The reaction mechanism of PHE on $Pt/TiO_2/Cu$ and the exact location of the reaction sites are still ambiguous, even when considering those proposed for the catalytic hydrogenation of ethylene.[22] A collective effect of Pt and Cu codeposited on TiO_2 particles was observed in the selective production of C_2H_6 in PHE, i.e., the hydrogenation of ethylene assisted by the photocatalytic production of hydrogen atoms from water. A further study in the collective effect of $Pt/TiO_2/Cu$ on PHE is now under way.

Heteronuclear NMR Studies of Cobalamins. 6. The Nucleotide Loop of Base-Off Cobalamins and the Nature of the Base-Off Species[1]

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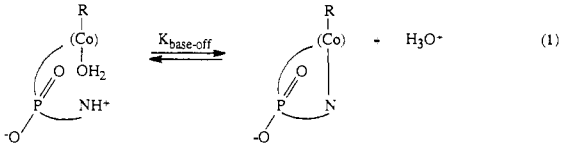
Abstract: The acid–base properties of α-ribazole-3′-phosphate, the detached axial nucleotide of cobalamins, have been studied by potentiometric and spectrophotometric techniques at 25.0 ± 0.1 °C, ionic strength 1.0 M. The microscopic pK_a for proton dissociation from the benzimidazolium moiety of the zwitterion (relevant to the base-on/base-off protonic equilibrium of cobalamins) is 5.54 and the isoelectronic point 2.94. The ^{13}C NMR resonances of the nucleotide loop carbons of a series of five protonated, base-off alkylcobalamins have been directly compared with those of the zwitterion of the free nucleotide in order to detect any noncovalent interactions between the pendent, protonated axial nucleotide and the remainder of the structure in the base-off forms. The comparison shows no significant differences along the top of the benzimidazole moiety (B9, B4, B10) or at R1 or R5, but significant differences at B5, B6, B7, B8, R2, R3, and R4. Considering the available X-ray structures of base-on cobalamins, these chemical shift perturbations do not readily suggest an interaction between the pendent nucleotide and the remainder of the structure. A similar comparison between the nucleotide loop ^{13}C resonances of two base-off, but benzimidazole deprotonated, alkyl(cyano)cobalamins and the dianion of the free nucleotide shows significant chemical shift perturbations at all benzimidazole carbons except B7, strongly suggesting an interaction between the pendent, but benzimidazole deprotonated, nucleotide and the remainder of the structure in the deprotonated base-off forms. A comparison between the ^{13}C NMR spectrum of base-off dicyanocobalamin and dicyanocobinamide (from which the axial nucleotide has been removed) suggests that the major interaction is formation of a hydrogen bond between the benzimidazole nitrogen (B3) and an amide hydrogen on the e propionamide side chain.

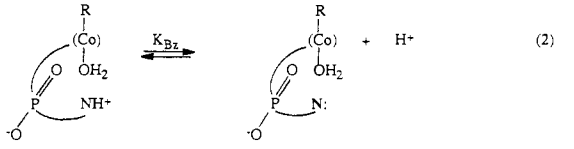
One of the most characteristic and perhaps one of the most thoroughly studied chemical properties of cobalamins is the so-called base-on/base-off reaction in which the axially coordinated dimethylbenzimidazole nucleotide is displaced by water and protonated (eq 1). This reaction is sometimes referred to as the



(1)

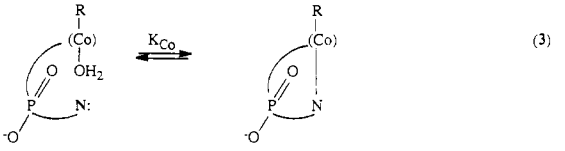
1 2

"red-yellow" shift due to the large changes in electronic spectrum attendant upon conversion of the base-on (**2**) to the base-off (**1**) form.[2] Many values of $pK_{base-off}$ (eq 1) have been determined (generally spectrophotometrically) and large tabulations of such values exist.[3] This protonic equilibrium is often conveniently viewed as the sum of two consecutive equilibria (eq 2 and 3)[1,4-14]



(2)

1 3



(3)

3 2

for which eq 4 relates the value of $K_{base-off}$ (eq 1) to the equilibrium

$$K_{base-off} = (1 + K_{Co})K_{Bz} \quad (4)$$

constants of eq 2 and 3. It is then often assumed that K_{Bz} (eq 2) is equivalent to the pK_a of the conjugate acid of the detached axial nucleoside (i.e., 1-α-D-ribofuranosyl-5,6-dimethylbenz-

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imidazole (α-ribazole), pK_a = 5.56 at 25 °C[13]). This then allows calculation of K_{Co} (eq 3) from eq 4, a critically important value as it represents the intrinsic affinity of the fifth ligand in this pentadentate system for the cobalt atom. Values of K_{Co} calculated in this manner have been shown to be strongly affected by the steric and inductive properties of the upper axial ligand[13], and ΔG_{Co} has been shown to be linearly related to the axial Co–N bond length[14] (for those cobalamins whose geometry is known from X-ray crystallography) and to the magnetic properties of the phosphorus atom of the nucleotide loop.[1,14] In addition, at various times it has been suggested that base-off cobalamin species participate in the catalytic cycle of adenosylcobalamin (AdoCbl)-requiring enzymes[15,16] and that binding of cobalamins to B_{12}-binding proteins is accompanied by displacement of the axial benzimidazole ligand by a histidine residue from the protein.[17,18]

Although the base-on/base-off equilibrium (eq 1) is very well known, the exact nature of the base-off species (**1** and/or **3**) is not at all clear. Basically the question involved is whether the detached benzimidazole ligand in the base-off forms remains associated with the remainder of the structure via noncovalent (and noncoordinative) forces or if the nucleotide loop, in fact, dangles freely in the base-off forms. If there is such a noncovalent association, do both the protonated (**1**) and deprotonated (**3**) base-off forms display it and does the nature and/or strength of the interaction depend on the nature of the upper axial ligand? If there is noncovalent interaction of the benzimidazole ligand in the base-off forms, then the assumption that K_{Bz} (eq 2) is equal to the pK_a of the conjugate acid of α-ribazole is incorrect, and values of K_{Co} calculated via eq 4 using this assumption may be in error.[1,4–14,19] Perhaps more importantly, even the *relative* values of K_{Co} so calculated may be in error if the strength of the interaction varies with the nature of the upper axial ligand.

