Proton Magnetic Resonance Studies of Vitamin B_{12}. Proton Magnetic Resonance Spectra of Some Cobalamins and Cobinamides*

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ABSTRACT: The conformation of vitamin B_{12} and the contribution of the various functional groups to its chemical properties were investigated by means of proton magnetic resonance studies of fifteen cobalamins and cobinamides. Ten Co(III) corrinoids produced well-resolved proton magnetic resonance spectra, as did the Co(I) derivative [B_{12s}]. Poorly resolved proton magnetic resonance spectra were obtained for four paramagnetic Co(II) corrinoids. B_{12s} was shown to be diamagnetic. Extensive resonance assignments were made for the Co(III) corrinoids, and employed to assess the interaction of the fifth and sixth cobalt ligands with the rest of the corrinoid. The methyl resonances of methylcobalamin and methyl-10-chlorocobalamin are virtually superimposable, implying little electronic coupling of the alkylcobalt ligand with the corrin ring. However, the resonance of the C-10 proton of dichloromethylcobalamin is shifted substantially to high field relative to the C-10 proton of methylcobalamin, suggesting that the converse may not be true. The differences between the proton magnetic resonance spectra of cobinamides and cobalamins are ascribed to changes in the conformation of groups extending from the corrin ring, with the corrin ring essentially unchanged. It was observed in cobinamides that the prochiral protons on the carbon bound to cobalt in alkyl ligands are not equivalent, and this is attributed to incomplete averaging of proton environments through rotation about the cobalt–carbon bonds. In four alkylcobinamides, resonances were detected that are attributed to water bound to the sixth coordination position of cobalt. A 7 Hz proton–proton nuclear spin coupling is assigned to coordinated H_2O. The four Co(II) derivatives of vitamin B_{12} are paramagnetic, and exhibit small shifts relative to diamagnetic vitamin B_{12}. Breadths of resonances of the paramagnetic derivatives are attributed to electron–nucleus dipolar relaxation. The nitrogen base, 5,6-dimethylbenzimidazole, is shown to be not coordinated to cobalt in cob(I)alamin [B_{12s}].

The diverse and unusual chemical reactions catalyzed by coenzyme B_{12} dependent enzymes possess as a common feature either the breaking of the preexisting carbon–cobalt bond, or the formation of a new carbon–cobalt bond *via* alkylation of a Co(I), Co(II), or mercapto species with accompanying redox changes (Hogenkamp, 1968). Extensive studies on the chemical and physical properties of vitamin B_{12} have brought much insight into the relationship between the structure and catalytic properties of its derivatives [Barker *et al.* (1960), Brodie (1969), Cockle *et al.* (1969), Firth *et al.* (1967), Hill *et al.* (1969a), Hogenkamp *et al.* (1965)]. One useful approach has been to compare the properties of a series of derivative compounds, cobalamins and cobinamides, in an attempt to assess the contribution of specific functional groups to the overall chemical and physical properties of the molecule. The proton magnetic resonance spectra of cobalamins and cobinamides have been particularly helpful in assessing the electronic state of the cobalt atom, the interaction of the fifth and sixth cobalt ligands with the rest of the molecule, and the rates of specific reactions such as hydrogen exchange at C-10. The proton magnetic resonance studies reported in this article extend previous ones (Hill *et al.*, 1965, 1968, 1969a) by making more complete assignments on a greater variety of compounds. "Cis" and "trans" ligand effects, the number of cobalt ligands, rates of configuration change, and the geometry of the environment of the cobalt ligands are considered in light of these data.[1]

Methods and Materials

All proton magnetic resonance spectra were obtained with a Varian 220 MHz proton magnetic resonance spectrometer. The signal-to-noise ratios of certain spectra were improved through use of a Varian C-1024 computer of average transients. Tetramethylsilane or the methyl resonance of the sodium salt of 2,2-dimethyl-2-silapentanesulfonic acid were employed as internal references. All chemical shifts are expressed in units of Hz or in parts per million of the polarizing field, with downfield shifts assigned positive values.

The proton magnetic resonance spectra of fifteen cobalamins and cobinamides were examined. Cyanocobalamin was purchased from Sigma Chemical Co., St. Louis, Mo.; the preparation and purification of the other B_{12} derivatives is given below. For proton magnetic resonance spectra about 30 mg of the cobalamin or cobinamide was dissolved in 1 ml of either $(CD_3)_2SO$ or D_2O. The four paramagnetic B_{12} derivatives were dissolved under a nitrogen atmosphere in $(CD_3)_2SO$ that had been extensively bubbled with oxygen-free nitrogen. Cob(I)alamin (B_{12s}) was prepared by adding excess

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[1] A preliminary account of the material appearing in this manuscript was presented to the Joint ACS-CIC National Meeting, Toronto, Canada, May 1970.

$NaBH_4$ to hydroxycobalamin dissolved in nitrogen-bubbled D_2O containing about 2 mM $(CH_3)_4NCl$. Full reduction was monitored visually. The seven derivatives containing carbon–cobalt bonds were dissolved in $(CD_3)_2SO$ in a darkened room and were stored in tubes wrapped with aluminum foil; these compounds were handled exclusively in darkened areas. Unless indicated otherwise spectra were obtained at 23°.

Hydroxycobalamin (B_{12a}) was prepared from cyanocobalamin [Hogenkamp and Rush (1968)]. Methylcobalamin was prepared from cob(I)alamin and methyl iodide [Mervyn and Smith (1968)]. Anaerobic photolysis of purified methylcobalamin followed by evaporation to dryness gave cob(II)alamin (B_{12r}). Methyl-10-chlorocobalamin was prepared from methylcobalamin and chloramine T [Dolphin *et al.* (1964)]. Anaerobic photolysis of this product gave 10-chlorocob(II)alamin. Dichloromethylcobalamin was prepared as recently described [Wood *et al.* (1968)].

Cyanocobinamide, prepared by $Ce(OH)_3$ hydrolysis [Friedrich and Bernhauer (1956)] of vitamin B_{12}, was the starting material for all cobinamide preparations. In contrast to the cobalamin series, it was found advantageous to prepare methylaquocobinamide *via* reduction of cyanoaquocobinamide and to purify the methylcobinamide on CM-cellulose. This was then photolyzed aerobically and the resultant diaquocobinamide used for further syntheses. The inclusion of this extra step facilitated the elimination of side products in the cobinamide preparations. Methyl-, *n*-propyl-, isopropyl-, and cyclohexylcobinamides were prepared from the corresponding alkyl bromide or iodide and diaquocobinamide. 10-Chlorocob(II)inamide was prepared by $Ce(OH)_3$ hydrolysis of 10-chloroaquocobalamin followed by preparation and anaerobic photolysis of the methyl-10-chlorocobinamide. Cob(II)inamide was prepared directly from methylcobinamide.

