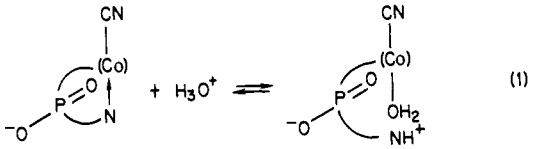
Heteronuclear NMR Studies of Cobalamins. 3. ^{31}P NMR of Aquocobalamin and Various Organocobalamins[1]

Kenneth L. Brown,*† Janette M. Hakimi,† and Donald W. Jacobsen‡

Contribution from the Department of Chemistry, The University of Texas at Arlington, Arlington, Texas 76019-0065, and the Division of Biochemistry, Department of Basic and Clinical Research, Scripps Clinic and Research Foundation, LaJolla, California 92037.
Received April 16, 1984

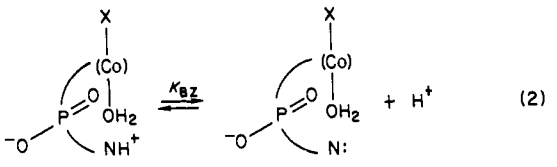
Abstract: ^{31}P NMR observations of aquocobalamin (H_2OCbl) in H_2SO_4/H_2O mixtures show that both the base-on and base-off species may be observed and quantitated. Correlation of the base-off ^{31}P chemical shifts with a generalized acidity function gives values of −1.59 and −0.09 for the two macroscopic pK_a's for phosphodiester deprotonation, virtually identical with the values previously obtained for base-off cyanocobalamin and base-off methylcobalamin. Similar studies of five alkylcobalamins show that all base-off cobalamins have the same phosphodiester pK_a's and chemical shifts. Correlation of the base-on ^{31}P-chemical shifts of H_2OCbl, however, shows that a different acidity function is followed, and the phosphodiester pK_a's are different from those of the base-off species. It is also shown that while all base-off cobalamins have the same ^{31}P chemical shift at both high and low ionic strength, the chemical shifts of ten base-on cobalamins vary in a regular way with the apparent free energy of coordination of the pendant axial 5,6-dimethylbenzimidazole ligand. These effects are interpreted in terms of variations of the axial Co–N bond length leading to progressive changes in the base-on phosphodiester conformation throughout a series of ten cobalamins.

In a recent report[2] we demonstrated that upon protonation and displacement of the axial dimethylbenzimidazole ligand of cyanocobalamin (CNCbl) in sulfuric acid/water mixtures (eq 1, $pK_a = 0.11$[2]), the chemical shift of the ^{31}P NMR resonance of the phosphodiester of the nucleotide loop undergoes an upfield shift of 35.7 Hz (at 81 MHz). This result confirmed that the earlier

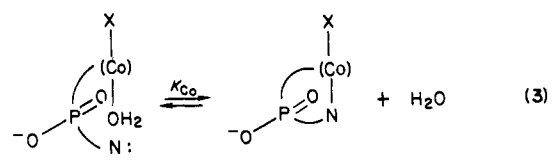


(1)

demonstration of an upfield shift of similar magnitude of the ^{31}P resonance of CNCbl upon reaction with excess cyanide ion[3-6] was indeed due to displacement of the axial nucleotide. However, we were unable to find any change in ^{31}P chemical shift of methylcobalamin (CH_3Cbl) upon displacement of its axial base by protonation ($pK_a = 2.89$).[2,6] As the chemical shifts of the protonated, base-off forms of CNCbl and CH_3Cbl are nearly identical (−37.40 and −38.47 Hz, respectively, relative to external 85% H_3PO_4),[2] the difference in behavior of the phosphorus chemical shift upon base displacement is entirely due to the difference in ^{31}P chemical shift of the two base-on cobalamins. One major difference between these two base-on cobalamins is the apparent affinity of the free base benzimidazole ligand for the cobalt atom. This may be appreciated by considering the base-on–base-off protonic equilibrium of eq 1 to be the sum of two consecutive equilibria (eq 2 and 3). This treatment readily leads to eq 4 which



(2)



(3)

relates the apparent K_a of the protonated, base-off species ($K_{base\text{-}off}$) to the K_a for proton dissociation of the pendant benzimidazolium ion of the base-off species (K_{Bz}) and the intrinsic affinity of the free base pendant benzimidazole ligand for the cobalt atom (K_{Co}).

