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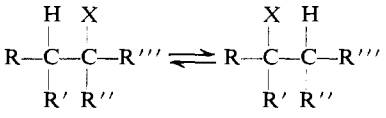
Proton Magnetic Resonance of Vitamin B_{12} Derivatives. Functioning of B_{12} Coenzymes†

Jonathan D. Brodie* and Martin Poe

ABSTRACT: Proton magnetic resonance studies of 5,6-dimethylbenzimidazole B_{12} coenzyme (CoB_{12}) or 5′-deoxyadenosylcobalamin in water at different pH values and temperatures and in dimethyl sulfoxide at different temperatures are presented. The 5,6-dimethylbenzimidazole base is shown to be longer coordinated to cobalt at acid pH in water or in dimethyl sulfoxide at 88°. This result is confirmed by optical measurements. CoB_{12} is about 15–20% "base off" in water at 85° and neutral pH. The pK at 23° for the protonation of the base on CoB_{12} is 3.28 ± 0.04. The rate constant for base dissociation is greater than 550 sec^{-1}. The prochiral protons on the cobalt-bound carbon in CoB_{12} are found to be nonequivalent for both "base-on" and "base-off" configurations. This nonequivalence is attributed to incomplete averaging of proton environments through rotation about the carbon–cobalt bond. Upon loss of 5,6-dimethylbenzimidazole coordination, CoB_{12} is suggested to exhibit a significant change in the average orientation of its 5′-deoxyadenosyl moiety. The loss of base coordination appears to have little effect on the cobalt–carbon R′5 (on 5′-deoxyadenosyl) bond. A brief discussion of the implications of these studies for the functioning of CoB_{12} in enzymatic reactions is presented.

The enzymatically active derivatives of vitamin B_{12} known as B_{12} coenzymes were initially isolated by Barker *et al.* (1958), and have been demonstrated to catalyze a number of unusual enzymatic transformations. The geometrical structure and chemical properties of the coenzymes are of particular interest to biochemists because the coenzyme serves as the prosthetic group in several enzyme-dependent reactions of the following type



X-Ray diffraction studies (Lenhert, 1968) have demonstrated three-dimensional molecular structure of 5,6-dimethylbenzimidazole B_{12} coenzyme (CoB_{12}).[1]

Several previous investigators (Brodie, 1969; Babior, 1970) have emphasized the importance of geometrical accommodations in the coenzyme that must accompany B_{12}-catalyzed enzymic reactions and a recent report (Law *et al.*, 1971) has pointed out that even in aqueous solution, the average orientation of the deoxyadenosyl moiety with respect to the macrocycle must vary appreciably from the crystalline state. We now report proton magnetic studies on CoB_{12} under conditions of varying pH and temperature that provide insight into those structural and electronic properties of B_{12} coenzymes in solution that might be relevant to enzymatic catalysis. These and earlier studies (Brodie and Poe, 1971; Cockle *et al.*, 1970; Hill *et al.*, 1965, 1968, 1969; Law *et al.*, 1971; Doddrell and Allerhand, 1971) also permit a detailed assessment of the relationship of geometrical and magnetic states in corrinoids.

Materials and Methods

CoB_{12} (kindly donated by Dr. L. Mervin, Glaxo, Ltd., England) was purified by chromatography on CM-cellulose in the dark. Methylcobalamin was prepared and purified as described previously (Brodie and Poe, 1971). Proton magnetic resonance spectra were run on either Varian 220 MHz or Varian HA-100 spectrometer, and referenced internally to $(CH_3)_4Si$ or to the methyl resonance of the sodium salt of 2,2-dimethyl-2-silapentanesulfonic acid. Chemical shifts were measured in hertz or parts per million, with downfield shifts assigned positive values. The temperature of the sample zone of the 220-MHz spectrometer was determined from the resonance frequencies of the hydroxyl group of ethylene glycol to an estimated accuracy of ±0.5°. The HA-100 was "locked" on either external $(CH_3)_4Si$ or benzene (C_6H_6). The signal-to-noise characteristics of certain spectra were improved using a Varian C-1024 computer of average transients.

For proton magnetic resonance studies about 30 mg of CoB_{12} was dissolved in 1 ml of either deuterated dimethyl sulfoxide, $(CD_3)_2SO$, or D_2O. About 50 mg of methylcobalamin was dissolved in 2 ml of D_2O. All handling operations were carried out in the dark; proton magnetic resonance tubes containing B_{12} solutions were kept wrapped in aluminum foil. pH measurements were made at 23° in the nuclear mag-

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: CoB_{12}, 5,6-dimethylbenzimidazole B_{12} coenzyme; pmr, proton magnetic resonance; pH, uncorrected glass-electrode pH meter readings in D_2O.

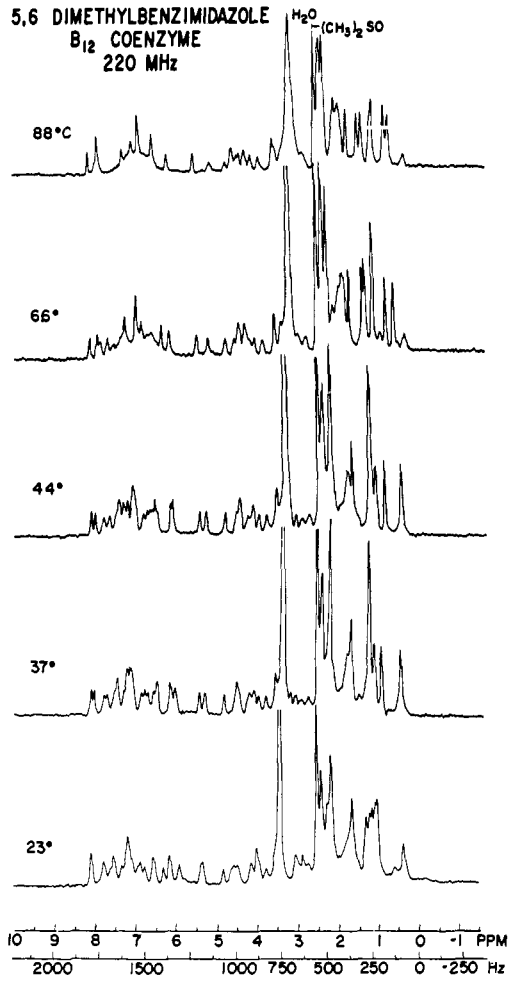

FIGURE 1: Temperature dependence of the pmr spectrum of CoB_{12} in $(CD_3)_2SO$.

netic resonance (nmr) tube with a Model 26c Radiometer pH meter (Radiometer A/S, Copenhagen, Denmark) equipped with a microelectrode immediately before and after recording spectra; unless the pH values agreed within 0.04, the spectra were disregarded. Either 0.1 M DCl or 0.1 M NaOD was used to adjust the pH of a sample. The pH values reported are the uncorrected glass-electrode meter readings for the D_2O solutions. When pH measurements are made in this way, pK values determined in D_2O are identical with pK values obtained in H_2O (Roberts *et al.*, 1969).

