of this statement (6) is largely circumstantial and by no means complete. However, if the species giving rise to resonances M does not have the structure suggested, it is very difficult to explain its presence in these systems on the basis of what is now known. Lastly, it would be a strange coincidence indeed if the appearance of M were unrelated to the transamination model reaction, in view of its apparent kinetic relationship to pyridoxamine formation, and its structural similarity to the aldimine as detected by nmr.

Carbon-13 Nuclear Magnetic Resonance Spectroscopy of Cobalamins and Cobinamides Selectively Enriched with Carbon-13[1a]

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Abstract: The cmr spectra of aqueous (D_2O) methylcobalamin, methylcobinamide, and cyanocobinamide, selectively enriched with ^{13}C in the ligands attached to cobalt, were recorded at 25.2 MHz and 32°. The cmr spectrum of [^{13}C]cyanocobinamide exhibits two well-resolved Co–^{13}CN resonances (chemical shift difference, 1.1 ppm) corresponding to the isomers: cyanoaquocobinamide and aquocyanocobinamide. The spectrum of di[^{13}C]cyanocobinamide at 32° shows only one rather broad Co–^{13}CN resonance, indicating that the chemical shift difference between the two sites is small. At 10°, two ^{13}CN resonances can be resolved partially (chemical shift difference <0.7 ppm) for di[^{13}C]cyanocobinamide. In contrast, the cmr spectrum of methylcobalamin exhibits one relatively sharp Co–$^{13}CH_3$ resonance broadened only slightly by the ^{59}Co nucleus, whereas the spectrum of methylcobinamide exhibits two Co–$^{13}CH_3$ resonances of approximately equal intensity. The chemical shift of the $^{13}CH_3$ moiety as well as the ^{13}C–H coupling constant are markedly affected by the nature of the trans ligand and a linear correlation has been established between the shift or the coupling constant and the β band of the visible spectrum. The electronegativity of the cobalt atom of methylcobalamin, estimated from the ^{13}C–H coupling constant and the carbon–cobalt bond length, varies from approximately 2.2 to 2.6 in the "base-on" and "base-off" form.

Deoxyadenosylcobalamin and methylcobalamin function as coenzymes in enzymatic reactions involving the transfer of hydrogen or of a methyl moiety, respectively. The most striking feature of these corrinoid coenzymes is the carbon–cobalt bond which is alternately cleaved and re-formed during the catalytic process.[2] The chemical and physical properties of the organometallic bond (X) are markedly influenced by axial ligands (Y) trans to it[3] (Figure 1). The electronic rearrangements in the carbon–cobalt bond that accompany coordination in the trans axial position are of interest both to the inorganic chemist concerned with ground state trans effects which are particularly large in these complexes, and to the biochemist concerned with the chemical changes that occur in the coenzyme during catalysis.

Because carbon-13 chemical shifts are remarkably sensitive to the electronic environment of the ^{13}C nucleus,[4] large trans effects should be evident in the ^{13}C nuclear magnetic resonance (cmr) spectra of the axial ligands in the cobalamins and cobinamides. This paper describes the cmr spectra of methylcobalamins, methylcobinamides, and cyanocobinamides selectively enriched with carbon-13 in the axial ligands.

Experimental Section

Materials. Cyanocobalamin was purchased from Sigma Chemical Co. Other corrinoids were prepared from cyanocobalamin by published procedures: aquocobalamin,[5] methylcobalamin,[6] diaquocobinamide,[3a] cyanoaquocobinamide,[7] and methylcobinamide.[3a] Carbon-13 methyl iodide, 61.8% enriched, was purchased from Prochem; [^{13}C]methyl iodide, 90% enriched, was prepared from [^{13}C]methanol (a gift from D. G. Ott of LASL) according to the method of Murray and Ronzio.[8]