There are several observations in the literature which suggest that there is, indeed, a noncovalent interaction of the benzimidazole ligand with the remainder of the corrin structure in the base-off cobalamins. Chemaly and Pratt[20] have presented electronic spectral arguments that in the base-off forms the benzimidazole ligand is held in contact with the corrin ring hydrophobically and have estimated values of pK_{Bz} (eq 2) to be 4.8–5.0 from the temperature dependence of the UV–visible spectra of alkylcobalamins in neutral solution. Similar measurements have been made on AdoCbl by Halpern et al.[21] and Finke and Han,[22] leading to estimates of 4.6 and 4.9, respectively, for pK_{Bz}. These values are substantially below the pK_a of 5.56 for the conjugate acid of detached α-ribazole[13] and consequently imply significant noncovalent interaction of the benzimidazole nucleotide with the remainder of the structure in either the protonated (**1**) or deprotonated (**3**) base-off forms, or in both. However, these measurements do not appear to have been controlled for the temperature dependence of the electronic spectra of the base-off forms which may be significant given the reversible temperature-dependent spectral changes previously observed for alkylcobinamides.[23] Thus, while the accuracy of these values of pK_{Bz} is not clear, they are in good agreement with a value of 5.0 for pK_{Bz} for dicyanocobalamin ($(CN)_2Cbl$), calculated from a thermodynamic cycle by Reenstra and Jencks.[24] It should be pointed out that direct determination of pK_{Bz} from studies of the pH dependence of the kinetics of the base-on/base-off reaction is not feasible owing to the very high rate of association of the free-base benzimidazole moiety with the cobalt atom.[25]

Jacobsen and co-workers[26] have prepared α-ribazole from cerrous hydroxide catalyzed phosphodiester hydrolysis of $(CN)_2Cbl$[27] and demonstrated that both the free-base and benzimidazole-protonated species are fluorescent. However, both the base-on and base-off forms of cobalamins are well known to be nonfluorescent.[26,28] For the base-on forms this is generally attributed to efficient intramolecular energy transfer from benzimidazole to cobalt. The obvious implication is that even in the base-off forms.the benzimidazole moiety is held close enough to the cobalt atom to allow for quenching via intramolecular energy transfer, although other explanations are, of course, possible.

Finally, Mishra and co-workers[29] have studied the ^{31}P spin–lattice relaxation times (T_1) of numerous cobalamins. All of the compounds studied for which the cobalt atom is formally in the +3 oxidation state had T_1 values close to 2.0 s, but T_1 values were decreased by nearly two orders of magnitude in cob(II)alamins because of the electron-nuclear interactions from the unpaired electron in the cobalt d_{z^2} orbital. As these authors concluded that the ^{31}P nuclear relaxation was dominated by intramolecular dipolar interaction of the paramagnetic Co(II) atom, they were able to calculate the cobalt to phosphorus distances for the cob(II)alamins from the paramagnetic contribution to the observed relaxation rates. Surprisingly, they found less difference than anticipated between the Co to P distance in base-on cob(II)alamin (pH 6) and the base-off species (pH 2) and consequently concluded that in the base-off form "the base may not hang loose but may remain situated relatively close to the corrin ring".[29]

In principle, two general types of methods may be used to detect and/or quantitate any noncovalent interactions of the axial nucleotide with the remainder of the structure in the base-off cobalamins: spectroscopic methods and thermodynamic methods. Suitable thermodynamic methods are currently under development and will be discussed in a subsequent article. Spectroscopic methods rely on the fact that both the detached axial nucleoside (α-ribazole) and the detached nucleotide (α-ribazole-3′-phosphate[30]) are available for spectroscopic study free of any influence of the remainder of the corrinoid. Comparison of the spectroscopic properties of the axial nucleotide of base-off cobalamins to those of the free nucleotide can then, in principle, detect any interactions in the base-off cobalamins which must be expected to influence the spectroscopic properties of the nucleotide. Conversely, since cobinamides (i.e., corrinoids from which the axial nucleotide has been removed) are also available, comparison of the spectral properties of the corrin (and its attachments) in base-off cobalamins and cobinamides should also reveal perturbations due to noncovalent interactions in the base-off species. Although many spectroscopic techniques could, in theory, reveal such interactions, ^{13}C NMR spectroscopy is ideal for several reasons. First, spectral features due to individual carbon atoms in the nucleotide loop and in the remainder of the structure are discernible and separable. In addition to overcoming the problem inherent in most other techniques of separating the spectral features of the nucleotide from those of the remainder of the corrinoid in base-off cobalamins, this allows the possibility of determining which atoms of each part of the structure interact and, hence, of characterizing the nature of the interaction. Second, as the complete ^{13}C NMR spectra of several cobalamins (both base-on and base-off) have now been assigned,[31–34] the converse experiment (of comparing

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(19) The magnitude of such errors in K_{Co} will depend on the strength of any such noncovalent interactions which may exist. Evaluation of such errors requires detailed thermodynamic measurements of these interactions. Such measurements are in progress and preliminary results show that in some cases, at least, the errors in K_{Co} values due to application of the assumptions inherent in eq 2–4 are quite small indeed.
(20) Chemaly, S. M.; Pratt, J. M. *J. Chem. Soc., Dalton Trans.* **1980**, 2267–2273.
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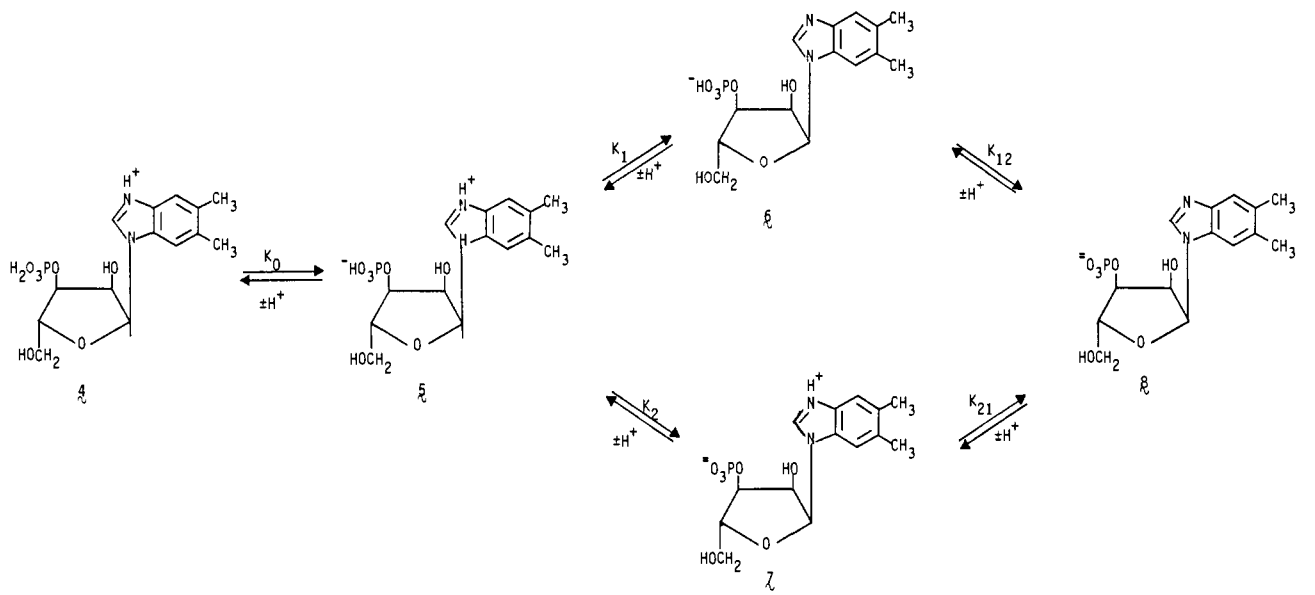
Scheme I