Results

The temperature dependence of the proton magnetic resonance spectrum at 220 MHz of 5,6-dimethylbenzimidazole B_{12} coenzyme in $(CD_3)_2SO$ is shown in Figure 1. CoB_{12} exhibits complex but moderately well-resolved spectra. The two intense resonances in the 23° spectrum at 2.55 and 3.45 ppm and corresponding resonances in the higher temperature spectra represent residual protons on solvent molecules, and a small amount of H_2O, respectively. The remaining resonances correspond to the 100 protons constitutively associated with CoB_{12}. For detailed calculations of resonance positions and intensities, 500-Hz wide spectral scans were taken at each temperature. An example of such a spectral scan is depicted in Figure 2, which represents the high-field region of resonance absorption of CoB_{12} at +66°.

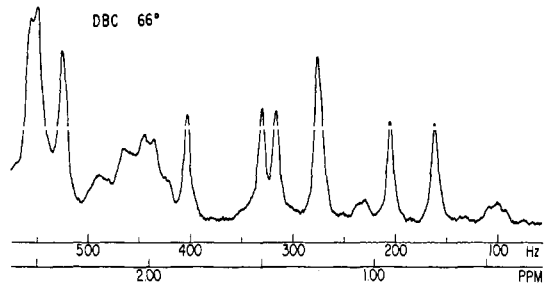

FIGURE 2: High-field portion of the pmr spectrum of CoB_{12} at +66° in $(CD_3)_2SO$.

The resonances of CoB_{12} that have chemical shifts between 0 and 2 ppm correspond primarily to nonaromatic methyl groups on the corrin. The peaks with chemical shifts between 5 and 8.5 ppm are predominantly single proton resonances. In the 23° spectrum of CoB_{12}, there are 37 resolved resonances exclusive of solvent and impurity peaks corresponding to 82 protons. All the protons on CoB_{12} are accounted for if the broad resonances between 1.5 and 2.5 ppm are taken to represent 18 protons, which is a value consistent with the area under the peak.

Proton magnetic resonance spectra of CoB_{12} dissolved in D_2O at 23 and 85° are shown in Figure 3. CoB_{12} in D_2O at 85° exhibits 31 resonances corresponding to 81 protons, and the region of resonance absorption to low field of the water resonance is particularly simple. In our assignments, the number system used will be that proposed by IUPAC–IUB (1967) (see Figure 4), and it will be assumed that the three dimensional molecular structure of CoB_{12} in solution at room temperature near pH 7 closely resembles its structure in the cyrstalline state (Lenhert, 1968).

Seventeen resonances, with intensities corresponding to 27 protons, exhibit chemical shifts greater than 4.85 ppm in CoB_{12} in $(CD_3)_2SO$ at 23° (see Figure 1). There are seven resonances corresponding to eight protons in the same spectral region for CoB_{12} in D_2O at 85°. These resonances have been fully assigned (Cockle *et al.* (1970) as modified by Law *et al.*

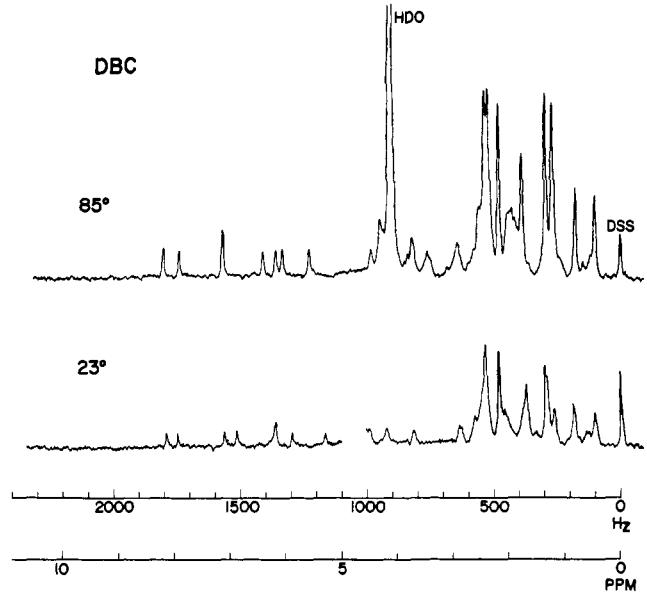

FIGURE 3: Temperature dependence of the pmr spectrum in D_2O.

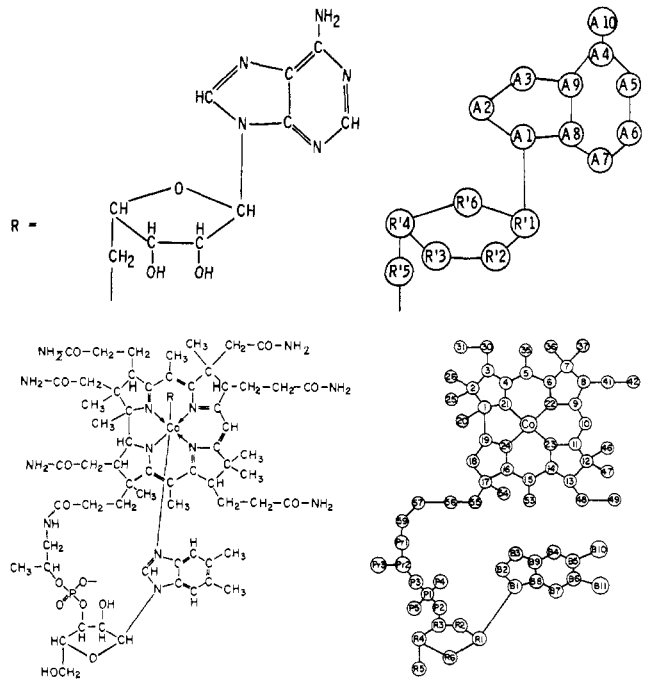

FIGURE 4: Corrinoid numbering system.