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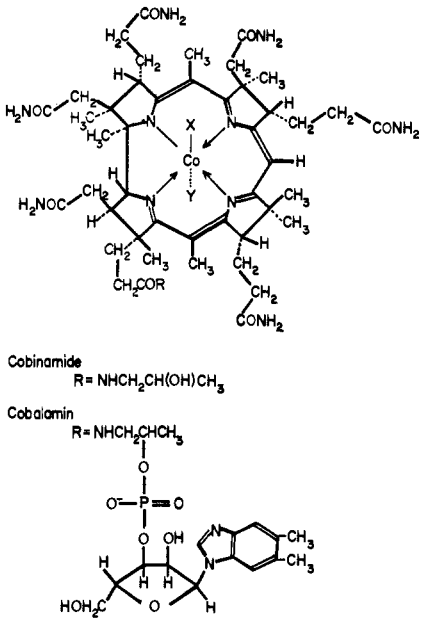

Figure 1. Structure of cobalamins and cobinamides.

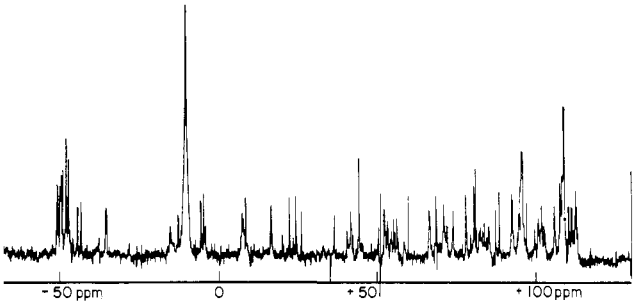

Figure 2. Cmr spectrum of di[^{13}C]cyanocobalamin.

The purity of the corrinoids was established by spectral analysis and by paper chromatography in three solvent systems.[9]

Methods. Pulse ^{13}C (25.2 MHz) nuclear magnetic resonance spectra were obtained at 32° using a Varian XL-100-15 spectrometer locked to the resonance (15.4 MHz) of internal D_2O and interfaced to a Supernova computer–magnetic disc system. The transients resulting from the application of 45-μsec pulses in a spectral width of 5000 Hz were accumulated as 8192 points in the time domain and transformed into a 4096-point real spectrum (frequency domain). The data acquisition time was 820 msec, and the spectra were obtained under conditions of simultaneous broad band (3000 Hz) proton noise decoupling. Peak positions were determined by computer examination of the final Fourier transformed spectrum. Chemical shifts were measured with respect to a benzene external standard. The cmr spectra of D_2O solutions in 12 mm o.d. nmr tubes were obtained for [^{13}C]methylcobalamin (61.8% ^{13}C), [^{13}C]-dicyanocobalamin (45% ^{13}C), [^{13}C]methylcobinamide (90% ^{13}C), and cyanoaquocobinamide at the following concentrations respectively: 10 m*M* at pH 7 and 100 m*M* at pH 2; 100 m*M*; 230 m*M*; and 150 m*M*. The cmr spectra of solutions of the [^{13}C]cyano-[^{13}C]methylcobinamide complexes were obtained by adding solid K^{13}CN (94% ^{13}C) to the methylcobinamide solutions (final cyanide concentrations 100, 260, and 380 m*M*). The cyanide ligand was then eliminated as hydrogen cyanide by adding a slight excess of concentrated aqueous hydrochloric acid. Next the solution was heated and purged vigorously with nitrogen. The cmr spectrum of the resulting [^{13}C]methylcobinamide solution indicated that only trace quantities of [^{13}]cyanide remained. To this solution pyridine was added in stepwise increments, giving formal pyridine concentrations of 60 m*M*, 170 m*M*, 270 m*M*, 550 m*M*, and 2070 m*M*.