All compounds other than the reduced cobalamins and cobinamides were purified by chromatography on DEAE- and CM-cellulose. When appropriate, cellulose phosphate columns were also employed. In addition, all compounds were tested for homogeneity by thin-layer chromatography on cellulose [Firth *et al.* (1968b)]. Methylcyanocobinamide was prepared by adding 50 μl of a 0.6 M KCN solution in D_2O to 1 ml of a $(CD_3)_2SO$ solution of about 30 mg of methylcobinamide.

Results

The proton magnetic resonance spectra of fifteen derivatives of vitamin B_{12} were obtained. Eight cobalamins: methyl-, dichloromethyl-, cyano-, hydroxy-, and methyl-10-chlorocobalamin, 10-chlorocob(II)alamin, cob(II)alamin (cobalamin r), and cob(I)alamin (cobalamin s) were studied along with seven cobinamides: methyl-, methylcyano-, *n*-propyl-, iso-propyl-, and cyclohexylcobinamide and 10-chlorocob(II)inamide and cob(II)inamide (cobinamide r).

The proton magnetic resonance spectra of the 11 diamagnetic cobalamins and cobinamides were generally well resolved at 220 MHz. It was therefore possible to identify resolved resonances with most of the constituent protons of these molecules. In fact, for these 11 derivatives, the resonances observed accounted for all the 75–100 protons present in the various compounds, provided that the broad resonances between 1.5 and 2.5 ppm in the proton magnetic resonance spectra were taken to represent about 10 protons. For example, in the proton magnetic resonance spectrum of methylcobinamide there are 34 resolvable peaks attributable to the cobinamide, with areas corresponding to 64 protons. There are 75 protons per molecule in this compound, excluding any protons of the sixth cobalt ligand. With appropriate selective deuteration procedures it would be possible to assign well over half the protons on the various derivatives to specific, resolved peaks in the proton magnetic resonance spectrum. Even without such an effort, it is possible to make a number of detailed assignments.

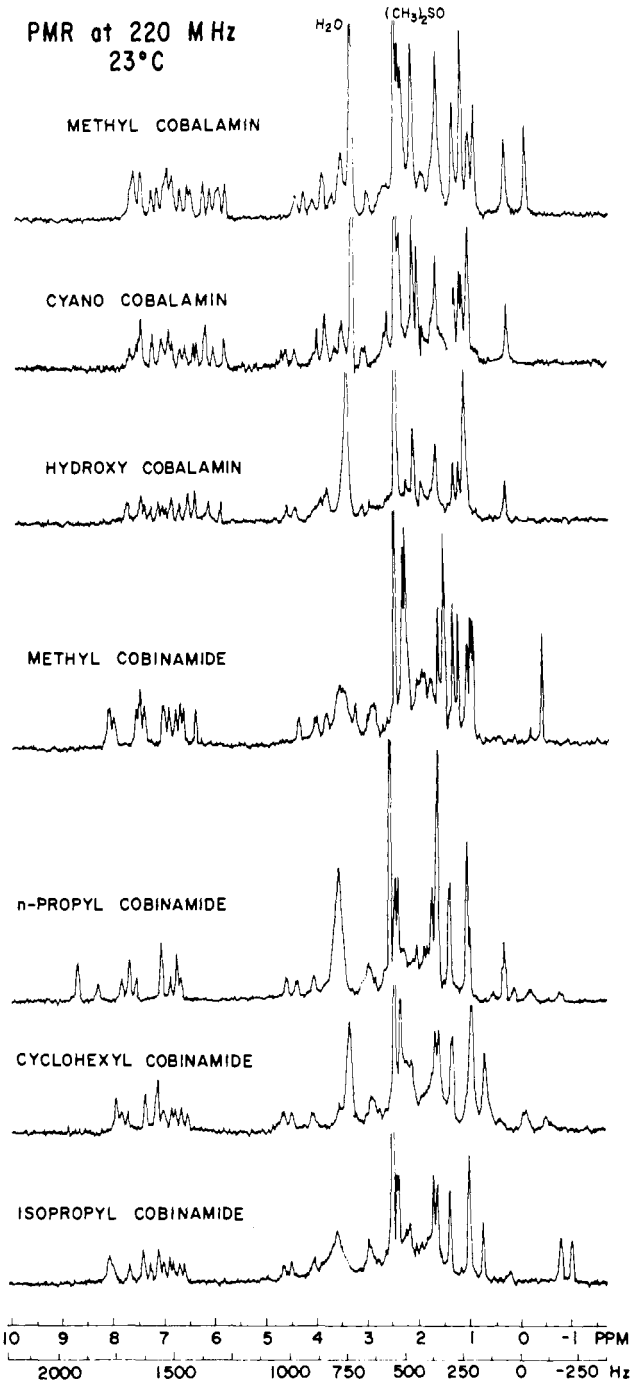

FIGURE 1: Proton magnetic resonance spectra (220 MHz) of seven cobalamins and cobinamides in $(CD_3)_2SO$. Concentrations in each case were approximately 30 mg/ml.

The proton magnetic resonance spectra of seven of the cobalamins and cobinamides studied are presented in Figure 1. The strong peak near 2.5 ppm in the seven spectra corresponds to residual protons of the $(CD_3)_2SO$. The other

TABLE I: Chemical Shifts of Protons on Alkyl Groups Bonded Directly to Cobalt in Diamagnetic Cobalamins and Cobinamides.