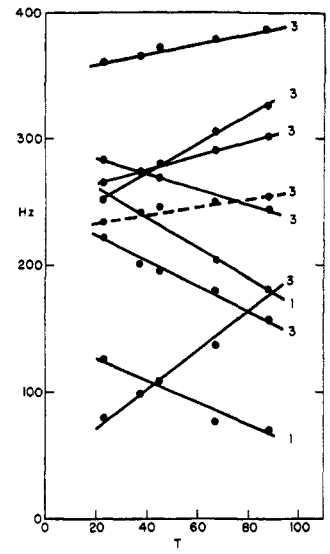

FIGURE 5: Temperature dependence of the chemical shifts of certain high-field resonances in CoB_{12} dissolved in $(CD_3)_2SO$. Numbers of protons corresponding to each resonance are given in the figure. The dotted line indicates a resonance showing spin–spin splitting.

(1971) and their chemical shifts at 23 and 85° are given in Table I. The 19 exchangeable protons doubtless represent the 19 nitrogen- and oxygen-bound protons on CoB_{12}, of which twelve protons are bonded to the six amide nitrogens, one to the peptide nitrogen, and two to the amino nitrogens. There are two hydroxyl protons, one on each of the deoxyribofuranosyl and ribofuranosyl rings of the deoxyadenosyl and benzimidazole moieties. These 19 protons are presumably shifted to low field through hydrogen bonding to $(CD_3)_2SO$ (Emsley *et al.*, 1965). Thus, it is possible to fully account for the resonances that exhibit chemical shifts greater than 4.85 ppm in CoB_{12} in $(CD_3)_2SO$ at 23°.

In the 88° spectrum of CoB_{12} in $(CD_3)_2SO$ there are ten narrow resonances and one broad resonance that exhibit chemical shifts greater than 4.75 ppm; the sharp resonances exhibit intensities corresponding to 14 protons. The resonances at 4.8, 5.15, 5.6, and 6.2 ppm are of unit intensity and exhibit spin–spin splittings. The 4.8-, 5.15-, and 5.6-ppm resonances are doublets with J near 5 Hz and the 6.2-ppm resonance is a poorly resolved multiplet that may be a triplet, with J of about 3 Hz. The resonances at 4.8 and 5.15 ppm decrease their chemical shifts with increasing temperature, while the resonances at 5.6 and 6.2 ppm increase the chemical shifts with increasing temperature. We tentatively suggest these four resonances are the four hydroxyl protons on the deoxyribofuranosyl and ribofuranosyl rings on DBC. The multiplet at 6.2 ppm would be the hydroxyl proton on carbon R-5, and the doublet at 5.6 ppm the hydroxyl proton on carbon R-2 on the ribofuranosyl ring. The doublets at 4.8 and 5.15 ppm would then be the two hydroxyl protons at carbons R′2 and R′3 on the deoxyribofuranosyl moiety of the deoxyadenosine. The other six sharp resonances in the low-field region of the 88° spectrum of CoB_{12} can be plausibly assigned to the eight carbon-bound protons and two nitrogen-bound protons expected to exhibit chemical shifts in this range. The broad resonance centered at 6.9 ppm in the 88° spectrum presumably corresponds to the thirteen other nitrogen-bound protons. The breadth of this resonance probably reflects exchange of these protons with the small amount of H_2O present. Consistent with this proposal is the observed broadening of the H_2O resonances.

TABLE 1: Chemical Shift of Certain CoB_{12} Protons, in Parts per Million Downfield from 2,2-Dimethyl-2-silapentane-5-sulfonate.

Proton	Chemical Shifts in D_2O	
	85°	23°
A2	8.28	8.38
A6	7.99	8.18
B7	7.22	7.34
B4	6.52	6.40
B2	7.22	7.11
R′1	5.67	5.55
R1	6.27	6.40
C10	6.15	6.10

The position of resonance absorption for the sharp, high-field three-proton resonances of CoB_{12} in D_2O and $(CD_3)_2SO$ are temperature dependent. The temperature dependence of resonance position in $(CD_3)_2SO$ of the seven methyl resonances with chemical shifts between 0 and 1.8 ppm are shown in Figure 5, as determined from the data presented in Figure 1. Also shown in this figure are the resonance positions of two single proton resonances; these protons are doubtless the two methylene protons at carbon R′5 on the deoxyribofuranosyl ring which is covalently linked to the cobalt atom (Cockle *et al.*, 1970; Law *et al.*, 1971). The single-proton resonances are best seen in the high field portion of 66° spectrum of CoB_{12} presented in Figure 2. These resonances at 0.34 and 0.93 ppm in Figure 2 are two and three times broader than the nearby methyl resonances; this breadth appears to be due to poorly resolved spin–spin coupling. This structure does not reflect an ABX pattern, since $(\nu_A - \nu_B)/J_{AB}$ is approximately 128 Hz/3 Hz or 43, but rather unresolved spin–spin

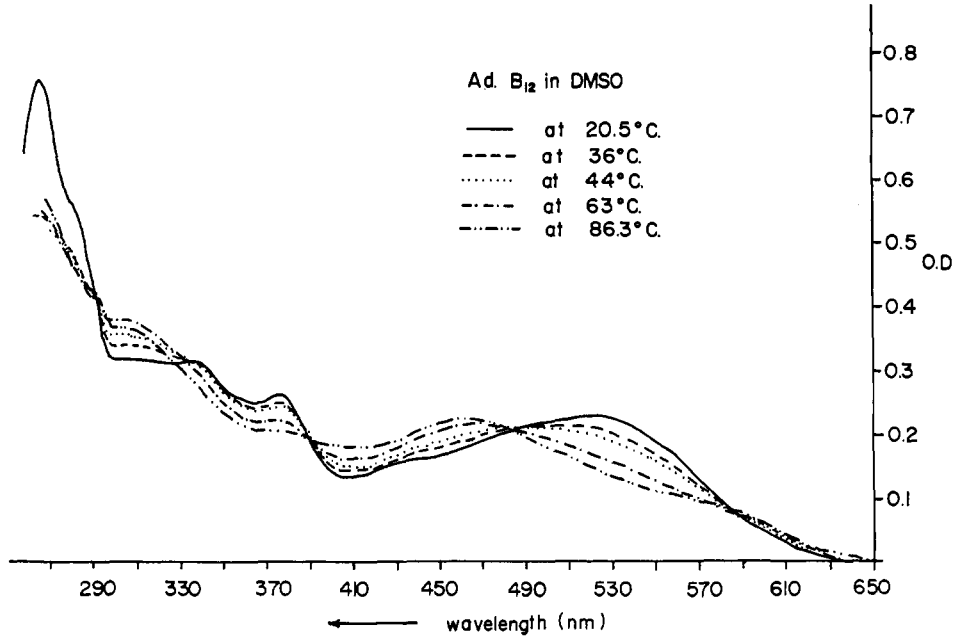

FIGURE 6: Temperature dependence of the optical absorbance of 5,6-dimethylbenzimidazole B_{12} coenzyme.

splittings between the vicinal protons. The resonance position of these two single-proton resonances shifts to higher field, *i.e.*, closer to the $(CH_3)_4Si$ resonance, with increasing temperature, as do two of the seven methyl proton resonances. The other five methyl proton resonances exhibit resonance positions that shift to lower field with increasing temperature. The data points connected by the dotted line in Figure 5 refer a doublet methyl peak with J of about 6 Hz; this is the methyl group on the propanolamine side chain that connects the corrin ring with the benzimidazole. The chemical shift of the methyl protons in the highest field resonance in DBC in $(CD_3)_2SO$ at 23° is 0.36 ppm. In cyano-, and hydroxycobalamin the pattern of chemical shifts for the high field methyls resembles the pattern for CoB_{12} at 23° (Brodie and Poe, 1971). In cyano- and hydroxycobalamin, the highest field methyl resonance, with a chemical shift of 0.31 and 0.34 ppm, respectively, was assigned to the protons of methyl C-20.