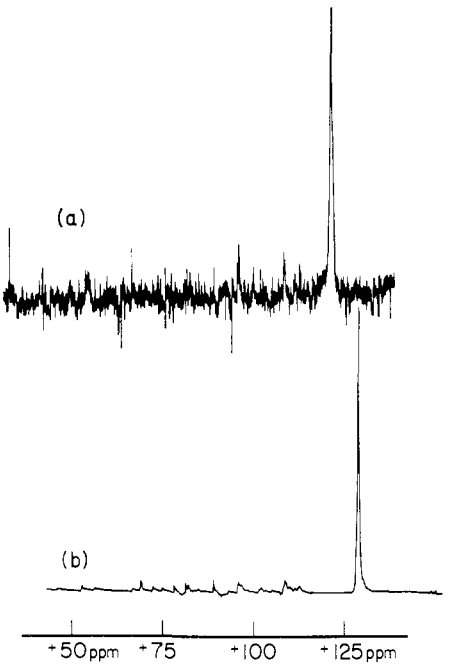

Figure 3. Cmr spectra of [^{13}C]methylcobalamin in the alkyl region at (a) pH 7 and (b) pH 2.

From the cmr spectra of these solutions, the ^{13}C chemical shifts of the [^{13}C]methylcobinamide–pyridine complexes were determined. In a similar fashion, the cmr spectra of solutions of the mono[^{13}C]-cyano- and di[^{13}C]cyanocobinamide complexes were obtained by adding increments of solid potassium [^{13}C]cyanide to solutions of cyanoaquocobinamide. All solutions were manipulated in subdued light and were stored in the dark at 5°. Proton magnetic resonance spectra were determined using a Varian HR-220 spectrometer. The chemical shift of the cobalt-bound methyl moiety of methylcobalamin was used as internal reference.

Results

The proton noise decoupled cmr spectrum of dicyanocobalamin (cyanide 45% ^{13}C) is reproduced in Figure 2. The intense resonance at −8.9 ppm is due to ^{13}CN ion coordinated to cobalt confirming the tentative assignment of Doddrell and Allerhand[10] in their natural-abundance cmr spectrum of dicyanocobalamin. The cmr spectra of [^{13}C]methylcobalamin at pH 7 and pH 2 are shown in Figure 3. The shift of the intense high field resonance which is due to the [^{13}C]methyl group bound to cobalt reflects the displacement of the 5,6-dimethylbenzimidazole moiety (Bz) by water at low pH.[11]

The effect of the addition of K^{13}CN to an aqueous solution of unlabeled cyanoaquocobinamide is shown in Figure 4. The intense signal at −9.4 ppm is assigned, on the basis of chemical shift comparisons and of the cyanide ion dependence of the spectra, to [^{13}C]cyanide in the dicyano complex. The set of higher field resonances (19.4 and 18.3 ppm, approximate relative intensity 60:40) is assigned to [^{13}C]cyanide occupying the upper and lower coordination positions (Figure 1). A similar "doubling" of the [^{13}C]methyl resonance (130.3 and 128.4 ppm; approximately equal relative intensities) is apparent in the cmr spectrum of [^{13}C]-methylcobinamide (Figure 5).

The [^{13}C]methyl chemical shifts of the methylcor-

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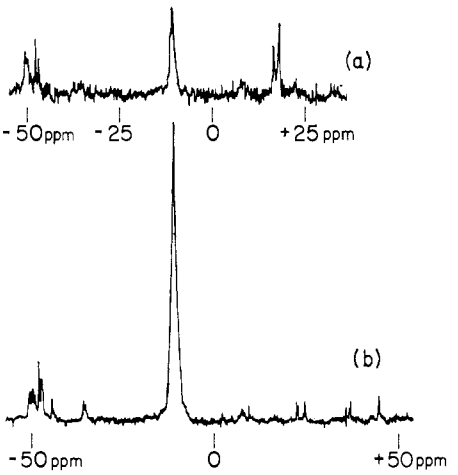

Figure 4. Cmr spectra in the CN region of aqueous 0.15 *M* cyanocobinamide in the presence excess of $^{13}CN^-$ ion: (a) 0.100 *M*; (b) 0.26 *M*.