base-off cobalamins and cobinamides) is feasible and enjoys the same advantages. Third, our recent ^{13}C NMR study of base-on cobalamins has led to evaluation of the magnetic anisotropy of the induced cobalt atom dipole for several cobalamins.[30] For cobalamins with electron-donating upper axial ligands these values are quite large, so one can anticipate significant dipolar shielding of any nucleotide loop carbons which remain relatively close to the cobalt atom in the base-off species. For example, using the value of the magnetic anisotropy of the cobalt atom of AdoCbl (-14.3×10^{-29} cm^3/molecule,[30] although this value will certainly be altered when the benzimidazole N-3 is no longer in the inner coordination sphere in the base-off forms), we can calculate values for the dipolar shielding of from +0.76 to −0.25 ppm (depending on the geometry) for any nucleotide loop carbon with 5 Å of the cobalt atom via application of McConnell's equation.[35] Evan at 7 Å radius, the calculated dipolar sheilding varies from 0.28 to −0.14 ppm, chemical shift differences which should be easily measurable.

Experimental Procedures

α-Ribazole-3′-phosphate was obtained by sulfuric acid catalyzed hydrolysis of the phosphodiester of cyanocobalamin (CNCbl) as previously described.[30] Cyano(aquo)cobinamide (CN(H_2O)Cbi) was obtained by cerrous hydroxide catalyzed hydrolysis of CNCbl according to a slight modification[13] of the method of Renz.[27] Final purification was effected by chromatography on SP-Sephadex[36,37] (sodium form), eluting with 0.05 M sodium acetate buffer, pH 5.0. AdoCbl, CH_3Cbl, CNCbl, and H_2OCbl were from Sigma. All other alkylcobalamins were synthesized from H_2OCbl by standard reductive alkylation procedures as previously described[13] except that all desalting was performed on Amberlite XAD-2 columns (Serva).[38] Purity was ascertained by high performance liquid chromatography.[39]

UV-visible spectral measurements were made on a Cary 219 recording spectrophotometer whose cell block was thermostated at 25.0 ± 0.1 °C. pH measurements were made on a Radiometer PHM 64 pH meter with samples, standards, and electrodes thermostated at 25.0 ± 0.1 °C. Ionic strength was maintained at 1.0 M with KCl throughout. NMR measurements were made on a Nicolet NT-200 wide-bore superconducting NMR spectrometer operating at 50.311 (^{13}C) or 80.988 (^{31}P) MHz. All samples were dissolved in water and the ionic strength was adjusted to 1.0 M with KCl. A deuterium lock signal was provided by D_2O in a concentric insert (Wilmad) which also contained 3-trimethylsilylpropionate (TSP) as a ^{13}C shift reference. ^{31}P spectra were referenced to external 85% H_3PO_4. The variable-temperature device on the NMR spectrometer was adjusted to provide an actual sample temperature (as determined by a thermistor temperature measuring device (Yellowsprings Instruments) immersed in the sample during pulsing and irradiation with the decoupling field) of 25 ± 1 °C. Generally this required setting the variable-temperature control to 22 to 23 °C to compensate for heating of the sample by the ^1H-decoupling field. ^{13}C NMR measurements on the zwitterion of α-ribazole-3′-phosphate were made on a supersaturated solution prepared by adjusting the pH of a solution of the dianion to the measured isoelectric point with HCl as described previously.[30]

Results and Discussion

Acid–Base Chemistry of α-Ribazole-3′-phosphate. In order to accurately compare the ^{13}C NMR spectra of the α-ribazole-3′-phosphate zwitterion to those of the nucleotide loop of protonated, base-off cobalamins, it is necessary to know the isoelectric point of the free nucleotide. In addition, it is of interest to obtain the appropriate microscopic pK_a for benzimidazolium ionization from the zwitterion species of α-ribazole-3′-phosphate for use as pK_{Bz} in eq 2 and 4. We have consequently investigated the acid–base chemistry of α-ribazole-3′-phosphate according to the abbreviated ionization scheme shown (Scheme I). This scheme is abbreviated in that the microscopic benzimidazolium ionization from the monocationic species (**4**) is omitted. This simplification has been introduced since this microscopic pK_a is of little interest and is not readily determinable. In addition, the macroscopic pK_a for ionization of the monocation (**4**) (pG_1) must surely be dominated by the microscopic ionization of the phosphate group (pK_0) so that pK_0 may be taken as equivalent to pG_1.

The upper two macroscopic pK_a's of α-ribazole-3′-phosphate, pG_2 (eq 5) and pG_3 (eq 6) were determined by potentiometric

$$G_2 = K_1 + K_2 \quad (5)$$

$$G_3 = K_1K_2/(K_1 + K_2) \quad (6)$$

titration of a solution of the dianion of the nucleotide with HCl. The data were fit to eq 7 where C is the concentration of α-ri-

$$\text{vol. 1.0 N HCl} = 2C - C(G_1/a_{H^+} + 2G_1G_2/a_{H^+}{}^2)/(1 + G_1/a_{H^+} + G_1G_2/a_{H^+}{}^2) \quad (7)$$

bazole-3′-phosphate (0.0435 M), by an iterative, nonlinear least-squares program using a simplex minimization algorithm to yield the values pG_1 = 5.25 and pG_2 = 6.27. The microscopic pK_a's were resolved by the spectrophotometric method of Benesch