In the region of proton magnetic resonance absorption between 1.75 and 4.75 ppm, resonances are difficult to study because of extensive overlap, frequent multiplet structure leading to breadth of resonances, and interference by the large solvent peaks. The vinyl protons at C-5 and C-15 on the corrin ring in CoB_{12} in D_2O and $(CD_3)_2SO$ probably contribute to the intense resonances between 2.2 and 2.4 ppm. The resonances do not have a large temperature dependence in their resonance position. In other cobalamins, the two methyl groups on the benzimidazole exhibited nonequivalent chemical shifts in the 3.5- to 4.0-ppm region of resonance absorption (Brodie and Poe, 1971). There are no three-proton resonances in this region for DBC in either D_2O or $(CD_3)_2SO$. The resonances with chemical shifts between 2.55 and 4.75 ppm had intensities corresponding to fifteen protons at all temperatures, provided it is assumed that the resonances masked by the intense H_2O resonances had the same intensity as when not masked at other temperatures. Exclusive of the six benzimidazole methyl protons, thirteen protons would be expected to exhibit chemical shifts in the range of 2.55–4.75 ppm. The eight carbon-bound protons at R-2, R-3, R-4, R-5 (2 protons), R'2, R'3, and R'4 on the furanosyl rings would be expected to have chemical shifts in this range, as would the four methylene protons on carbons 56 and Pr 1 which are α to the peptide bond, and the proton at carbon Pr 2. The extra two protons could be two methylene protons on the corrin ring which have been shifted downfield by the ring current of the adenine. A similar analysis holds for CoB_{12} in D_2O. Thus, it appears that the six methyl protons on the benzimidazole do not exhibit chemical shifts in the range 2.55–4.75 ppm. These protons most likely contribute to the intensity of the resonances near 2.4 ppm in CoB_{12} in both $(CD_3)_2SO$ and D_2O. The methyl protons on 5,6-dimethylbenzimidazole exhibit chemical shifts of 2.35 ppm (Hill *et al.*, 1965).

In order to determine the configuration of CoB_{12} at high temperature in dimethyl sulfoxide, the temperature-dependent spectra between 325 and 650 nm of a CoB_{12} solution were obtained. The results of these experiments are displayed in Figure 6. At ambient temperature, the native spectrum of CoB_{12} is obtained. At high temperature, the visible absorption maxima shift to shorter wavelength; this shift to shorter wavelength is also obtained upon protonation of the benzimidazole base (Ladd *et al.*, 1961). The shift in visible absorbance at high temperature toward a "base-off" configuration is also seen when H_2O is used as solvent (Fox *et al.*, 1968).

The protonated, or "base-off" form of CoB_{12} in which the dimethylbenzimidazole base is no longer coordinated to cobalt was studied in D_2O at 100 MHz and 32°. Portrayed in Figure 7 are the high- and low-field spectra of CoB_{12} at pH 2.00 (top), 3.60 (middle), and 4.95 (bottom). The dependence of chemical shift upon pH for the seven low-field resonances and the seven high-field resonances are presented graphically on the left-hand and right-hand sides of Figure 8, respectively. Chemical shifts were calculated from the pmr spectra shown in Figure 7, and from similar pmr data. The assignments noted for the low-field resonances are as in Table I. The dotted line corresponds to the propanolamine methyl; its resonance is a doublet with $J \cong 6$ Hz.

As may be seen in the right-hand side of Figure 7, the six proton resonance at 225 Hz of CoB_{12} at neutral pH, probably

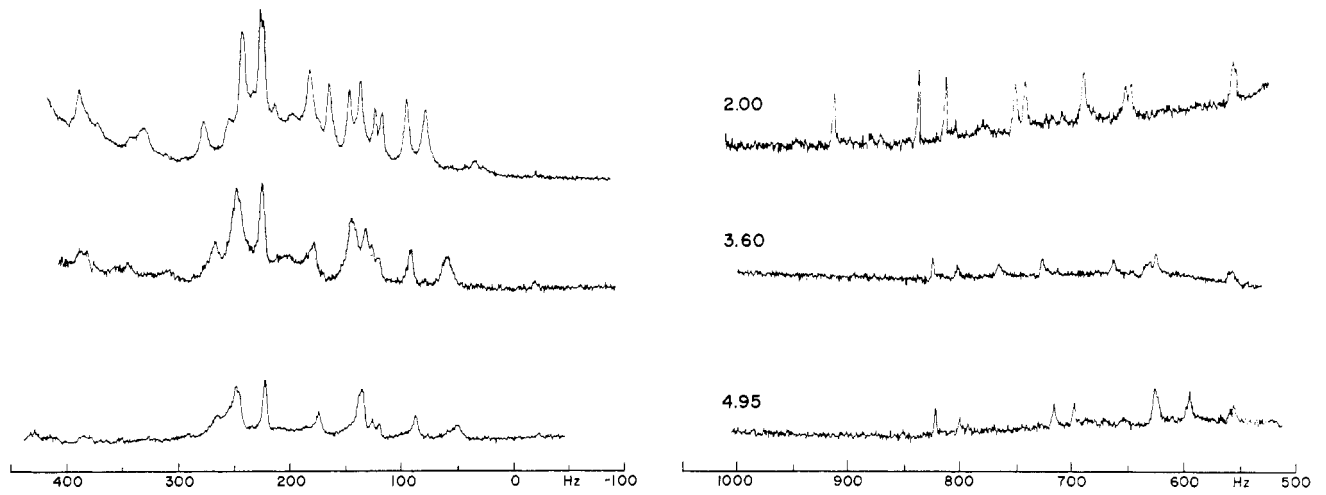

FIGURE 7: Low-field and high-field region of resonance absorption for three CoB_{12} solutions at 100 MHz in D_2O at 32°. The pH of the CoB_{12} solutions is indicated at upper left; vertical scales on the six spectra are not directly intercomparable.