Compound	Substituent	Chemical Shift: Actual (ppm)	Chemical Shift: Adjusted[a] (ppm)	
Methylcobalamin	CH_3	−0.06	0.97	
Methyl-10-chlorocobalamin	CH_3	+0.01	0.90	
Dichloromethylcobalamin	$CHCl_2$	−0.27		
Methylcobinamide	CH_3	−0.36	1.27	
Methylcyanocobinamide	CH_3	−0.04	0.95	
n-Propylcobinamide	$CH_3CH_2CH_2$	+0.26	0.65	
	$CH_3CH_2CH_2$	+0.05	1.30	1.20
		+0.46	0.89	
	$CH_3CH_2CH_2$	−0.85	2.20	1.90
		−0.25	1.60	
Cyclohexylcobinamide	$C_6H_{11}CH$	−0.50		
	3 ring methylenes	+0.01		
	1 ring methylenes	+0.49		
	Ca. 2 ring methylenes	+0.65		
	Ca. 2 ring methylenes	+1.01		
Isopropylcobinamide	$(CH_3)_2CH$	−1.02	1.93	1.80
		−0.75	1.66	
	$(CH_3)_2CH$	+0.20	1.94	

[a] The adjusted chemical shift is the difference between the actual chemical shift given in column three and the chemical shift of the substituent when it is in the alkanes formed by replacing the cobalt with a methyl group [Ferguson and Marquardt (1964)].

strong peak at about 3.5 ppm is H_2O. The strong, sharp resonances at high field between 0 and 2 ppm in these spectra are mostly methyl groups. The low-field resonances between +5.8 and +8.8 are predominantly single proton resonances. In searches to higher and lower field, no proton resonances other than those shown in the spectra of Figure 1 were observed between −25 to +40 ppm.

The chemical shifts of those peaks that derive from methyl groups of seven of the diamagnetic cobalamins and cobinamides studied are given in Figure 2, as derived from the spectra of Figure 1. In Figure 2, a line of unit height corresponds to a single methyl group; the abscissa is in parts per million downfield from internal $(CH_3)_4Si$. The dotted lines correspond to methyl groups on alkyl substituents directly bonded to cobalt and are listed in Table I. A striking feature of Figure 2 is that each of the three cobalamins and each of the four cobinamides exhibit very similar methyl group chemical shift patterns. This leads to immediate identification of methyl groups on cobalt-bound alkyl groups as those which exhibit resonances to extreme high field.

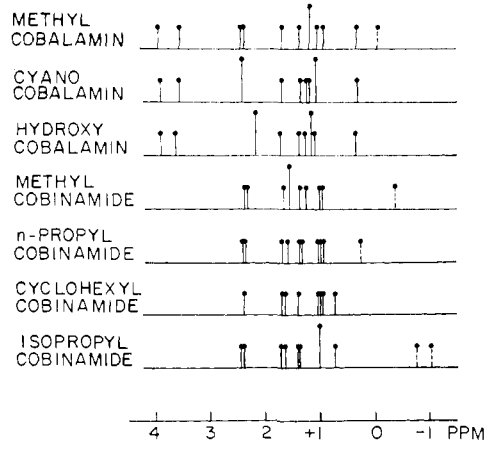

FIGURE 2: Schematic representation of the chemical shifts of methyl resonances of the seven diamagnetic corrinoids whose proton magnetic resonance spectra are presented in Figure 1. A line one unit high corresponds to a single methyl group, or three protons. The dotted lines are methyl groups of alkyl ligands bound to cobalt.

The pair of methyl groups observed in the three cobalamins between 3.6 and 4 ppm are the methyl groups on the benzimidazole six-carbon aromatic ring. The benzimidazole methyl protons exhibit chemical shifts about 1 ppm to low field when compared with 5,6-dimethylbenzimidazole [Hill *et al.* (1965)] and as expected are nonequivalent. The pairs of methyl resonances between 2.15 and 2.5 ppm in the seven derivatives (except cyclohexylcobinamide) are the vinyl methyls at C-5 and C-15 on the corrinoid ring [Hill *et al.* (1968)]. These assignments are based on comparisons with model compounds. The remaining seven methyl groups of each of these B_{12} derivatives are found between 0.3 and 1.8 ppm. Of these methyls, only that attached to the propanolamine side chain would be expected to exhibit appreciable spin–spin structure; the 5-Hz doublet with an intensity equivalent to three protons at 1.07 ppm in methylcobalamin is assigned to this methyl group. In other derivatives, however, it is not possible to observe this expected doublet structure directly because of overlap with other peaks, as for example in isopropylcobinamide.

The assignments of the vinyl and aromatic methyls and the cobalt-bound alkyl methyls on B_{12} derivatives are reasonably straightforward. A tentative assignment of a number of the methyl groups of these seven derivatives is given in Table II. The assignments in columns three, four, and five are as discussed above. The other assignments draw heavily on the high degree of homology in the chemical shift patterns

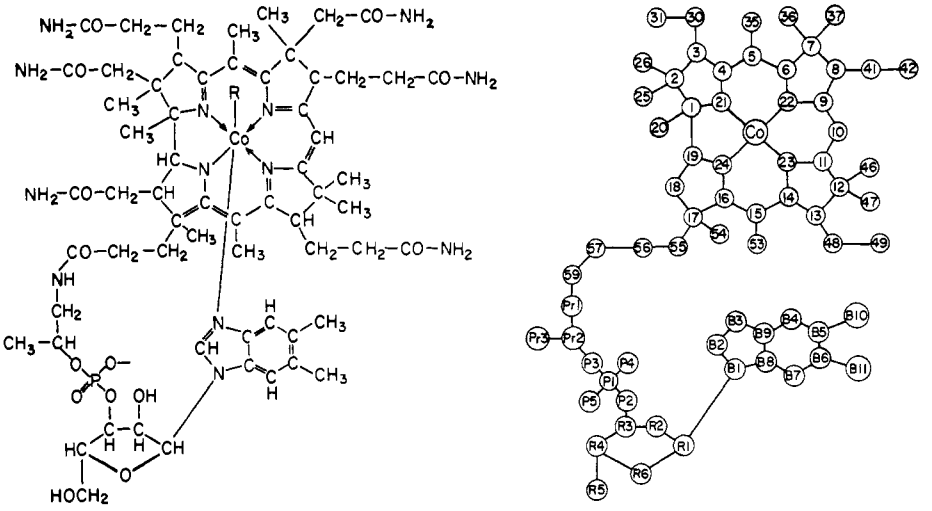

FIGURE 3: Corrinoid numbering system.

TABLE II: Tentative Assignment of Certain Methyl Groups on Corrinoid Ring, Propanolamine Side Chain, and Benzimidazole Ring of Certain Cobalamins and Cobinamides.[a] Unresolved Ambiguities Are Indicated by Commas.