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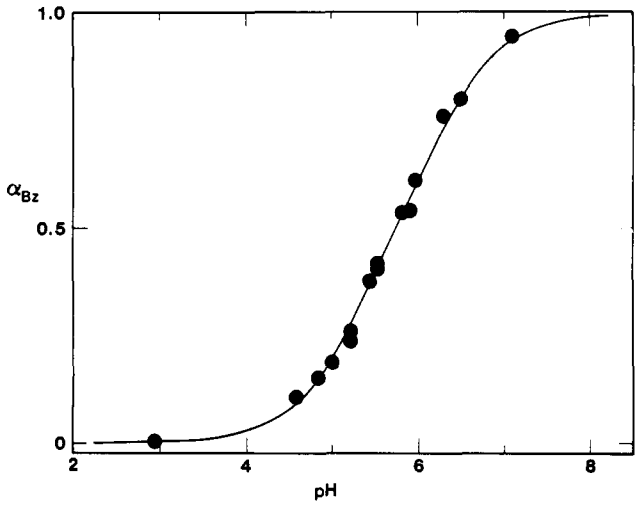

Figure 1. Plot of α_{Bz} (eq 8 and 9) vs. pH for α-ribazole-3′-phosphate, 25.0 ± 0.1 °C, ionic strength 1.0 M (KCl). The solid line was calculated from eq 10 and the values $K_1 = 2.88 \times 10^{-6}$, $G_1 = 5.62 \times 10^{-6}$, and $G_2 = 5.37 \times 10^{-7}$.

and Benesch.[40] Spectrophotometric determination of the fraction of benzimidazole deprotonated species, α_{Bz} (eq 8), was made from

$$\alpha_{Bz} = ([6] + [8])/([5] + [6] + [7] + [8]) \quad (8)$$

absorbance measurements at 285.2 nm, where α_{Bz} at pH's from 4.6 to 7.1 was calculated via eq 9, where $A_{obsd}^{285.2}$, $A_5^{285.2}$, and

$$\alpha_{Bz} = (A_5^{285.2} - A_{obsd}^{285.2})/(A_5^{285.2} - A_8^{285.2}) \quad (9)$$

$A_8^{285.8}$ are the absorbances at 285.2 nm of solutions at constant concentration of α-ribazole-3′-phosphate at pH_x, pH 3.0, and pH 8.3, respectively.[41] These data were used to determine the microscopic ionization constant K_1 (Scheme I) via eq 10 which is

$$\alpha_{Bz} = (K_1/a_{H^+} + G_1G_2/a_{H^+}^2)/(1 + G_1/a_{H^+} + G_1G_2/a_{H^+}^2) \quad (10)$$

readily derived from the scheme. Thirteen individual values of α_{Bz} were used in conjunction with G_1 and G_2 to calculate an average value of $K_1 = 2.88 \pm 0.24 \times 10^{-6}$, via eq 10. The data are shown graphically in Figure 1. The resulting microscopic pK_a's are $pK_1 = 5.54$, $pK_2 = 5.56$, $pK_{12} = 5.98$, and $pK_{21} = 5.95$. We note the the microscopic ionization constant pK_1, the ionization of relevance to the base-on/base-off reaction of cobalamins (eq 2–4), is nearly identical, at least under these conditions (25.0 °C, ionic strength 1.0 M) to the value previously determined for the pK_a of α-ribazole (5.56).[13]

The lower macroscopic pK_a of α-ribazole-3′-phosphate ($pG_1 = pK_0$, Scheme I) was determined from the pH dependence of the frequency of the ^{31}P resonance between pH 2.9 and 0.3. The data were analyzed via eq 11, where ν_4 and ν_5 are the frequencies

$$\nu^5{}_{^{31}P} - \nu^{obsd}{}_{^{31}P} = (\nu^5{}_{^{31}P} - \nu^4{}_{^{31}P}) - K_0\,(\nu^5{}_{^{31}P} - \nu^{obsd}{}_{^{31}P})/a_{H^+} \quad (11)$$

of the ^{31}P resonances of species **4** and **5**, respectively, and ν_{obsd} is the frequency of the ^{31}P resonance of α-ribazole-3′-phosphate at pH_x. A least-squares fit (Figure 2) using the value $\nu_5 = 16.16$ Hz gave $\nu_4 = -50.03$ Hz, and $K_0 = 0.235 \pm 0.009$. Consequently $pK_0 = 0.63$ and the isoelectric point of α-ribazole-3′-phosphate is 2.94.

^{13}C NMR of α-Ribazole-3′-phosphate. In order to precisely compare the ^{13}C resonances of the base-off cobalamins to those of α-ribazole-3′-phosphate, the ^{13}C NMR spectra of the zwitterion and dianion species of the detached nucleotide, which had previously been measured in D_2O at zero ionic strength,[30] were determined in water, at ionic strength 1.0 M (KCl), 25 ± 1 °C

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(41) The method requires the assumption that the molar extinction coefficients of the benzimidazole moiety are not appreciably affected by the state of ionization of the phosphate moiety.

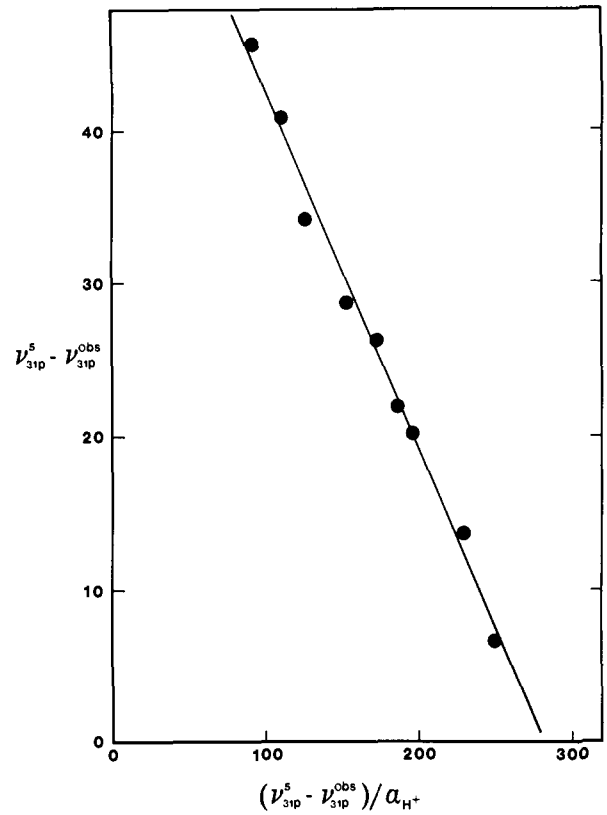


Figure 2. Plot of $\nu^5{}_{^{31}P} - \nu^{obsd}{}_{^{31}P}$ vs. $(\nu^5{}_{^{31}P} - \nu^{obsd}{}_{^{31}P})/a_{H^+}$ for the ^{31}P resonance of α-ribazole-3′-phosphate according to eq 11. The solid line is a least-squares fit, intercept = 66.19 ± 1.55 Hz, slope = −0.235 + 0.009 M.