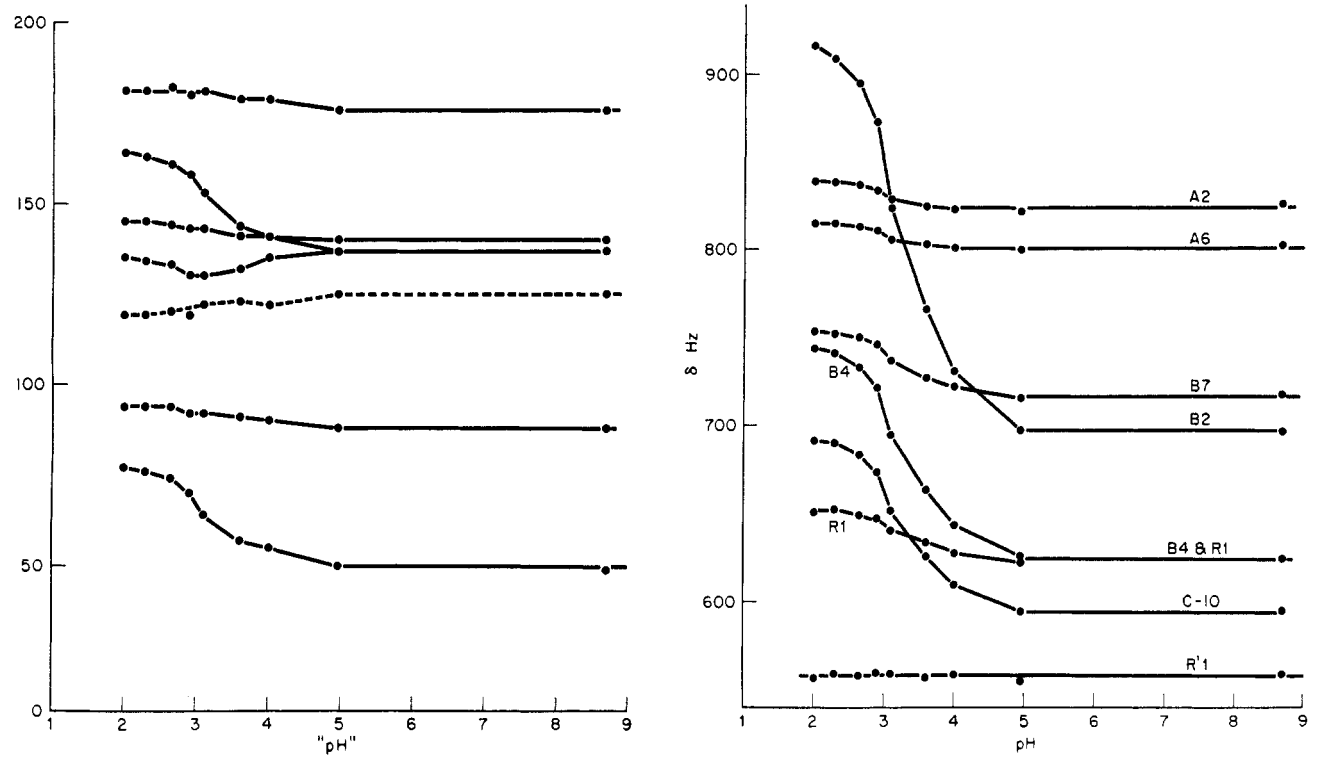

FIGURE 8: Dependence of chemical shift upon pH for certain resonances of CoB_{12} at 100 MHz and 32° in D_2O. Chemical shifts from spectra of Figure 7 and similar data. The left-hand portion presents data on assigned low-field resonances; the right-hand portion on partially assigned high-field resonances.

corresponding to the C-5 and C-15 methyl protons, becomes slightly resolved at pH 2.00. Further, the broad slightly resolved three-proton resonances at 247 and 250 Hz for CoB_{12} at pH 4.95, which probably correspond to the two methyls on the dimethylbenzimidazole, are a single resonance at 241 Hz at pH 2.00. The two methylene protons of carbon R'5 are difficult to resolve in most of the spectra obtained. The proton at higher field exhibits a resonance with a chemical shift of about 60 Hz at pH 4.95. At pH 2.00 this resonance is somewhat better resolved; it is a poorly resolved triplet centered at 32 Hz with $J \cong 8$ Hz with an intensity corresponding to 1.2 ± 0.4 protons when standardized against the highest-field methyl resonance.

If the base-on–base-off equilibrium can be described by a single proton ionization reaction of the type $HA^+ \rightleftharpoons H^+ + A$ then a pK value can be calculated from the dependence of chemical shift upon pH. If a proton on A exhibits a chemical shift δ_a and on HA^+ exhibits a chemical shift δ_b then a fast-exchange situation (Emsley *et al.*, 1965) with a chemical shift scale chosen so that δ_a equals zero defines a chemical shift δ of that proton as: $\delta = \delta_b([H^+]/[H^+] + K)$. Thus the plot of $1/\delta$ *vs.* $1/[H^+]$ determines K and δ_b. When this analysis