Compound	Methyl Group at				
	C-20	C-47	C-5 and C-15	Prop	Benzimidazole (2)
Methylcobalamin	0.34	1.21	2.40, 2.46	1.07	3.58, 3.96
Methyl-10-chlorocobalamin	0.35	1.13	2.42, 2.49	1.08	3.57, 4.01
Cyanocobalamin	0.31	1.08	2.45	1.20	3.58, 3.92
Hydroxycobalamin	0.36	1.16	2.18	1.10	3.65, 3.90
Methylcobinamide	1.57		2.34, 3.28	1.02	
Methylcyanocobinamide	1.35,	1.40	2.20, 2.23	1.03	
n-Propylcobinamide	1.35,	1.60	2.37, 2.42	1.01	
Cyclohexylcobinamide	1.41,	1.65	1.05, 2.40	1.01	
Isopropylcobinamide	1.40,	1.65	2.42, 2.45	1.02	

[a] All chemical shifts in the table are in ppm downfield from $(CH_3)_4Si$.

of the cobinamides and cobalamins. The proton magnetic resonance spectra of the three cobalamins are strikingly similar, as are those of the four cobinamides. In these assignments, it will be presumed that the three-dimensional structure of the corrinoid ring as determined by X-ray diffraction [Lenhert (1968), Hodgkin (1965)] is retained virtually unchanged in the seven B_{12} derivatives. The numbering system used for the corrinoids is the same as outlined by IUPAC–IUB in 1966 and given in Figure 3. If the chemical shifts of the methyl groups in methylcobinamide are compared with those found in methylcobalamin, there are two significant differences: (a) the two benzimidazole methyl peaks are absent in the cobinamide spectra, and (b) the peak at 0.34 ppm and one-half of the peak at 1.21 ppm in methylcobalamin are replaced by a single peak at 1.57 ppm in methylcobinamide. The methyl group at carbon one (C-20) and one of the methyl groups at carbon twelve (C-47) project out of the corrin plane on the same side of the plane as the benzimidazole, and would be shifted to high field by the benzimidazole's ring-current field. The C-20 methyl probably would be shifted more strongly, and therefore the cobalamin's methyl resonance near 0.3 ppm is assigned to C-20. The rest of the assignments for C-20 and C-47 are made on the basis of the homology of the cobalamins and cobinamides. Methyls C-25, C-36, C-46, and C-54 are assigned to the remaining four resonances that exhibit chemical shifts between 0.74 and 1.74 ppm in the nine corrinoids listed in Table II. As noted before, the vinyl methyls at C-5 and C-15 display similar chemical shifts in all seven B_{12} derivatives, except for one of these methyls in cyclohexylcobinamide.

The proton magnetic resonance spectra of seven diamagnetic cobalamins and cobinamides dissolved in $(CD_3)_2SO$ (Figure 1) contain a large number of resonances between 5.8 and 8.8 ppm. In this region, the four cobinamides display resonances corresponding to 14 protons, hydroxycobalamin, to 18 protons, and cyanocobalamin and methylcobalamin, to 20 protons each. Figure 4 schematically presents the chemical shifts of these protons. None of these seven compounds exhibit any resonance absorption between 4.7 and 5.8 ppm. For those cobalamins in which nitrogen- and oxygen-bound protons are replaced with deuterons, the low-field region of the proton magnetic resonance spectrum consists of five peaks; in exchanged cobinamides there is only one peak. Thirteen of the low-field peaks may be attributed to nitrogen-bound protons on the six amide groups and the propanolamine nitrogen. These protons are presumably

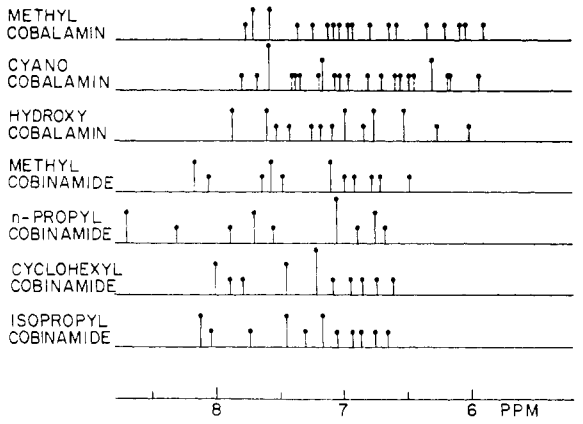

FIGURE 4: Schematic representation of the chemical shifts of low-field resonances of the seven diamagnetic corrinoids whose proton magnetic resonance spectra are presented in Figure 1. A line one unit high corresponds to a single proton. The dotted lines are the chemical shifts of the five protons observed when cyanocobalamin is examined in D_2O.

shifted to this low-field position by hydrogen bonding to $(CD_3)_2SO$ molecules. There are two hydroxyl protons on the ribofuranosyl ring of cobalamins which would have chemical shifts in the 5.8–8.8 ppm range when hydrogen bonded to $(CD_3)_2SO$ molecules. In cobinamides, however, there is only one hydroxyl group at the termination of the propanolamine side chain. Thus, for these seven B_{12} derivatives, it is possible to correlate the number of protons with chemical shifts between 5.8 and 8.8 ppm with the number of aromatic, vinyl, oxygen-bound, and nitrogen-bound protons in the molecule, except that one proton in the cobinamides and two protons in hydroxycobalamin do not exhibit chemical shifts in this range. The two missing resonances in hydroxycobalamin may possibly reflect exchange of the protons involved with water. It is clear that the pairs of protons on at least some of the amide nitrogens are not equivalent.

The dotted lines in Figure 4 for cyanocobalamin are the chemical shifts of the five single proton resonances between 5.8 and 8.8 ppm that are observed when cyanocobalamin is examined in D_2O. In the proton magnetic resonance spectrum of the low-field region of cyanocobalamin in D_2O (Figure 5), the two lowest field resonances may be ascribed to the aromatic protons at B-4 and B-7 on the benzimidazole, the next lowest field resonance to the benzimidazole B-2 proton, the slightly broadened resonance to the vinyl proton at C-10 on the corrin ring, and the final resonance to the hydrogen on the ribofuranosyl ring carbon R1 bonded directly to the benzimidazole [Hill *et al.* (1965, 1968, 1969a)]. In dichloromethylcobalamin, four of these five protons have identical chemical shifts within 0.05 ppm; the resonance corresponding to the vinyl C-10 proton is shifted upfield by 0.6 ppm.