Table I. ^{13}C NMR Data for α-Ribazole-3′-phosphate, 25.0 °C, Ionic Strength 1.0 M[a]

	dianion (pH 8.36)[b]		zwitterion (pH 2.94)[c]		pH 0.6[d]	
atom	$\delta_{^{13}C}$, ppm	J,[e] Hz	$\delta_{^{13}C}$, ppm	J,[c] Hz	$\delta_{^{13}C}$, ppm	J,[e] Hz
B2	145.461		140.839		n.o.[f]	
B4	121.109		116.461		116.612	
B5	135.007		139.564		139.795	
B6	134.091		139.732		139.974	
B7	113.574		114.977		115.253	
B8	135.972		131.184		131.341	
B9	142.668		131.383		131.434	
B10	22.207		22.327		22.414	
B11	21.922		22.127		22.274	
R1	88.356		89.625		89.748	
R2	74.059	1.69	74.060	4.41	74.031	4.09
R3	75.687	4.20	76.725	4.74	77.185	5.18
R4	85.967	5.64	88.246	4.55	88.088	5.30
R5	64.000		63.784		63.718	

[a] Ionic strength maintained with KCl. All chemical shifts in ppm from internal TSP. [b] $\nu_{^{31}P}$ = +354.5 Hz (relative to external 85% H_3PO_4), $J_{H\text{-}P}$ = 5.76 Hz. [c] $\nu_{^{31}P}$ = +16.16 Hz (relative to external 85% H_3PO_4), $J_{H\text{-}P}$ = 7.22 Hz. [d] Net charge +0.49. Phosphorus–carbon coupling constant. [f] Not observed.

(Table I). For convenience, the numbering scheme for the cobalamin nucleotide loop (Figure 3) has been used for the free nucleotide. The results are quite similar to those previously obtained in D_2O at zero ionic strength although there is a small, but apparently real, ionic strength effect. It should be pointed out that under the current conditions of high ionic strength, the B2 carbon resonance is quite broad, possibly because of exchange of the B2 proton with solvent.[30] In addition, we have now interchanged the assignments of B5 and B6 of the zwitterion from our previous work[30] based on careful observation of the changes in these chemical shifts with pH at pH's between 8.3 and 3.0. In addition, Table I also shows the ^{13}C NMR chemical shifts of the

Table II. ^{13}C NMR Data for the Nucleotide Loop of Protonated, Base-Off Cobalamins, 25.0 °C, Ionic Strength 1.0 M[a]

	δ, ppm (J, Hz)[b]					
R	$CH_3CH_2^-$	$CH_3(CH_2)_2^-$	Ado$^-$	$NC(CH_3)^-$	CH_3^-	av ± std dev
$pK_{base\text{-}off}$	4.16[c]	4.10[d]	3.67[e]	3.50[d]	2.89[d]	
pH	2.08	2.06	2.04	2.02	2.02	
$\alpha_{base\text{-}on}$	0.008	0.009	0.023	0.032	0.119	
atom						
B2	140.554	141.033	141.610	140.827	141.162	141.037 ± 0.394
B4	116.466	116.503	116.548	116.462	116.668	116.529 ± 0.085
B5	139.206	139.253	139.281	139.277	139.203	139.244 ± 0.038
B6	139.473	139.541	139.583	139.571	139.501	139.534 ± 0.046
B7	115.436	115.492	115.546	115.482	115.406	115.472 ± 0.054
B8	131.464	131.402	131.456	131.312	131.517	131.430 ± 0.078
B9	131.516	131.473	131.517	131.455	131.526	131.497 ± 0.031
B10	22.285	22.129	22.303	22.256	22.271	22.249 ± 0.069
B11	22.390	22.462	22.371	22.367	22.339	22.385 ± 0.047
R1	89.716	89.772	89.874	89.723	89.710	89.759 ± 0.069
R2	74.155 (3.5)	74.254 (3.3)	74.389 (3.6)	74.228 (3.3)	74.122 (4.0)	74.230 ± 0.104
R3	77.378 (5.1)	77.405 (4.8)	77.342 (4.9)	77.329 (5.1)	77.277 (4.8)	77.346 ± 0.049
R4	88.703 (3.1)	88.801 (4.0)	88.736 (4.4)	88.645 (4.0)	88.732 (3.5)	88.723 ± 0.057
R5	63.903	63.945	63.894	63.849	63.815	63.881 ± 0.050
Pr1	47.087 (4.3)	47.142 (4.5)	47.194 (4.5)	47.032 (4.1)	47.123 (4.4)	47.116 ± 0.061
Pr2	74.749 (5.0)	74.803 (5.2)	74.832 (5.2)	74.720 (5.5)	74.749 (5.3)	74.771 ± 0.046
Pr3	21.155 (3.4)	21.162 (3.6)	21.215 (3.5)	21.076 (3.6)	21.107 (3.9)	21.143 ± 0.054

[a] Ionic strength maintained with KCl. All chemical shifts are in ppm from internal TSP and have been corrected for the presence of the base-on species via eq 12, with the exception of CH_3CH_2Cbl. [b] P–C coupling constant. [c] Reference 1. [d] Reference 13. [e] Reference 14.

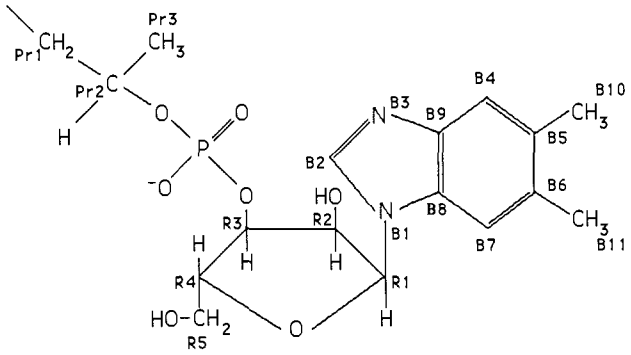


Figure 3. Structure and numbering system from the nucleotide loop of cobalamins.

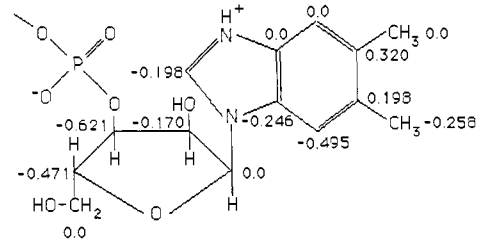
nucleotide at p*H* 0.64, i.e., a nearly equimolar mixture of the zwitterion and the monocationic species (**4**, Scheme I). These data show the expected substantial effect of the final phosphate protonation on the position of the R3 resonance. While the effect of such protonation is much smaller on the remaining ribose resonances and on the benzimidazolium resonances, it is still significant on several of these resonances. At this pH, the B2 resonance is not observed.