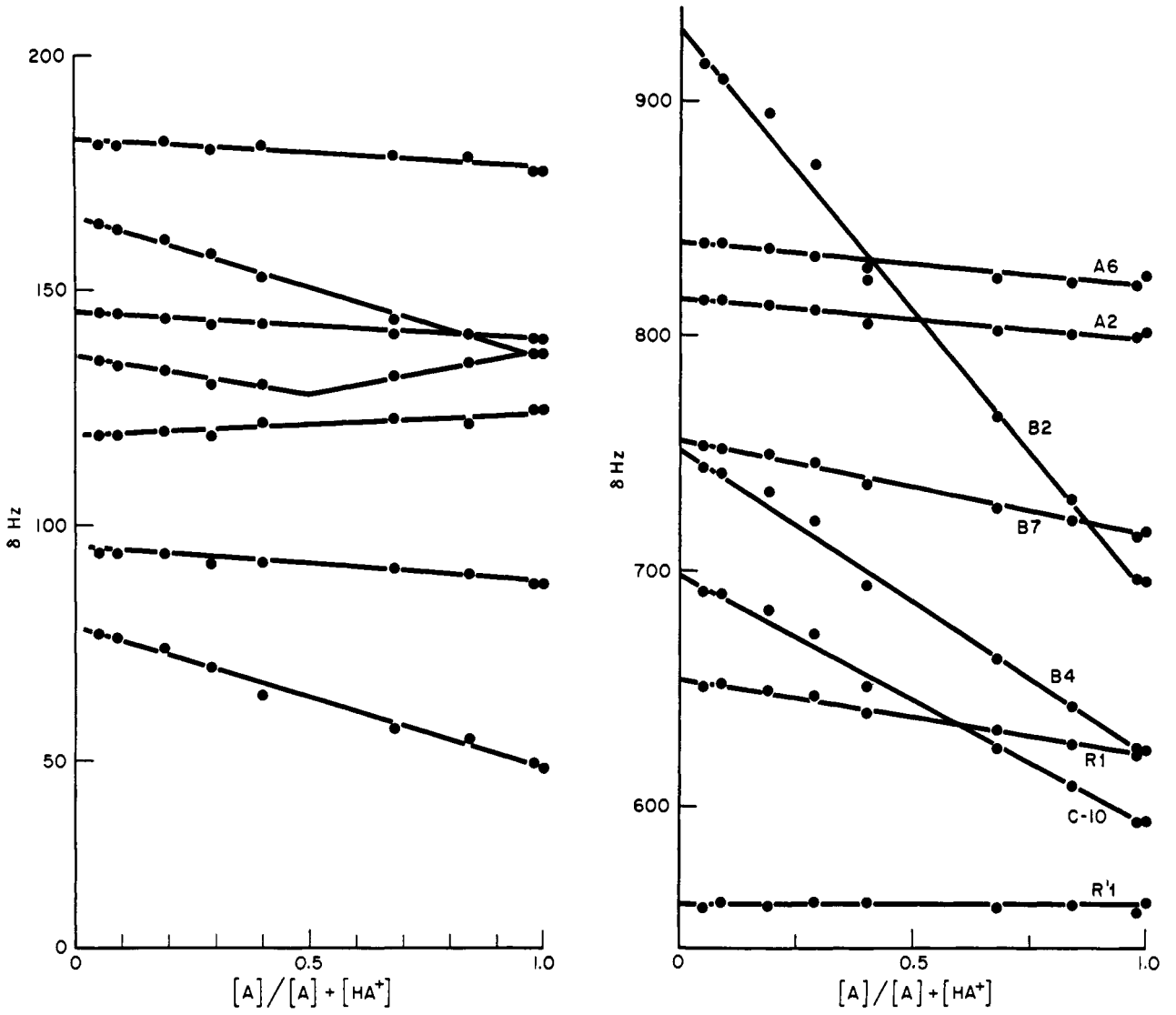

FIGURE 9: Dependence of chemical shift upon proportion of unprotonated form of CoB_{12} for certain resonances of CoB_{12}. The left-hand portion portrays low-field resonances; the right-hand portion high-field methyl resonances.

was performed for resonance B2, a straight line was obtained and a pK of 3.28 ± 0.04 was determined. Similar plots of the data for C10 and B4 gave an identical pK. The dependence of chemical shift upon pH for resonances B2, B4, and C10 is in Table II. It has earlier been noted that pK values determined in D_2O are coincidentally identical with pK values determined in H_2O (Roberts *et al.*, 1969). Thus, this pK determination is in excellent agreement with the pK of 3.3 reported by Hill *et al.* (1962), but is somewhat lower than the value of 3.52 reported by Dolphin *et al.* (1964).

It is of interest to know whether all the changes in the pmr spectrum of CoB_{12} noted upon protonation of the benzimidazole also reflect the calculated pK. To examine this question, it was assumed that the pK for the protonation was exactly 3.28 and the relative amounts of CoB_{12} in the protonated [HA^+] and unprontonated [A] form at a particular pH calculated on the assumption of a simple proton ionization. When the chemical shifts portrayed in Figure 8 are plotted *vs.* the values of [A]/[A] + [HA^+] thus calculated, the plots displayed in Figure 9 result. For both the high-field methyls and the low-field resonances, except for one methyl resonance, the plots obtained are linear. It appears that virtually the entire pH dependence of pmr spectrum of CoB_{12} in D_2O in the pH range of 2–9 may be explained by invoking only two conformational states (unprotonated and protonated CoB_{12}) in rapid equilibrium with one another.

To clarify the effect of base protonation upon the R'5 methylene protons of CoB_{12}, a partial pH titration of methylcobalamin was performed. The high-field portion of the 100-MHz pmr spectrum of methylcobalamin in D_2O at 32° is given in Figure 10 for pH 2.73 (bottom), 2.47 (middle), and 2.24 (top). As with CoB_{12}, the resonances appear to move continuously with pH. A calculation which was entirely analogous to that performed for CoB_{12} in the data summarized on Figure 9 was carried out for the eight high-field methyl resonances of methylcobalamin. The assumed pK value was 2.72 (Hogenkamp, 1965). The results are portrayed in Figure 11. Extrapolation of the data in Figure 11 for the cobalt-bound methyl, which corresponds to the resonance at highest field, indicate that the methyl resonance shifts upfield about 30 Hz or 0.3 ppm upon protonation of the base. It is noteworthy that the plots are linear for all eight resonances.

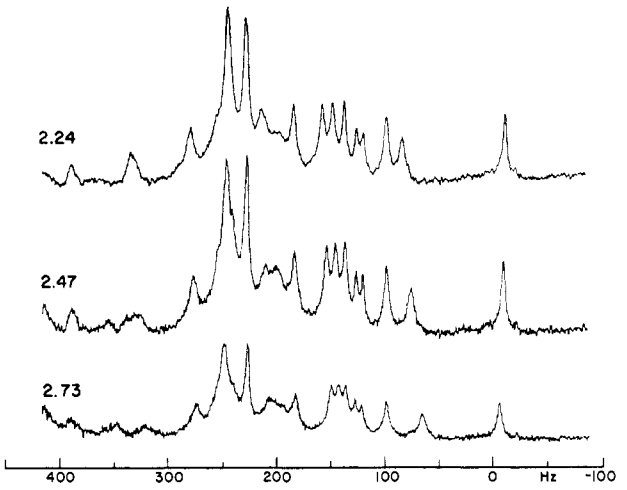

FIGURE 10: High-field region of resonance absorption for three solutions of methylcobalamin at 100 MHz in D_2O at 32°. The pH of the solutions are indicated above and to the left of the spectra; the vertical scales of the spectra are not directly comparable.