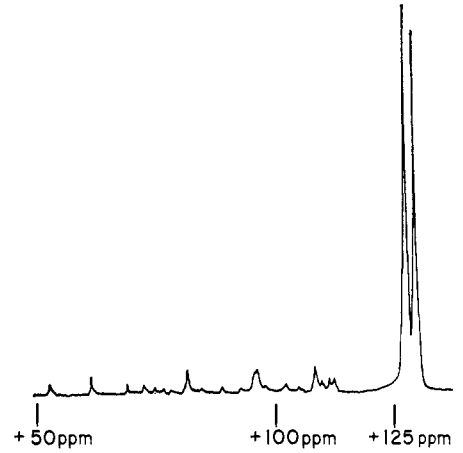

Figure 5. Cmr spectrum of aqueous [^{13}C]methylcobinamide in the alkyl region.

rinoids are summarized in Table I and the [^{13}C]cyanide chemical shifts of the cyanocorrinoids are summarized in Table II. The data for systems which involve a

Table I. Methyl Resonance Chemical Shifts

Complex	Chemical shift[a]	Trans group
[^{13}C]Methylcobinamide	130.3	H_2O
	128.4	H_2O
[^{13}C]Methylcobalamin at low pH	129.7	H_2O
[^{13}C]Methylpyridinecobinamide	123.9	Pyridine
	122.8	Pyridine
[^{13}C]Methylcobalamin	122.0	Bz
[^{13}C]Methyl[^{13}C]cyanocobinamide	116.3	CN^-
	114.8	CN^-

[a] Ppm (±0.2) upfield from external benzene.

Table II. Cyanide Resonance Chemical Shifts

Complex	Chemical shift[a]	Trans group
[^{13}C]Cyanocobinamide	+19.4	H_2O
	+18.3	H_2O
Di[^{13}C]cyanocobalamin	−8.9	CN^-
Di[^{13}C]cyanocobinamide	−9.4	CN^-
[^{13}C]Methyl[^{13}C]cyanocobinamide	−33.0	CH_3

[a] Ppm (±0.2) from external benzene; a positive sign denotes an upfield shift.

rapid exchange of ligand trans to the "fixed" axial ligand were obtained from titration plots such as that shown in Figure 6. For example, the $^{13}CH_3$ chemical shift of aqueous methylcobinamide at large molar ratios of CN^- ion to cobinamide (Figure 6) is ascribed to the complex cyanomethylcobinamide, whereas the $^{13}CH_3$ chemical shift at lower ratios is due to a weighted average of the ^{13}C chemical shifts of aquomethylcobinamide and cyanomethylcobinamide which are in rapid chemical exchange equilibrium. The ^{13}C resonance of the corrin ring and its substituents are also subject to axial ligation effects which will not be covered in this paper.

Most of the $^{13}CH_3$ and ^{13}CN resonances are surprisingly narrow, 11 ± 2 Hz full width at half maximum intensity. The width of the lines for methylcobalamin at pH 7, and for dicyanocobalamin and dicyanocobinamide, are somewhat larger: 20, 25, and 25 Hz, respectively.

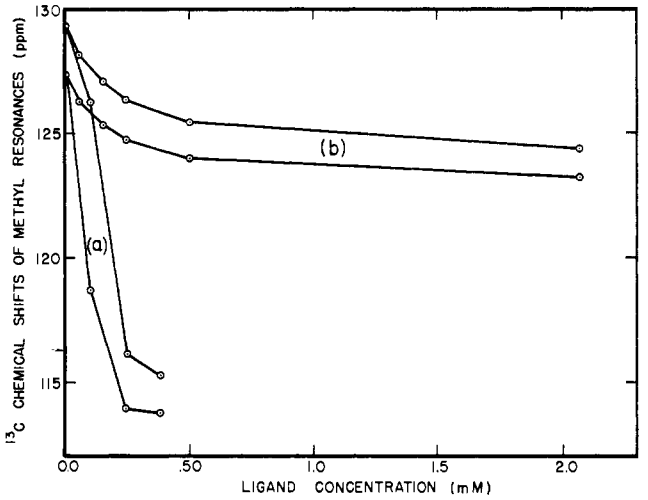

Figure 6. Dependence of the ^{13}C chemical shifts of aqueous 0.23 *M* [^{13}C]methylcobinamide upon added ligands: (a) cyanide ion; (b) pyridine.

