylene carbon region of the spectra of the cobalamins. The base-on → base-off conversion of the organocobalamins in acid affects only resonances C-48 and C-49, the methylene carbons of the e propionamide side chain. The protonation of the 5,6-dimethylbenzimidazole moiety of adenosylcobalamin and its replacement by water are accompanied by upfield shifts of C-48 (0.7 ppm) and C-49 (2.7 ppm), while the resonances of C-41 and C-42, the methylene carbons of the d propionamide side chain, undergo only very small upfield shifts (0.1 and 0.4 ppm, respectively).

Glusker (1982) has compared the conformations of the acetamide and propionamide side chains of a series of corrins. She noted that the conformations of the a, b, f, and g side chains attached to the "western" half of the corrin ring are remarkably constant, while the conformations of the c, d, and e side chains are variable. In accord with our ^{13}C NMR data, the greatest variability was observed in the conformation of the e propionamide side chain. Brown & Hakini (1982) reported that the base-on → base-off conversion of methylcobalamin in acid is *not* accompanied by a shift of its ^{31}P resonance, implying that the magnetic environment of the phosphorus atom in the base-on and base-off forms is very similar. However, our ^{13}C–^{31}P coupling data presented in Table II demonstrate that the sugar moiety of the lower ligand and the 2-propanolamine arm do undergo considerable conformational changes during the base-on → base-off conversion.

It has been well documented that, except for ribonucleotide reductase, the adenosylcobalamin-dependent enzymes such as diol dehydrase, ethanolamine ammonia-lyase, glutamate mutase, and methylmalonyl-CoA isomerase form very tight complexes with the coenzyme (Toraya & Fukui, 1982; Babior, 1982; Switzer, 1982; Retey, 1982). In the holoenzymes, the carbon–cobalt bond is protected and not cleaved by light or by cyanide ion. Toraya et al. (1979) and Krouwer et al. (1981) have presented evidence suggesting that the interaction of the coenzyme with the enzyme labilizes the carbon–cobalt bond of the coenzyme with respect to the homolytic cleavage needed to initiate the catalytic process. The diol dehydrase–adeno-

sylcobalamin complex is very tight, and the complex does not dissociate at a detectable rate. In contrast, several analogues of adenosylcobalamin modified at the b, d, or e propionamide side chains form complexes with diol dehydrase that dissociate readily. These results demonstrate that these side chains play an important role in apoenzyme–coenzyme interactions. These earlier findings and the results presented in this paper suggest that the interaction of the apoenzymes with adenosylcobalamin, methylcobalamin, and (carboxymethyl)cobalamin causes conformational changes in the corrin ring (predominantly at C-5, C-8, C-9, C-10, C-11, and C-15) that facilitate the cleavage of the carbon–cobalt bond of the coenzymes. Our results also suggest that the e propionamide side chain is a key interaction site for the apoenzyme. It should be noted that the earlier identification of the three cobalaminmonocarboxylic acids was incorrect (Anton et al., 1980), and thus the conclusions from studies before 1980 using those analogues may be invalid.

Registry No. H_2OCbl, 13422-52-1; AdoCbl, 13870-90-1; CH_3Cbl, 13422-55-4; CMCbl, 14517-61-4.

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