Discussion

The cleavage of the carbon–cobalt bond of adenosyl-B_{12} prosthetic group appears to be a common feature of enzymic rearrangements involving this coenzyme (Frey *et al.*, 1967; Rétey *et al.*, 1966; Miller and Richards, 1969). Yet the potentiation of this carbon–cobalt bond scission, identification of catalytically important intermediates and the role of the cobalamin residue, are still quite unclear. Indeed, it has only recently been recognized that several proposed intermediates such as a substrate–cobalamin (Brodie, 1969; Schrauzer *et al.*, 1970) or a Cob(I)alamin (Brodie and Poe, 1971) require the removal of dimethylbenzimidazole from the coordination sphere. While other possible intermediates such as a Cob(II)alamin (Law *et al.*, 1971; Babior and Gould, 1969) do not require removal of the base from the cobalt they are compatible with its displacement by another ligand as well as N–Co band shortening as deduced from hyperfine interactions (Bayston *et al.*, 1970).

Because of this functional significance of the cobalt coordination sphere, our discussion of nuclear magnetic resonance assignments will emphasize the effects of removing the dimethylbenzimidazole group from the cobalt ligand field by elevating temperature or lowering pH.

TABLE II: Dependence of Chemical Shift upon pH for Certain CoB_{12} Protons.

	Chemical Shift for Proton (ppm)		
pH	B2	B4	C10
8.68	6.96	6.24	5.94
4.95	6.97	6.25	5.94
3.99	7.31	6.43	6.09
3.60	7.66	6.63	6.25
3.11	8.24	6.94	6.51
2.90	8.73	7.21	6.73
2.65	8.95	7.33	6.83
2.29	9.09	7.41	6.90
2.00	9.16	7.44	6.91

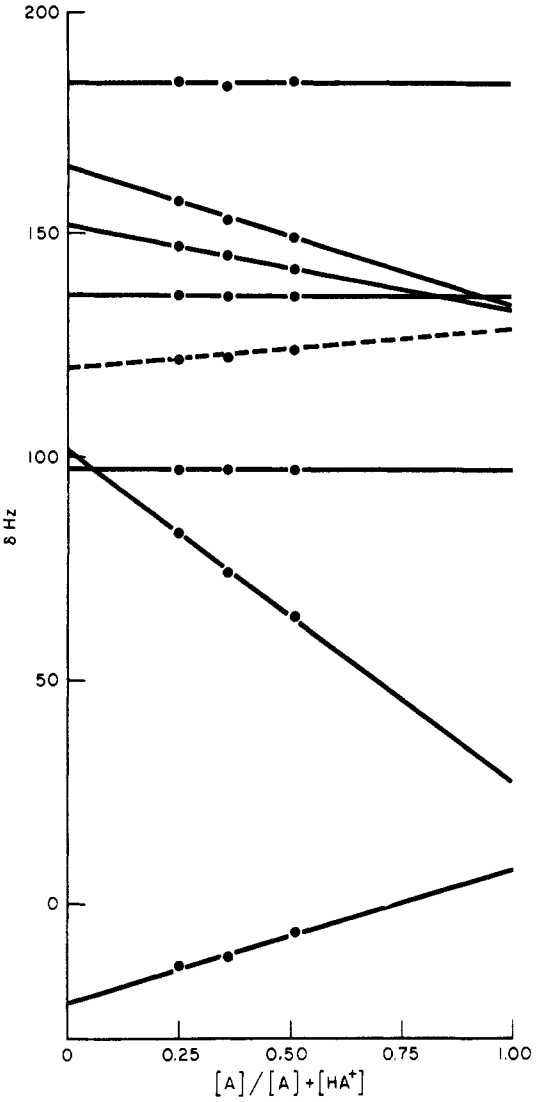

FIGURE 11: Dependence of chemical shift upon proportion of unprotonated form of methylcobalamin for high-field methyl resonances.

The protons whose chemical shifts are portrayed in Figures 5 and 8 have chemical shifts that vary continuously with increasing temperature and increasing pH, respectively. One low-field resonance of COB_{12} in D_2O changes its resonance position by 220 Hz between pH 2.00 and 8.68. Since this resonance exhibits a narrow line width and continuously varies its position of resonance absorption with temperature, the process of base dissociation and reassociation must be fast on the nuclear magnetic resonance time scale (Pople *et al.*, 1959). The first-order rate constant for the breaking of the cobalt–benzimidazole coordination must be somewhat larger than $(2\pi)^{1/2}$ (220 sec^{-1}) or 550 sec^{-1}. The sharpness of the methyl resonances in protonated CoB_{12} in D_2O and in CoB_{12} at 88° in $(CD_3)_2SO$ also indicates the absence of significant paramagnetism associated with the "base-off" form of CoB_{12}; paramagnetism would broaden the resonances of nearby protons (Poe *et al.*, 1970; Phillips *et al.*, 1970).

It is noteworthy that the prochiral protons on carbon R′5 are not equivalent in either D_2O or $(CD_3)_2SO$. The large difference in chemical shift between the two protons infers that there is a highly asymmetric environment above the corrin ring and that rotation about the cobalt–carbon R′5 bond

is restricted in such a way as to prevent the averaging of this environment. One such plausible restriction would be if the cobalt–carbon R′5 bond were not orthogonal to the corrin plane. Brodie and Poe (1971) proposed that the nonequivalence noted for analogous prochiral protons in some secondary cobinamides be attributed to incomplete averaging of proton environments. The bulky 5′-deoxyadenosyl moiety in CoB_{12} should exhibit even more restrictions on its rotation about the carbon–cobalt bond (Law *et al.*, 1971).

Upon conversion to the "base-off" form of CoB_{12}, the R′5 methylene protons exhibit an upfield shift of about 0.3 ppm. The cobalt-bound methyls of methylcobinamide or of acidified methylcobalamin are about 0.3 ppm to high field of the cobalt-bound methyl of "base-on" methylcobalamin. Since both methylcobalamin and methylcobinamide contain hexacoordinated cobalt (III), the corresponding upfield shift in CoB_{12} is probably due to loss of the ring-current field of the benzimidazole base.

The pmr spectra of the various versions of "base-off" CoB_{12} are not superimposable. For example, the C-20 methyl resonance (the highest field methyl in Figure 7) in CoB_{12} at pH 2.00 in D_2O is at 0.77 ppm, at 88° in $(CD_3)_2SO$ is at 0.82 ppm, but at 85° in D_2O at neutral pH is at 0.50 ppm. The pattern and absolute value of chemical shifts of protons in protonated CoB_{12} indicate that the benzimidazole base is no longer coordinated to cobalt. The equivalence of the benzimidazole methyls and the benzimidazole single protons (B4 and B7) as well as the absence of a ring-current shift in the resonance for methyl C-20 are the strongest evidence supporting this contention. CoB_{12} in $(CD_3)_2SO$ at 88° is doubtless also in the fully base-off configuration, as reflected by the magnitude of the upfield shift of the R′5 methylene protons, the resonance position of methyl C-20 and the change in absorption spectrum. However, there are small differences in the resonance positions of the corrinoid methyls for acidified CoB_{12} in D_2O when contrasted to CoB_{12} at 88° in $(CD_3)_2SO$ (see Table III), which probably reflects somewhat different average configurations for the 5′-deoxyadenosyl moiety in the two base-off configurations. In D_2O at neutral pH and 85°, CoB_{12} still exhibits an upfield shift for methyl C-20 and exhibits chemical shifts for its eight low-field resonances roughly equal to those at pH 4.95 (see Table I and Figure 8). Thus, CoB_{12} appears to be only partially (about 15–20%) in the base-off configuration in D_2O at 85° and neutral pH.