The 220-MHz proton magnetic resonance spectrum of [^{13}C]methylcobalamin in D_2O shows three resonances upfield from the C(20) methyl resonance position at 0.17, −0.14, and −0.45 ppm. The resonance at −0.14 ppm has been assigned to the cobalt-bound methyl group, while the resonances at 0.17 and −0.45 ppm can be assigned to the ^{13}C methyl moiety (Figure 7), $J_{^{13}C-H}$ = 138 Hz.

Upon acidification with deuterioacetic acid, the C(20) methyl resonance of [^{13}C]methylcobalamin is shifted to lower field[12] while the ^{13}C–H coupling constant increases to 141 Hz. The pmr spectrum of [^{13}C]methylaquocobinamide is identical with that of "base off" [^{13}C]methylcobalamin in the region around δ 0. However, the nature of the trans ligand in the methylcobinamides does affect the ^{13}C–H coupling constant. In Figure 8 is shown the correlation between the ^{13}C–H coupling constant and the maximum of the β band in the visible spectrum.

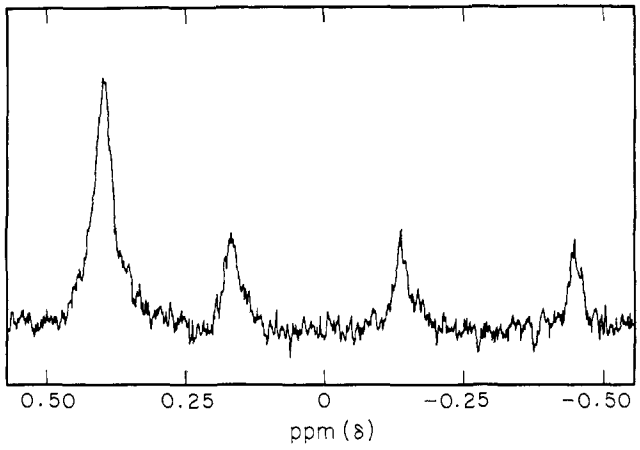

Figure 7. The 220-MHz pmr spectrum of [^{13}C]methylcobalamin in D_2O around δ 0.

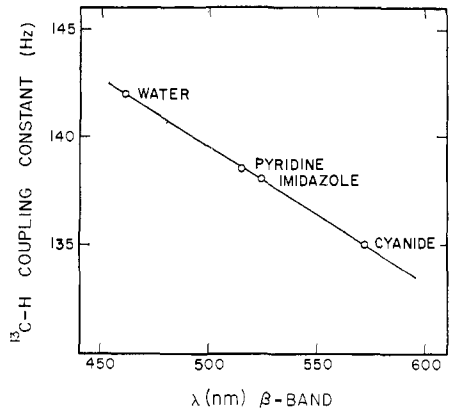

Figure 8. Correlation between the ^{13}C–H coupling constant and the maximum of the β band in the visible spectra of some methylcobinamides.

Discussion

It has been well established that the ligands attached to cobalt in the corrinoids have a marked effect on each other.[9,11-13] For instance, this trans effect is readily discernible in the alkylcobalamins, where the pK_a of the 5,6-dimethylbenzimidazole moiety is determined by the nature of the trans alkyl ligand.[14] On the one hand, the electronegativity of the alkyl group (the upper axial ligand) has a large effect on the strength of coordination of the lower (trans) base. On the other hand, the nature of the trans base greatly influences the reactivity of the cobalt atom and the cobalt-bound alkyl group. When an alkylcobalamin acts as a coenzyme, it has been postulated that, in the coenzyme–enzyme–substrate complex, the 5,6-dimethylbenzimidazole moiety is alternately coordinated and uncoordinated (the trans coordination position perhaps being occupied by another ligand donated by the enzyme), thus greatly altering the reactivity of the carbon–cobalt linkage at different stages of the reaction.[9,15]