Protonated Base-Off Cobalamins. In order to avoid protonation of the phospodiester of the base-off cobalamins ($pK_a = -0.04$[14] and the attendant changes in chemical shift (Table I), ^{13}C NMR spectra of protonated, base-off cobalamins were obtained at pH's of 2.00 or above, and at ionic strength 1.0 M, 25 °C. As few alkylcobalamins have $pK_{base\text{-}off}$ values significantly above 4.0,[1,13] this necessitated a correction of the observed chemical shifts (and coupling constants) for the presence of a small amount of base-on species, whose ^{13}C NMR spectra had previously been determined.[30] The correction was made by use of eq 12, where δ^{obsd} is the

$$\delta^{base\text{-}off} = (\delta^{obsd} - \alpha_{base\text{-}on}\delta^{base\text{-}on})/(1 - \alpha_{base\text{-}on}) \quad (12)$$

observed chemical shift at the measured pH, $\delta^{base\text{-}on}$ and $\delta^{base\text{-}off}$ are the chemical shifts for the base-on and base-off species, respectively, and $\alpha_{base\text{-}on}$ is the fraction of base-on species present, which was calculated from the value of $pK_{base\text{-}off}$[1,13] and the measured pH. Observed phosphorus–carbon coupling constants were corrected by an analogous equation. In order to minimize the magnitude of such corrections, measurements were restricted to five alkylcobalamins (R = CH_3CH_2, $CH_3(CH_2)_2$, Ado, NC-$(CH_2)_3$, and CH_3) whose values of $pK_{base\text{-}off}$ were ≥2.89 (thus the maximum correction was for 11.9% base-on species, R = CH_3). The corrected chemical shifts and P–C coupling constants for the nucleotide loops of these protonated, base-off cobalamins are shown in Table II, along with the average value (and standard deviation) for each chemical shift. The assignments are based on the work of Hogenkemp and co-workers[31,32] and more recent work[33,34] in which some of the earlier assignments have been changed based on two-dimensional NMR techniques which unambiguously establish connectivity. With the exception of B2, which has a broad resonance under these conditions and is difficult to locate accurately, all of the chemical shift standard deviations are ≤0.10 ppm. In addition, the relatively good agreement of the phosphorus–carbon coupling constants across the series (within the limits of precision of determining these values, as previously discussed[30]) leads to the conclusion that the chemical shifts of the nucleotide loops of all five protonated, base-off cobalamins are probably the same; i.e., they are unaffected by the upper axial ligand. Consequently, the average chemical shift values may be directly compared to those of the α-ribazole-3′-phosphate zwitterion for evidence of interaction between the protonated, pendent nucleotide and the remainder of the structure. A criterion for significance of any differences found may also be established from the data in Table II. Since the average of the standard deviations of the chemical shifts of all of the nucleotide loop carbons (with the exception of B2 which inappropriately distorts the data) is 0.059 ppm, application of the Student's *t* test gives an 0.15 ppm 98% confidence limit. It consequently seems reasonable to assume that any difference in chemical shift between the free nucleotide zwitterion and the average value of the five pendent, zwitterionic nucleotides that exceeds 0.15 ppm is significant.

The comparison is shown in structure **9** as the signed difference



9

in chemical shift between the free, zwitterionic nucleotide and the protonated, base-off nucleotide loop. The comparison shows no significant differences in chemical shift along the top of the

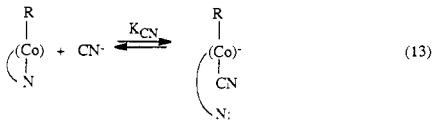
Table III. Formation Constants, K_{CN}, for Alkyl(cyano)coblamins[a]

R	K_{CN} (M^{-1})
CF_2H	3.27 ± 0.11
$NCCH_2$	63.8 ± 1.4
$^-OOCCH_2$	1.74 ± 0.07
$NC(CH_2)_3$	0.640 ± 0.050
CH_3CH_2	0.665 ± 0.066
$CH_3CH_2CH_2$	1.30 ± 0.14
CN	3.0×10^3 [b]

[a] 25.0 ± 0.1 °C, ionic strength 1.0 M. [b] Reference 24.

benzimidazole moiety (B9, B4, B10), but significant differences at B5, B6, B7, B8, B11, R2, R3, and R4. Given the difficulty in accurately locating the B2 resonance of the protonated, base-off cobalamins and the large standard deviation for this chemical shift (Table II), it is not clear if the small difference in B2 chemical shift should be considered significant or not. Careful examination of three-dimensional representations of the X-ray crystal structures of cobalamins[42] does not readily suggest a mode of interaction between the protonated, pendent nucleotide and the remainder of the structure that would fail to affect the chemical shifts along the top of the benzimidazole moiety. However, the possibility of such an interaction cannot be specifically excluded based on the current data. On the other hand, the pattern of chemical shift differences in **9** suggests the possibility of a difference in conformation about the N-glycosidic bond, especially in view of the significant chemical shift differences at R2, R3, and R4, but not at R1 and R5. The conformation about the N-glycosidic bond is not known in either the base-off cobalamins or in the free nucleotide, but only in the base-on cobalamins, in which the exo conformation has been observed in all X-ray crystal structures to date.[43-46] However, recent two-dimensional NMR studies of base-off AdoCbl[34] show unequivocally that the benzimidazole nucleotide is in the exo conformation, and NOE measurements suggest that the benzimidazole is "upside-down" in base-off AdoCbl. This reorientation of the benzimidazole nucleotide presumably accounts for the chemical shift differences seen in **9** and may indicate an interaction between the underside of the benzimidazole moiety and the corrin ring.[34]

Deprotonated Base-off Cobalamins. It is of great interest to use these techniques to try to determine if there is an interaction between the pendent but uncoordinated nucleotide and the remainder of the structure in the deprotonated, base-off cobalamins (i.e., species **3**). Unfortunately, no matter how the thermodynamics of the base-on/base-off reaction (eq 1) are treated, it is clear that this species is at best a minor contributor at neutral pH whose NMR spectrum cannot be directly determined. We have consequently attempted to model such species by displacement of the axial benzimidazole of base-on cobalamins by cyanide. Although some alkylcobalamins (including AdoCbl) undergo cyanolysis of the upper axial Co–C bond in excess cyanide in the dark,[47-50] we have found that a number of alkylcobalamins undergo ligand displacement to form stable alkyl(cyano)cobalamins (R-(CN)Cbl, eq 13). Values for the equilibrium constants for



(13)