TABLE III: Chemical Shifts of High-Field Methyl Resonances of CoB_{12} in D_2O at pH 2.0 and 32° and in $(CO_3)_2SO$ at 88°.

Solvent	Resonance Positions (ppm)						
D_2O	0.77[a]	0.94	1.19[b]	1.35	1.45	1.64	1.81
$(CD_3)_2SO$	0.82[a]	0.71	1.16[b]	1.11	1.37	1.48	1.76

[a] C-20 methyl. [b] Propanolamine methyl.

It should be noted that six of the eight corrin methyls of methylcobinamide and methlycobalamin exhibit very similar patterns of resonance absorptions (Brodie and Poe, 1971), which demonstrates that these six corrin methyls are not significantly shifted by the ring current field of cobalt-coordinated benzimidazole. However, the corresponding six corrin methyls in CoB_{12} exhibit a marked temperature dependence in their position of resonance absorption (see Figure 5). This temperature dependence is therefore probably not directly attributable to the temperature-dependent degree of benzimidazole coordination. A reasonable explanation for the temperature dependence of resonance position for the six corrin methyls is that the loss of benzimidazole coordination is accompanied by a change in average orientation of the 5′-deoxyadenosyl moiety, with consequent changes in its ring-current field.

In base-off CoB_{12} at 88° in $(CD_3)_2SO$, no evidence was found for coordinated water at the sixth ligand position of cobalt, *i.e.*, there is neither a shift in the bulk water resonance nor evidence for a coordinated water. This is in contrast to the case in alkylcobinamides at 23° in $(CD_3)_2SO$ (Brodie and Poe, 1971). It is possible that at 88° the rate of exchange of coordinated water with the rest of the water in $(CD_3)_2SO$ is so rapid as to preclude the observation of separate resonances.

The 5′-deoxyadenosyl moiety in CoB_{12} is quite bulky, but there appears to be appreciable rotation about the carbon–cobalt bond.[2] The R′5 protons exhibit resonances with intensities corresponding to an integral proton each. The steric asymmetry of the cobalt environment (Lehnhert, 1968) appears sufficient to give rise to different rotamer populations so as to make these two protons nonequivalent, even if rotation is indeed unrestricted as would be the case if the cobalt were somewhat out of the corrin plan toward the deoxyadenosyl moiety. The relatively weak coordination of the dimethylbenzimidazole base to cobalt in CoB_{12} at room temperature and neutral pH, as evidenced by the easy accessibility of the base-off configuration and the resonance position of the benzimidazole methyls near their free solution values, is consistent with this situation.

It is clear that CoB_{12} has the capacity for facile ligand displacement at the fifth and sixth coordination positions of the cobalt. Removal of both ligands, *i.e.*, a square-planar cobalt, would be a necessary requirement for any catalysis involving Co(I) such as a hydride transfer. Removal of only the deoxyadenosyl moiety would result in an octahedral cobalt, but replacement of the deoxyadenosyl with substrate in the course of enzymic catalysis would result in displacement of the trans base and a change in the average orientation of the cobalt with respect to the corrin ring. Indeed, the resemblance of thermally excited CoB_{12} to secondary alkylcobinamides and -cobalamins as reflected in their pmr spectra suggests that the first step in CoB_{12} mediated enzyme catalysis, the destabilization of the C–Co σ bond, possibly accompanied by a replacement for the dimethylbenzimidazole moeity is effected by means of formation of a labile pentacoordinate, square-pyramidal coenzyme.

[2] There is great similarity with regard to the resonance separation of the prochiral α-carbon protons on *n*-propylcobinamide (Brodie and Poe, 1971) and the R′5 protons of CoB_{12}. Although one might expect that the methylene protons in CoB_{12} would exhibit greater nonequivalence than the equivalent protons in *n*-propylcobinamide, this need not be the case if the deoxyadenosyl group still interconverts rotamers swiftly on an nmr time scale. The change in relative population of rotamers which might accompany introduction of a bulkier substituent need not be reflected in a greater nonequivalence of the prochiral protons in the larger group (Mislow and Raban, 1967). Therefore, we attribute the nonequivalence of the prochiral protons in alkylcobalamins and -cobinamides to the asymmetry of the environment above cobalt due to side chain projection and aplanarity of the macrocycle and the fact that these C–H bonds do not rotate about the *z* axis of the cobalt, probably due to the deformation of the d_{z^2} orbital.

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Reduction of Flavins by Molybdenum(V)†

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ABSTRACT: The mechanism of the reduction of flavins by molybdenum(V) in tartrate buffer (pH 2.50–5.25) has been investigated as a model for molybdenum–flavin reactions in enzymes. The reaction reaches a pH-dependent equilibrium, and the rate of reduction is independent of flavin concentration and dependent on molybdenum(V) concentration to the first power. A two-electron reduction mechanism involving molybdenum(IV) as a reactive intermediate has been developed and applied to the reaction using a computer curve-fitting program to obtain rate constants. No evidence was found for the involvement of flavosemiquinone or molybdenum–flavin complexes in the reduction. A second, much slower reaction between reduced flavin and molybdenum(VI) producing a molybdenum(V)–flavosemiquinone complex was observed. The implications for the reactions of molybdenum-containing enzymes are discussed.

Molybdenum is now well established as a necessary constituent of several redox flavoenzymes (Bray *et al.*, 1967; Spence, 1969). Current evidence suggests a redox role for the metal, as in xanthine oxidase, where it appears to transfer electrons from substrate to flavin: xanthine → Mo(VI) → FAD → Fe(III) → O_2. Although it is generally thought to function between the +6 and +5 oxidation states, some recent work indicates lower states, particularly +4, may be involved (Palmer and Massey, 1969; Massey and Edmondson, 1970). Whether the reaction between molybdenum and flavin is a one- or two-electron-transfer process is unknown, either in enzymatic or model systems.

Preliminary studies of the reaction between the flavin and molybdenum(V,VI) redox systems have been reported

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