There are between five and nine resolvable resonances in the 2.6- to 4.8-ppm region of the proton magnetic resonance spectra of the seven nonparamagnetic cobalamins and cobinamides presented in Figure 1. Several of these resonances exhibit structure arising from nuclear spin–spin coupling. From consideration of model compounds [Bovey (1967), Emsley *et al.* (1965)], each of the three cobalamins whose proton magnetic resonance spectra are portrayed in Figure 1 would be expected to possess 15 protons exhibiting chemical shifts between 2.6 and 4.8 ppm. There are four protons on the two methylene groups α to the peptide bond in the propanolamine side chain, and five carbon-bound protons at R2, R3, R4, and R5 (two protons) on the ribofuranosyl ring. These nine protons would be expected to exhibit chemical shifts near 3.0 ppm. Chemical shifts of the two benzimidazole methyl groups are near 3.6 and 4.0 ppm (see Table II). Resonances corresponding to 14 of the 15 protons are found in the 2.6- to 4.8-ppm region for the three cobalamins; the fifteenth proton is possibly under the broad water peak.

TABLE III: Chemical Shifts of Protons of Cobalt-Bound Water in Certain Alkylcobinamides.

Derivative	Chemical Shifts (ppm)	
Methylcobinamide	3.89	4.42
n-Propylcobinamide	4.39	4.59
Cyclohexylcobinamide	4.57	4.73
Isopropylcobinamide	4.60	4.69

Resonances corresponding to six protons should be present for the five cobinamides whose 2.6- to 4.8-ppm regions of resonance absorption are portrayed in Figure 6. The five ribofuranosyl protons and the six benzimidazole methyl protons are absent in cobinamides, but two additional protons with chemical shifts near 3.0 ppm will be present. One of these is the oxygen-bound or nitrogen-bound proton that is not shifted to low field through hydrogen bonding (see above). The other is the proton on the carbon with the lone hydroxyl group in cobinamides (Pr 2) that should possess a chemical shift near 3 ppm. In the proton magnetic resonance spectra of Figure 6 resonances are present that correspond to eight protons, except in methylcyanocobinamide; the two additional protons exhibit chemical shifts near 4.5 ppm. Six of the protons in this region exhibit similar chemical shifts in all five cobinamides if it is assumed that the peaks obscured by the H_2O peak possess the same chemical shifts as in the other cobinamides. We propose that the two extra proton resonances in the spectra of each of the four alkylcobinamides arise from a water molecule bound at the sixth coordination site of cobalt. This assignment is based not only on the fact that the resonance positions are reasonable for bound water, but on the following experiment: methylcyanocobinamide was prepared from methylcobinamide by the addition of KCN, a procedure known to result in cyanide addition at the sixth coordinate position (Brodie, 1969). The total proton magnetic resonance spectrum of methylcyanocobinamide is remarkably similar to the proton magnetic resonance spectrum of methylcobinamide, except that the two peaks at 3.89 and 4.42 ppm are missing in methylcyanocobinamide (see Figure 6 and Table II). Furthermore, when the $(CD_3)_2SO$ solution of *n*-propylcobinamide is diluted with an equal volume of D_2O, these two resonances are no longer present. Thus the assignment is reasonable; the chemical shifts of the two resonances ascribed to the protons of coordinated water in alkylcobinamides are given in Table III.

If the above assignment of resonances in the 4.0- to 4.7-ppm region of resonance absorption of the alkylcobinamides to coordinately bound water is correct, the somewhat surprising observation of intramolecular nonequivalence of water protons with a nuclear spin coupling of about 7 Hz must be explained. Restriction of rotation about the cobalt–

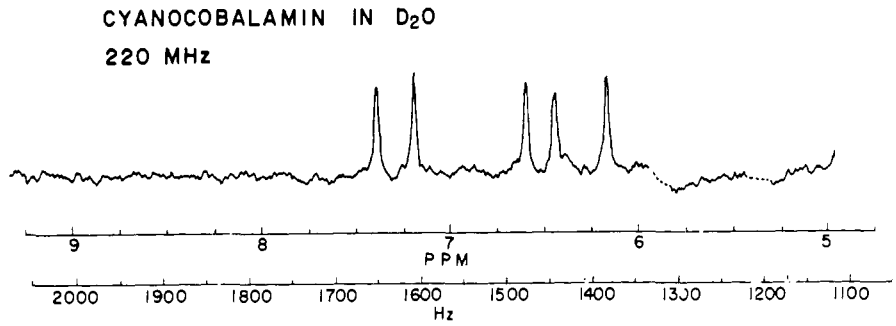

FIGURE 5: Low-field proton magnetic resonance spectrum of cyanocobalamin in D_2O. The dotted lines represent information derived from spectra obtained at different sample spinning rates.

oxygen bond, perhaps through strong intramolecular hydrogen bonds, involving one or both water protons, could account for the nonequivalence of the protons of the bound water. Another, intuitively more likely explanation is that rotation about the cobalt–oxygen bond is fast and not restricted, but that the shielding environments of the two water protons are not averaged to the same value because of different rotomer populations. The very high asymmetry about cobalt in the corrinoid ring qualitatively would appear sufficient to produce the requisite difference in rotomer populations to yield the observed nonequivalence of the water protons. A similar nonequivalence in certain CH protons of alkyl groups bound to cobalt in the alkylcobinamides has been observed and will be discussed in a later section.

The above appears to be the first determination of H–H nuclear spin–spin coupling in the water molecule. The value of 7 Hz for J_{HH} in H_2O should be compared with 12.4 Hz for J_{HH} in methane [Karplus *et al.* (1957)]. However, J_{HH} of geminal protons is a sensitive function of HCH angle in alkanes [Gutowsky *et al.* (1959)], and is likely to be a sensitive function of the HOH angle in water. Thus, J_{HH} for the bound water observed here could be substantially different from the J_{HH} of "free" water.