To date, the most useful probes of the nature (*e.g.*, nitrogen, sulfur, or carbon ligand) of the trans group in these complexes have been electronic absorption spectroscopy and epr methods. The large ^{13}C chemical shifts that accompany the coordination of different trans ligands to cobalamins and cobinamides (Tables I and II) suggest that cmr spectroscopy will provide an additional, extremely sensitive technique for studying the trans coordination site in enzyme–coenzyme complexes. The large cmr trans effects, observed in this study, are especially welcome in view of a recent study[16] which shows that electronic absorption spectroscopy can be insensitive to drastic changes in the nature of the trans ligand. Of course, epr spectroscopy can be applied only to paramagnetic (Co^{II}) systems whose resonances frequently are superimposed on other radical signals in enzyme systems. The cmr technique can be applied both to diamagnetic and paramagnetic systems.[17]

The magnitude of the effect of the trans ligands on the ^{13}C shifts of the methyl and cyano ^{13}C resonances of the cobalamin and cobinamide complexes increases in the order: H_2O < pyridine < benzimidazole < CN^- < CH_3. This is the same order of trans effects that have been deduced from studies on the C–N stretching frequencies of cyanocobinamides[18] and from the formation constants for the substitution of coordinated water in aquocobalamin.[19] The origin of the large downfield shifts of the $^{13}CH_3$ and ^{13}CN resonances that occur when strong trans nucleophiles are coordinated to cobalt is not entirely clear (*e.g.*, compare the $^{13}CH_3$ shifts of [^{13}C]methylaquocobinamide and [^{13}C]methylcyanocobinamide, Table I). Steric interactions between the ligands in the axial positions and the corrin ring substituents do not appear to be the cause of this large downfield shift. First, steric interactions between the lower axial group and the C(20) methyl group appear to be small (see below) and second, a pronounced steric interaction between these groups in a strong Y–Co–CN (or CH_3) complex should lead to a large upfield shift rather than a downfield shift.[20]

Indeed, the excellent correlation between the position of the β band of the visible spectrum and the ^{13}C–H coupling constants (Figure 8) and the ^{13}C chemical shifts (Figure 9) suggests that the interactions are primarily electronic in nature. A similar correlation has been shown between the chemical shift of the C(10) hydrogen of the corrin ring and the β band of the spectrum.[12] Molecular orbital calculations suggest that the shift of the β band to high energy when a strong field ligand (CN^-) is substituted by a weak field one (H_2O) is associated with an increased net charge on the Co atom[21] and with decreased overlap integrals

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between the axial ligand orbitals and the cobalt $4p_z$ orbital.[22] The ^{13}C–H coupling constants, which provide a measure of the degree of hybridization of the carbon atom bonded to cobalt and of the electronegativity of the cobalt atom,[23,24] are not inconsistent with this suggestion. Assuming a carbon–cobalt bond length of 2.05 Å in methylcobalamin,[25] the electronegativity of the cobalt atom in methylcobalamin can be estimated from the ^{13}C–H coupling constant using the equation derived by Muller and Pritchard.[24] This electronegativity (E_{Co} = 2.23) is much larger than that determined by Hill, Pratt, and Williams (1.90)[26] from the chemical shift of the methyl hydrogens of methylcobalamin in trifluoroacetic acid. From the $J_{^{13}C-H}$ of methylcobalamin in the "base off" form we estimate an electronegativity of approximately 2.6 (assumed r[C–Co] = 1.9 Å).