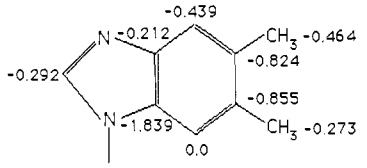
Table IV. ^{13}C NMR Data for the Nucleotide Loop of Base-Off Alkyl(cyano)cobalamins, R(CN)Cbl, 25.0 °C, Ionic Strength 1.0 M[a]

	δ, ppm (J, Hz)[b]	
R atom	CN^-	$NCCH_2^-$
B2	145.141	145.198
B4	121.563	121.532
B5	135.839	135.822
B6	134.984	134.908
B7	113.556	113.591
B8	134.133	134.133
B9	142.891	142.868
B10	22.670	22.672
B11	22.171	22.218
R1	88.266	88.251
R2	73.669 (n.o.)	73.668 (1.9)
R3	76.664 (4.9)	76.520 (5.2)
R4	85.590 (5.2)	85.561 (5.4)
R5	63.460	63.502
Pr1	47.521 (7.0)	47.556 (6.9)
Pr2	74.937 (6.1)	74.893 (6.0)
Pr3	21.252 (3.9)	21.335 (3.5)

[a] All chemical shifts in ppm from internal TSP. Ionic strength maintained with 1.0 M KCN. Chemical shifts for $NCCH_2(CN)Cbl$ have been corrected for the presence of 1.54% base-on $NCCH_2Cbl$ via an equation analogous to eq 12. [b] P–C coupling constant.

formation of the base-off alkyl(cyano)cobalamins (K_{CN}) were determined by spectrophotometric titration of the alkylcobalamins found to be stable toward cyanolysis at wavelengths of 580–600 nm corresponding to formation of a new d–d transition band common to all such species. The values are listed in Table III along with the value for R = CN (i.e., formation of dicyanocobalamin, $(CN)_2Cbl$) previously determined by Reenstra and Jencks.[24] Unfortunately, for most of the compounds the formation constants for the alkyl(cyano)cobalamins are too low to provide sufficient amounts of these species in 1.0 M KCN. However, the formation constants for dicyanocobalamin and cyanomethyl(cyano)cobalamin are sufficiently high to provide 100 and 98.5%, respectively, of these species in 1.0 M KCN. The ^{13}C NMR resonances of the nucleotide loop of these two species are shown in Table IV, in which the chemical shifts of $NCCH_2(CN)Cbl$ have been corrected for the presence of 1.5% base-on $NCCH_2Cbl$ via an equation analogous to eq 12. Although only two compounds are available, it is clear from the data that the chemical shifts for the two compounds are very similar and it seems reasonable to conclude that the nucleotide loops of both compounds are magnetically identical.

Structure **10** shows the comparison of the chemical shifts of



10

the benzimidazole moiety of these base-off, but deprotonated alkyl(cyano)cobalamins to that of the dianion of α-ribazole-3′-phosphate as the signed difference between the nucleotide dianion chemical shift and that of the average of the two R(CN)Cbl species. As we have previously shown that the presence or absence of phosphate at R3 in the free nucleoside has very little effect on the benzimidazole carbon chemical shifts,[30] this comparison is relevant. Using the previously developed criterion of significance (0.15 ppm), the comparison shows significant, and often very large chemical shift differences at all of the benzimidazole carbons except B7. It thus seems impossible to escape the conclusion that a significant interaction occurs between the free base benzimidazole moiety and the remainder of the structure in the benzimidazole deprotonated, base-off species.

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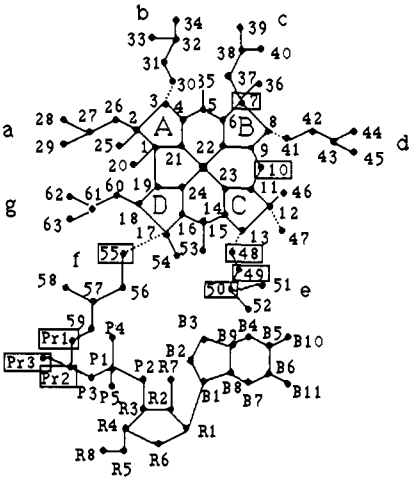

Figure 4. Numbering scheme for the cobalamins, highlighting the chemical shift differences between $(CN)_2Cbl$ and $(CN)_2Cbi$ (Table V).

In order to attempt to determine where in the remainder of the structure the interaction occurs and perhaps the nature of this interaction, the ^{13}C NMR spectra of dicyanocobalamin, which contains a pendent deprotonated but uncoordinated benzimidazole nucleotide, and dicyanocobinamide, from which the nucleotide has been removed, were compared. The data are shown in Table V and the chemical shift differences are highlighted in Figure 4, which also shows the standard cobalamin numbering scheme. The assignments were originally based on the assignments for $(CN)_2Cbl$ of Bratt and Hogenkamp.[32] However, while this report was being prepared, we became aware of a number of reassignments in the ^{13}C NMR spectrum of base-on and base-off AdoCbl recently made by Summers et al.[33,34] who used two-dimensional NMR techniques to unambiguously establish connectivity and rigorously assign both the ^{13}C and ^{1}H NMR spectra of base-on and base-off AdoCbl. The ^{13}C NMR spectra in Table V have consequently been reassigned based on the new assignments of Summers et al. for AdoCbl[33,34] and relative chemical shifts of AdoCbl and $(CN)_2Cbl$ of Bratt and Hogenkamp.[32] It is, however, important to point out that these changes in assignment in no way affect the conclusions to be drawn from the data in Table V as the resonances for the e and f side chains have not been reassigned by the more recent work. The data show that the ^{13}C NMR spectra of $(CN)_2Cbl$ and $(CN)_2Cbi$ are remarkably similar, only 8 of 48 resonances showing significant differences. Within the corrin ring only C7 shows a barely significant chemical shift difference (using the 0.15-ppm criterion). Very significant differences are seen in the pendent isopropanolamine moiety as would be expected owing to detachment of the axial nucleotide and the attendant changes in electronic effects and, presumably, isopropanolamine conformation. Extending back into the f propionamide side chain, to which the isopropanolamine is attached, the C55 methylene group shows a large chemical shift difference while the C56 methylene group shows none. However, we cannot be certain from these data that the assignments of C55 and C56 in the cobinamide spectrum are not in fact reversed, which would give rise to large chemical shift differences at both methylene carbons in the f side chain. This again suggests a major conformational change in the f side chain upon detachment of the axial nucleotide. Importantly, the chemical shift of the f side chain carbonyl carbon (C57) is not changed as would be expected since the conformationally rigid sp^2-hybridized carbon is too remote from Pr2 to experience any significant electronic effect due to loss of the phosphate moiety. The only other place in the molecule where significant chemical shift differences are seen is in the e propionamide side chain where both of the methylene groups (C48 and C49) *and* the carbonyl carbon (C50) show significant effects. Thus, the likely site of interaction of the pendent, but deprotonated benzimidazole in the base-off alkyl(cyano)cobalamins is the e side chain. Since the conformationally rigid carbonyl carbon of the e side chain is also affected, an electronic interaction is indicated.