A comparison of the proton magnetic resonance spectrum of methyl-10-chlorocobalamin with that of methylcobalamin is presented in Figure 7. The ring-chlorinated methylcobalamin is somewhat less soluble in $(CD_3)_2SO$ than methylcobalamin, but clearly exhibits a similar proton magnetic resonance spectrum. The assigned methyl resonances of the two derivatives exhibit chemical shifts that vary by no more than 0.08 ppm (Table II). In fact, all the methyl resonances of methyl-10-chlorocobalamin and methylcobalamin exhibit chemical shifts that vary by no more than 0.1 ppm in the two derivatives. Particularly noteworthy is the fact that the position of resonance absorption for the cobalt-bound methyl group differs only 0.07 ppm in the two derivatives (Table I). The intermediate region of the spectrum from 2.6 to 4.8 ppm is almost identical for the two compounds while the low-field region is moderately different. In the chlorinated corrinoid, the peaks with shifts greater than 4.8 ppm are displaced as a group roughly 0.1 ppm to lower field, are rearranged slightly, and correspond in intensity to only 19 protons as compared with 20 for the unchlorinated corrinoid. This additional proton is presumably the vinyl proton at C-10 that is abstracted to form methyl-10-chlorocobalamin. On the other hand, chlorination of the alkyl group, *e.g.*, dichloromethylcobalamin, produces a proton magnetic resonance spectrum which differs significantly from methyl-

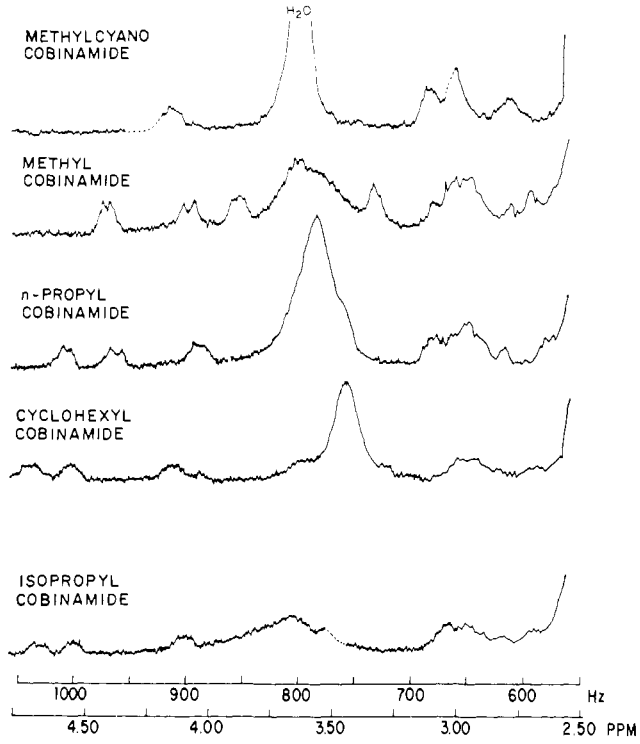

FIGURE 6: Central portion of the proton magnetic resonance spectra of five cobinamides in $(CD_3)_2SO$.

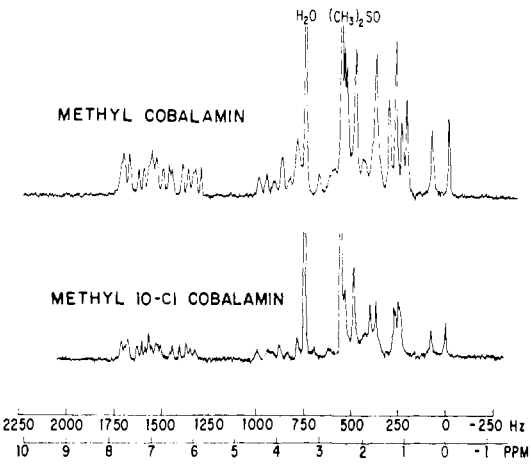

FIGURE 7: Proton magnetic resonance spectra of methylcobalamin and methyl-10-chlorocobalamin in $(CD_3)_2SO$.

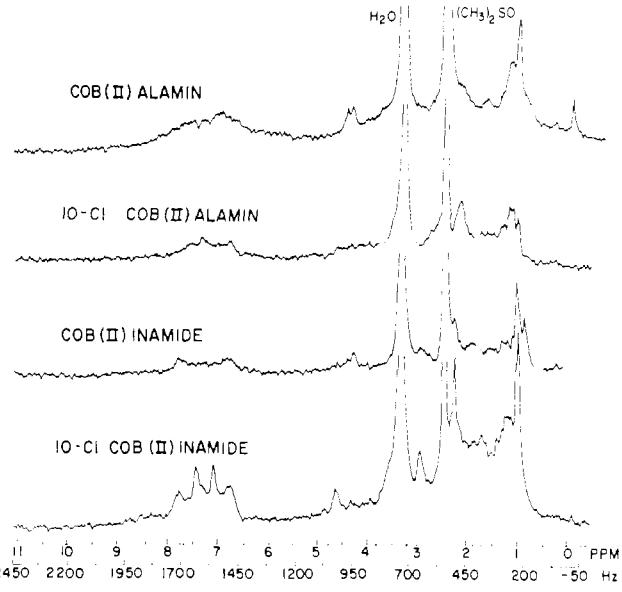

FIGURE 8: Proton magnetic resonance spectra of four paramagnetic corrinoids in $(CD_3)_2SO$.

cobalamin in that the C-10 proton is shifted to higher field by approximately 0.6 ppm.

The proton magnetic resonance spectra of the four paramagnetic derivatives of vitamin B_{12} that were studied are presented in Figure 8. The proton magnetic resonance spectra of the paramagnetic compounds possess a number of common features. Excepting those of the solvent, all resonances of these paramagnetic derivatives are considerably broader than in the corresponding diamagnetic B_{12} derivatives. No resonances other than those of Figure 8 were found for cob(II)inamide in the -30- to $+45$-ppm region of resonance absorption. Thus, we have been unable to identify any large contact-shifted resonances such as have been observed for heme [Yamane *et al.* (1970)] and iron–sulfur proteins [Poe *et al.* (1970)]. The absence of contact shifts and the electron–nucleus dipolar interaction responsible for the broadening of proton resonances in these paramagnetic cobalt complexes are consistent with a relatively long electronic relaxation time [Eaton and Phillips (1965)].[2] The sharp, structured electron spin resonance absorptions observed for the species even at room temperature [Schrauzer and Lee (1968) Bayston *et al.* (1970)] indicate that the electronic relaxation times, indeed, are sufficiently long to account for the observed proton magnetic resonance characteristics. Thus, if contact-shifted resonances are present in the paramagnetic B_{12} derivatives, they must be broadened to the point that detection is not possible under our experimental conditions. The lack of resolution in the proton magnetic resonance spectra of the paramagnetic compounds is most pronounced in the low field peaks. Cob(II)alamin and cob(II)inamide however are relatively well resolved in their methyl regions. The vinyl methyl groups at C-5 and C-15 in cob(II)inamide and the aromatic methyl groups on the benzimidazole in cob(II)alamin are displaced upfield and downfield, respectively, relative to their position in the diamagnetic cyanocobalamin, with the vinyl methyls experiencing a smaller shift than the aromatic methyls. The other methyls apparently all are at slightly higher field in the paramagnetic compounds.