The interpretation of the trans effects on the ^{13}C chemical shift data is not as straightforward. As a first approximation one might expect that as the net charge on the Co atom is reduced, the $^{13}CH_3$ and ^{13}CN resonances would move to high field as a consequence of the buildup of negtive charge on the *carbon* atom. However, the opposite trend is observed (strong field ligands induce downfield shifts of the ^{13}CN and $^{13}CH_3$ resonances). Refined molecular orbital calculations on small molecules[27–30] indicate that factors other than charge density have an important influence on ^{13}C chemical shifts including: variations in spin pairing between p electrons; the extent of p orbital occupation; changes in the radial distance of electrons from screened nuclei; and variations in excitation energies for "mixing" ground with excited state wave functions. A more detailed insight into the origin of the trends in the $^{13}CH_3$ and ^{13}CN shift data will probably require calculations on corrin derivatives which explicitly incorporate parameters like those listed above.

The relative intensities of the ^{13}C resonances of the two stereoisomers of the [^{13}C]methyl- and [^{13}C]cyanocobinamide complexes are nearly equal, suggesting that a large site preference for the apparently less hindered upper coordination position does not exist. A similar lack of a large preference for the upper coordination position was noted in the *Co*-methylcobyric acids and the *Co*-methylcobinamidemonocarboxylic acids.[31] The shape of the titration curves (Figure 6) also suggests that large steric interactions are absent in the formation of the pyridine and cyano complexes from the isomers of [^{13}C]methylaquocobinamide. From the relative curvature of the plots, we estimate that the

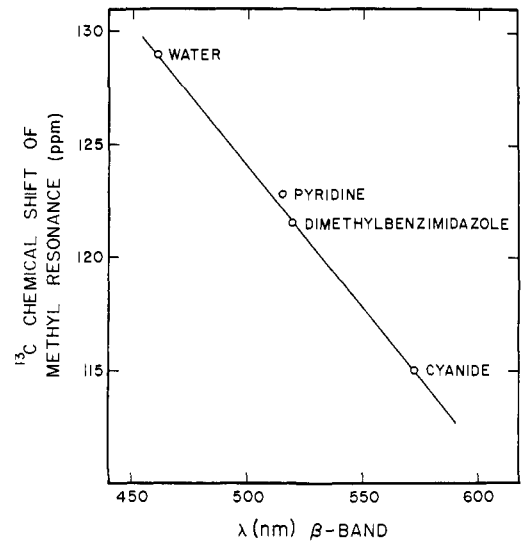

Figure 9. Correlation between the $^{13}CH_3$ chemical shift and the maximum of the β band in the visible spectra of some methylcorrinoids.

equilibrium constants, for formation of the pyridine complexes of the two isomers of methylcobinamide, are approximately equal.[32] A similar calculation for the formation of the cyanomethylcobinamide complexes indicates a larger degree of discrimination by the upper and lower isomers (equilibrium constant ratio, ~7). In contrast, polarographic studies[9] have shown that the ratio of the concentrations of the two cyanoaquocobinamide isomers varies between 0.67 at pH 6.3 to approximately 1.5 at pH 12.4. Thus, on the one hand, the slightly more crowded methylcyanocobinamide complex shows a greater preference for one isomer (the one corresponding to the downfield methyl resonance) than does the less crowded cyanoaquocobinamide. On the other hand, the steric requirements of pyridine should be larger than those for the cyanide ion, yet the isomers of methylpyridinecobinamide are formed in nearly equal quantities. Additional studies with a more extensive series of ligands are required to differentiate between steric and electronic effects.