Table V. ^{13}C NMR Chemical Shifts for Dicyanocobalamin and Dicyanocobinamide, 25.0 °C, 1.0 M KCN

	δ (ppm)		
atom	$(CN)_2Cbi$	$(CN)_2Cbl$	$(CN)_2Cbi - (CN)_2Cbl$
	Corrin and Corrin Methyls		
1	85.587	85.590	−0.003
2	48.836	48.767	0.068
3	58.823	58.920	−0.097
4	181.283	181.245	0.038
5	107.651	107.715	−0.064
6	165.433	165.531	−0.098
7	51.716	51.863	−0.147
8	57.792	57.891	−0.099
9	174.531	174.580	−0.049
10	93.544	93.672	−0.128
11	179.325	179.267	0.058
12	49.361	49.422	−0.061
13	55.741	55.812	−0.071
14	165.928	165.891	0.037
15	105.611	105.655	−0.044
16	181.043	181.104	−0.061
17	61.303	61.376	−0.073
18	41.534	41.490	0.044
19	77.553	77.549	0.004
20	21.644	21.731	−0.090
25	20.034	19.954	0.080
35	17.780	17.868	−0.088
36	21.338	21.432	−0.094
46	34.456	34.409	−0.047
47	24.265	24.236	−0.029
53	17.623	17.663	−0.040
54	19.102	19.043	0.059
	a Side Chain		
26	46.377	46.438	−0.061
27	178.451	178.537	−0.086
	b Side Chain		
30	28.285	28.278	0.007
31	37.565	37.628	−0.063
32	180.031	180.066	−0.035
	c Side Chain		
37	44.856	44.848	0.008
38	177.644	177.661	−0.017
	d Side Chain		
41	27.414	27.339	0.075
42	34.456	34.409	−0.047
43	179.869	179.823	0.046
	e Side Chain		
48	29.177	29.326	−0.149
49	35.105	35.579	−0.474
50	180.597	180.329	0.268
	f Side Chain		
55	34.134	34.737	−0.603
56	33.063	33.128	−0.065
57	177.745	177.837	−0.092
	Isopropanolamine		
Pr1	48.836	47.521	1.315
Pr2	68.865	73.669	−4.804
Pr3	22.189	21.252	0.937
	g Side Chain		
60	35.105	35.067	0.038
61	178.434	178.441	−0.007

It thus seems likely that in the base-off, but benzimidazole deprotonated species, benzimidazole B3 is hydrogen bonded to one of the amide hydrogens on the e side chain. If this is correct, one might expect the pattern of chemical shift differences between the free nucleotide dianion and the base-off R(CN)Cbl's (structure **10**) to resemble the pattern of chemical shift differences due to protonation of the free nucleotide or nucleoside at B3.[30] In fact, the signs of these patterns of chemical shift differences are similar except at B7, where the difference in **10** is negligible, and at B9

and B4 where the signs are reversed in **10** compared to the difference between the deprotonated and protonated nucleoside and nucleotide. This suggests that in addition to hydrogen bonding between B3 and an e side chain amide hydrogen another interaction may be occurring involving the top edge of the benzimidazole moiety. This additional interaction could be a hydrophobic interaction between the top edge of the benzimidazole moiety and the e side chain methylene groups, although we cannot rule out the possibility that dipolar shielding from the cobalt atom is responsible for this effect, as neither the exact geometry of the hydrogen-bonded species nor the magnetic anisotropy of the cobalt atom in any base-off cobalamin is known.

Attempts are currently in progress to evaluate the equilibrium constant for formation of the hydrogen-bonded base-off complex and thus to reinterpret the thermodynamics of the base-on/base-off reaction. These results will be reported in a subsequent communication.

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Chlorophyll Model Compounds: Effects of Low Symmetry on the Resonance Raman Spectra and Normal Mode Descriptions of Nickel(II) Dihydroporphyrins

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Abstract: Resonance Raman (RR) spectra are reported for a series of nickel(II) dihydroporphyrins with excitation in the B, Q_x, and Q_y absorption regions. The molecules include *trans*-octaethylchlorin, γ,δ-deuteriated *trans*-octaethylchlorin, 9-deoxomethylmesopyropheophorbide *a*, methylmesopyropheophorbide *a*, and methylpyropheophorbide *a*. These molecules represent a series in which the structural complexities of chlorophyll *a* (reduced pyrrole ring, isocyclic ring, 9-keto group, and 2-vinyl group) are systematically added to the basic tetrapyrrole structure. All of the observed in-plane chlorin skeletal modes and vibrations of the isocyclic ring and vinyl group are assigned. Assignments are then proposed for chlorophyll *a* based on analogy to those of the Ni(II) complexes. In addition to the spectral assignments, normal coordinate calculations are performed on the various nickel(II) dihydroporphyrins. These calculations indicate that the forms of the normal coordinates of the metallochlorins bear little resemblance to those of the parent metalloporphyrins. In the low-symmetry environment which characterizes the reduced pyrrole pigments, a number of the vibrations are localized in semicircles or quadrants of the macrocycle rather than being delocalized over the entire ring. This vibrational localization is due to geometrical changes in the π-bonded system which occur as a result of reduction of one of the pyrrole rings and addition of the isocyclic ring.

I. Introduction

Metallodihydroporphyrins (metallochlorins) are ubiquitous in nature, occurring in various chlorophyll pigments,[1] in the marine worm pigment, bonellin,[2] and in the green heme proteins, myeloperoxidase,[3,4] sulfmyoglobin,[5-7] sulfhemoglobin,[7b,8] and microbial hemes *d* and d_1.[3,9-12] The biological rationale for the presence of a prosthetic group which contains one reduced pyrrole ring (or two in the case of bacteriochlorophyll) is not presently understood. However, it is clear that modification of the basic tetrapyrrole structure significantly alters the photophysical, redox, and ligand binding properties of metallochlorin vs. metalloporphyrin systems.[13-16] The altered nature of these physical properties is presumably responsible for the evolutionary selection of the metallodihydroporphyrin moiety as the active unit in the protein.

Resonance Raman (RR) spectroscopy has proven to be an extremely useful probe of the vibrational and electronic structure of metalloporphyrins and heme proteins.[17-20] More recently, metallochlorins and chlorophyll have come under increased scrutiny by the RR technique.[3,4,6,21-27] The RR spectra of me-

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