The magnetic susceptibility of cob(I)alamin (B_{12s}) was measured by a nuclear magnetic resonance method [Dickinson (1951), Bartle *et al.* (1968)] using $(CH_3)_4NCl$ and $(CH_3CH_2)_4NCl$ as references; B_{12s} possesses no significant paramagnetism, *i.e.*, is diamagnetic. The proton magnetic resonance spectrum of B_{12s} is presented in Figure 9. The pattern of chemical shifts clearly indicates that B_{12s} is in the "base off" configuration. The nine corrinoid methyl groups do not show the ring-current shifts present in other cobalamins. The benzimidazole methyl groups are shifted about 1 ppm to high field relative to the cobalamins listed in Table II and exhibit chemical shifts near those of 5,6-dimethylbenzimidazole [Hill *et al.* (1965)]. The B-10 and B-11 benzimidazole protons are seen to be nearly equivalent at 7.2 ppm when the four lowest field resonances in B_{12s} are assigned as in cyanocobalamin (see Figure 5). We conclude from the equivalence of the B-10 and B-11 benzimidazole protons, the position of resonance absorption of the benzimidazole methyls, and the absence of ring current shifts on the corrin methyl groups that the benzimidazole base is not coordinated to cobalt in cob(I)alamin.

Discussion

The prior studies of the physical and chemical properties of vitamin B_{12} carried out by Hill *et al.* (1965, 1969a) have included observations on the interaction of the fifth and sixth cobalt ligands with the rest of the corrinoid, penta- *vs.* hexacoordinated cobalt, the interactions in the paramagnetic state, and the nature of the alkyl–cobalt bond. The proton magnetic resonance studies reported in the present work were specifically addressed to analysis of these properties.

The nature of the interaction of the alkyl cobalt ligand with the corrin in B_{12} derivatives [the cis effect] may be examined by noting the striking homology of the chemical shift patterns of the methyl groups on methylcobalamin and methyl-10-chlorocobalamin. The cobalt-bound methyl groups exhibit chemical shifts of -0.06 ppm in the methylcobalamin and $+0.01$ in methyl-10-chlorocobalamin which would indicate similar cobalt–carbon bonding situations in the two molecules. Thus, the dramatic shift in the optical spectrum of B_{12} derivatives upon chlorination at C-10 apparently does not affect markedly the environment of the alkyl ligand on the cobalt, at least insofar as this is reflected in chemical shifts of alkyl proton resonances. No clear evidence exists then from this particular result for a cis ligand effect, but it should be noted that displacement reactions on 10-halocobalamins [Wagner (1965), Tamao *et al.* (1968)] clearly indicate that changes at C-10 do indeed alter the charge residing on cobalt and, thus, the nature of the cobalt–alkyl bond. On the other hand, a large change in the proton magnetic resonance spectrum can be seen in the case of dichloromethylcobalamin where the C-10 proton is shifted 0.6 ppm upfield relative to the unchlorinated analog. This shift may be due to steric rather than electronic factors, since the dichloromethyl group would tend to distort the corrinoid system. Changes in conformation of the side chains, which might mediate a cis effect, could not be studied in this experiment, since technical considerations necessitated the use of D_2O as solvent.

The interaction of the sixth cobalt ligand, which is a benz-

[2] It has been pointed out (H. A. O. Hill, private communication) that the absence of large contact shifts is also reasonable if cobalt(II) cobalamin is low-spin d^7 with the single unpaired electron well described by d_{z^2}, in view of the small anisotropy of the g tensor (added in proof).

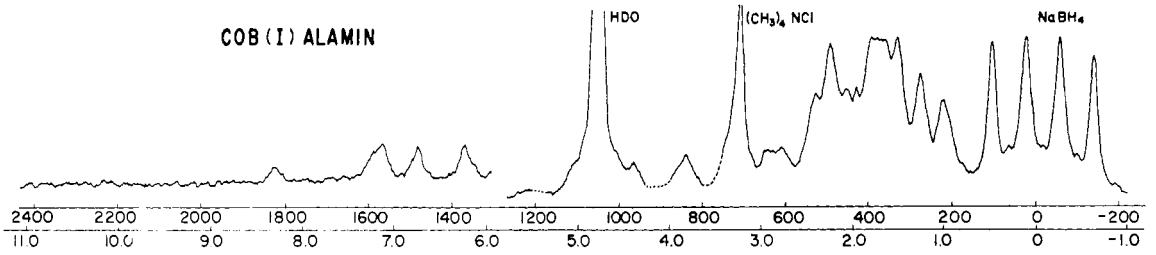

FIGURE 9: Proton magnetic resonance spectra of cob(I)alamin [B_{12s}] in D_2O. The four highest field peaks correspond to unreacted borohydride ion. The two strong peaks in the middle of the spectrum correspond to $(CH_3)_4NCl$ and the protons of HDO. The right-hand portion of the proton magnetic resonance spectrum was accumulated on a computer of average transients for 15 passes; the low-field, left-hand portion for 25 passes.

imidazole nitrogen in cobalamins, with the rest of the corrinoid may also be investigated through the proton magnetic resonance studies reported here. When the chemical shifts of the nonaromatic methyl protons of methylcobalamin are compared with those of methylcobinamide, we see that the chemical shifts of only two of the methyl groups are appreciably different in the two compounds. Both may be assigned to methyl groups displaced to high field by the ring current of the benzimidazole present only in cobalamins. Thus, the conformations of the corrinoid rings in methylcobinamide and cobalamin appear to be very similar. However, the cobalt-bound methyl is more strongly shielded by 0.3 ppm in methylcobinamide than in methylcobalamin. The low-field resonances of methylcobinamide are to lower field than those of methylcobalamin. This may be ascribable, in part at least, to the ring-current field of the benzimidazole, but there is probably also a contribution by stronger hydrogen bonding for certain amides, particularly those that project toward the volume the benzimidazole occupies in cobalamins. Certainly the increased shielding of the methyl protons in methylcobinamide is *not* due to increased electron donation by cobalt to the carbon as compared with methylcobalamin. The downfield shift of the cobalt methyl in methylcobalamin relative to methylcobinamide is probably due to the ring-current field of the benzimidazole. It appears from the above that changing the trans ligand of a cobalt which bonds a methyl group as its fifth ligand does not affect the protons of the corrinoid ring appreciably, but does affect the side chains projecting from the corrin. If the trans effects noted in the comparison of methylcobalamin and methylcobinamide are generally true for all cobalamins and cobinamides, then the conformational changes associated with trans ligand substitution are primarily confined to the fringes of the molecules and are small. The significant part of the trans ligand effects, which lead to enormous reactivity differences in corrinoids [Hayward *et al.* (1965)], would be the change in the electronic character of the bond between the cobalt and any single axial ligand that is reflected in stretching frequencies, bond length, and proton magnetic resonance shielding effects [Hill *et al.* (1969b)].