Friedrich[33] first demonstrated that corrinoids with different axial ligands exist in solution as a mixture of two stereoisomers. Cyanoaquocobinamide could be separated by chromatographic techniques into the two stereoisomers aquocyanocobinamide and cyanoaquocobinamide. The equilibrium between these two isomers is established at a slow rate, so that each isomer can be reduced separately at the dropping mercury electrode.[9] More recently, Doddrell and Aller-

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(32) The $^{13}CH_3$ chemical shifts of the mixtures of methylcobinamide complexes with water and pyridine which are in rapid chemical exchange equilibrium conform to the equations: $\delta^i = P^i\delta_0{}^i$, where δ^i is the measured exchange averaged shift for the ith isomer; δ_0 is the $^{13}CH_3$ shift of the methylaquocobinamide with respect to the methylpyridinecobinamide, and P is the mole fraction of the cobinamide in the form of the aquocomplex; and K = [methylpyridinecobinamide]/[pyridine]-[methylaquocobainamide]. Consequently, between the extremes of very low and very high pyridine concentrations, the relative equilibrium constants for the association of the upper (u) and lower (l) methylcobinamide complexes with pyridine are a sensitive function of the chemical shift ratio: $[\delta^u/\delta_0{}^u]:[\delta^l/\delta_0{}^l]$. Specifically, $K^u/K^l = [\delta^l/\delta^u][(\delta_0{}^u - \delta^u)/(\delta_0{}^l - \delta^l)]$.
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hand[34] were able to detect differences in the ^{13}C chemical shifts of the isomers of aquocyanocobyric acid. Using cyanoaquocobinamide highly enriched in ^{13}C in the cyanide moiety, separate sharp resonances in the cmr spectrum are readily detected (Figure 4). At ambient probe temperature (25°), only one [^{13}C]-cyanide resonance was distinguished for the dicyanocobinamide complex even though the two cyanide environments are not equivalent. Because the cmr data demonstrate that exchange between free cyanide ion (or hydrogen cyanide) and dicyanocobinamide or cyanoaquocobinamide is slow, the appearance of only one peak cannot be due to a rapid averaging of the two cyanide environments of dicyanocobinamide *via* exchange. Instead it appears that the chemical shift difference between the sites is small, a suggestion which is not inconsistent with the observation that the cyanide resonance in this complex is twice as broad as those of the monocyano complexes. Indeed, at lower temperatures (*ca.* 10°), two resonances are partially resolved with a chemical shift difference of about 0.7 ppm.

Two isomers of methylcobalamin and methylcobinamide have also been described. Methylcobalamin *a* with the methyl moiety in the lower axial position was isolated[33] while methylation of Factor I (Cobinamide-phospho-ribose) yielded both diastereoisomers. Furthermore, heating of a mixture of *Co*-[^{14}C]methylcobalamin and unlabeled methylcobinamide gave *Co*-[^{14}C]methylcobinamide.[33] These results indicate that axial isomerization is a general property of the corrinoids. Although only one isomer of [^{13}C]-methylcobalamin could be demonstrated, the cmr spectra of [^{13}C]methylcobinamide shows two separate resonances, differing in chemical shifts by 1.9 ppm.

All the resonances for ^{13}C atoms bonded to cobalt in the complexes studied are quite narrow suggesting that the quadrupolar contribution strongly dominates the spin-lattice relaxation rate of ^{59}Co (100% abundance, $I = 7/2$) resulting in an extremely effective obliteration of the Co–C spin–spin splittings. These effects are not unexpected in molecules such as these having large static electric field gradients at the ^{59}Co nucleus and being capable of large rapid fluctuations of those gradients. Although a more detailed study of the chemical exchange effects on the ^{13}C resonances of the complexes is still in progress, we could note that under the conditions of the experiments reported here, we could discern no chemical exchange contributions to the ^{13}C line widths. From the number of ^{13}CN and $^{13}CH_3$ resonances and their $^{13}CN^-$ concentration dependence, it is clear that the ^{13}CN ligand in cyanomethylcobinamide exchanges rapidly. For ^{13}CN trans to any other group but CH_3 in these complexes, ^{13}CN exchanges only slowly. From the chemical shift data, it can be estimated that the former complex exchanges CN ligands at rates much greater than 4×10^3 sec^{-1}. These dramatic effects of the trans ligand on the chemical exchange rate can be elucidated in much more detail by studies of the temperature dependence of the cmr spectra.

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