It has been suggested by Firth *et al.* (1968a) that alkylcobinamides may have partial pentavalent cobalt character, with increasing temperature and increasing electron donation by alkyl ligands favoring a higher proportion of pentavalent character. The pentavalent state is thought to be diamagnetic because of the apparent absence of contact shifts and of broadened resonances (Firth *et al.*, 1968a). While there is no reason to doubt this conclusion, expected contact shifted resonances frequently are not observed because of unfavorable nuclear and electronic relaxation times. There are a number of properties which correlate well with the degree of pentavalent character in cobinamides [Hill *et al.* (1969a); Firth *et al.* (1968a)]. As already discussed, it appears from the proton magnetic resonance spectra of cobinamides in $(CD_3)_2SO$) that a water molecule is tightly bound to these molecules. The protons of this water molecule exchange with those of bulk H_2O (in $(CD_3)_2SO$) at a rate slower than about 10^2 sec^{-1}, since separate proton magnetic resonance signals for the two types of water are observed. It is difficult to see how such a water molecule could be tightly bound to a cobinamide except as the sixth cobalt ligand. Model building excludes the possibility that the water protons could be protons of pendant amides.[3]

Chemical shifts of CH protons of alkyl groups on alkylcobalt corrinoids relative to internal $(CH_3)_4Si$ are listed in column 3 of Table I. These shifts are adjusted in column 4 to reflect the shift in these resonances that could be attributed to replacement in the appropriate alkane of a terminal methyl group by cobalt upon incorporation into the alkylcobalamin or cobinamide. The α-CH_2 protons of the *n*-propylcobinamide and the CH proton of isopropylcobinamide exhibit similar "adjusted" high-field shifts of 1.9 ppm, while the CH_3 protons of methylcobinamide are shifted by only 1.3 ppm. The positions of the CH_3 resonances in methylcobinamide and methylcyanocobinamide are appreciably different, but because of the interplay of poorly understood factors in producing such shifts, any attempt at interpretation in terms of trans-ligand effects would seem premature.

A notable feature of the proton magnetic resonance spectra of the alkylcobinamides are the nonequivalences of the two α-CH_2 and β-CH_2 protons of the *n*-propyl derivative and of the two CH_3 groups of the isopropyl derivative (see Figure 10). A similar nonequivalence has been noted for the cobalt-bound CH_2 protons in deoxyadenosylcobalamin (J. D. Brodie, M. Poe, and W. D. Phillips, in preparation). These nonequivalences are reminiscent of those discussed earlier for the bound water of the cobinamides.

The nonequivalences of the α-CH_2 protons of the *n*-propyl group of *n*-propylcobinamide and of the two CH_3 groups of the isopropyl group of isopropylcobinamide are attributed to incomplete environmental averaging despite fast rotation about the cobalt–carbon bond, a situation apparently anal-

[3] The suggested equilibrium between pentavalent and hexavalent cobalt in alkylcobinamides has been found to be solvent dependent [S. A. Cockle, Part II, Dissertation, Oxford (1968); S. A. Cockle, H. A. O. Hill, J. M. Pratt, and R. J. P. Williams, unpublished]; alkylcobinamides in dimethyl sulfoxide are fully hexavalent. Thus the suggested identification of water as the sixth cobalt ligand in alkylcobinamides in $(CD_3)_2SO$ does not illuminate the nature of the proposed pentavalent state (added in proof).

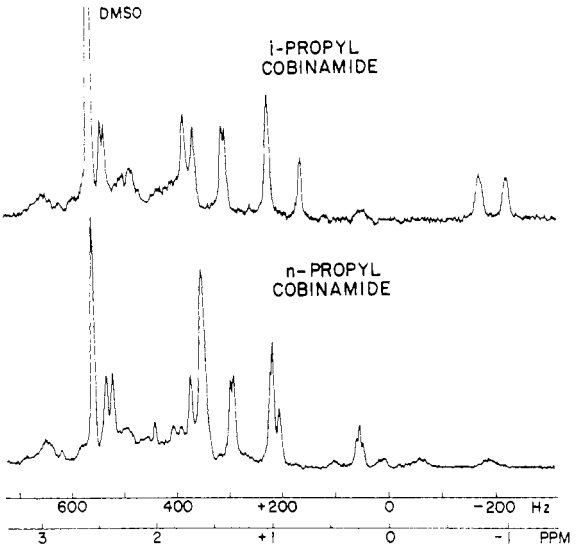

FIGURE 10: High-field portion of the proton magnetic resonance spectrum of *n*-propyl- and isopropylcobinamide.

ogous to the previously discussed, cobalt-bound water. If rotation about the cobalt–carbon bond were highly restricted, which would lead to a fixed rotomer configuration for the alkyl group, then the same qualitative proton magnetic resonance manifestation of nonequivalence would occur. However, molecular models indicate that rotation about the cobalt–carbon bond should be essentially unrestricted, at least for the simple alkylcobinamides. On the other hand, deoxyadenosylcobalamin could well have restricted rotation about the cobalt–carbon bond.

The observed nonequivalence in proton magnetic resonance of the β-CH_2 protons of the *n*-propyl group in *n*-propylcobinamide is of some additional interest. Given fast rotation about the Cα–Cβ bond, such nonequivalence is unexpected. However, models appear to indicate that 360° rotation about the Cα–Cβ bond in this cobinamide is not possible because of contact between the terminal methyl group and the corrinoid ring. Such restriction of rotation should be sufficient to produce the large nonequivalences observed between the β-CH_2 protons.

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