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

If we assume, as is often done, that pK_{Bz} is equivalent to the pK_a of the conjugate acid of the detached benzimidazole nucleoside (1-α-D-ribofuranosyl-5,6-dimethylbenzimidazole or α-ribazole, $pK_a = 5.56$ at 25 °C, ionic strength 1.0 M[7,8]), we can show that the free base benzimidazole ligand of base-on CNCbl is bound nearly three orders of magnitude more tightly than that of CH_3Cbl. On the basis of the work of Gorenstein and co-workers,[9-11] who have shown that phosphate ester and phosphodiester ^{31}P chemical shifts are largely, if not completely, determined by conformation effects, it seemed reasonable that the difference in ^{31}P chemical shift between base-on CNCbl and CH_3Cbl was due to differences in phosphodiester conformation which in turn were due to the difference in tightness with which the axial base is coordinated to the cobalt atom. If this hypothesis is correct, several predictions can be made about the ^{31}P NMR behavior of cobalamins in general. First, all base-off cobalamins should have the same ^{31}P chemical shift, if indeed all such species have the same phosphodiester conformation. Second, aquocobalamin (H_2OCbl) in which the axial base is bound some two orders of magnitude more tightly than that of CNCbl ($pK_{base\text{-}off} = -2.4$[12]) should have an even more distorted phosphodiester conformation than CNCbl and its base-on ^{31}P chemical shift should be even further downfield than that of CNCbl. Third, a series of organocobalamins whose $pK_{base\text{-}off}$ values vary from above 4.0 to below 2.0[8] should show a regular progression of ^{31}P chemical shifts as variations in K_{Co} (eq 3) cause progressive distortions of the phosphodiester conformation. We consequently have examined the ^{31}P NMR properties of H_2OCbl plus a series of eight organocobalamins, and the results are the subject of this report.

† The University of Texas at Arlington.
‡ Scripps Clinic and Research Foundation.

(1) For Part 2, see ref 2.
(2) Brown, K. L.; Hakimi, J. M. *Inorg. Chem.* **1984**, *23*, 1756–1764.
(3) Satterlee, J. D. *Biochem. Biophys. Res. Commun.* **1979**, *89*, 272–278.
(4) Satterlee, J. D. *Inorg. Chim. Acta* **1980**, *46*, 157–166.
(5) Mishra, P. K.; Gupta, R. K.; Goswami, P. C.; Venkatasubramanian, P. N.; Nath, A. *Biochim. Biophys. Acta* **1981**, *668*, 406–412.
(6) Brown, K. L.; Hakimi, J. M. *Inorg. Chim. Acta* **1982**, *67*, L29–31.
(7) The wisdom of this assumption has recently been discussed.[8]
(8) Brown, K. L.; Hakimi, J. M.; Nuss, D. M.; Montejano, Y. D.; Jacobsen, D. W. *Inorg. Chem.* **1984**, *23*, 1463–1471.
(9) Gorenstein, D. G. *J. Am. Chem. Soc.* **1975**, *97*, 898–900.
(10) Gorenstein, D. G.; Kar, D. *Biochem. Biophys. Res. Commun.* **1975**, *65*, 1073–1080.
(11) Gorenstein, D. G. *J. Am. Chem. Soc.* **1977**, *99*, 2254–2258.
(12) Hayward, G. C.; Hill, H. A. O.; Pratt, J. M.; Vanston, N. J.; Williams, R. J. P. *J. Chem. Soc.* **1965**, 6485–6493.

0002-7863/84/1506-7894$01.50/0

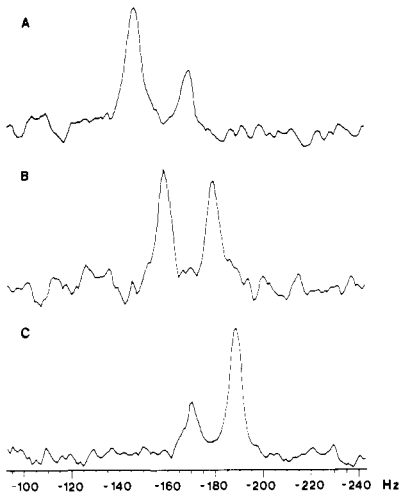


Figure 1. ^{31}P NMR spectra of H_2OCbl in sulfuric acid/water mixtures at 25 ± 1 °C. Chemical shifts are relative to external 85% H_3PO_4. A: $[H_2SO_4]$ = 3.9098 M, $\delta^{base\text{-}on}$ = −146.40 Hz, $\delta^{base\text{-}off}$ = −168.87 Hz, $\alpha_{base\text{-}on}$ = 0.659. B: $[H_2SO_4]$ = 4.5148 M, $\delta^{base\text{-}on}$ = −159.37 Hz, $\delta^{base\text{-}off}$ = −179.81 Hz, $\alpha_{base\text{-}on}$ = 0.528. C: $[H_2SO_4]$ = 5.2495 M, $\delta^{base\text{-}on}$ = −170.70 Hz, $\delta^{base\text{-}off}$ = −188.98 Hz, $\alpha_{base\text{-}on}$ = 0.328.

Experimental Section

5′-Deoxyadenosylcobalamin (AdoCbl), CNCbl, H_2OCbl, and CH_3Cbl were from Sigma. All other alkylcobalamins were synthesized by reductive alkylation of H_2OCbl with $NaBH_4$ as reducing agent and the appropriate alkyl halide as alkylating agent.[8] These products were purified by extraction through phenol,[13] chromatography on SP-Sephadex,[8,14,15] and crystallization from aqueous acetone. All alkylcobalamins were characterized by their UV-vis spectra in neutral (base-on) and acidic (base-off) solution, conversion to the spectrum of H_2OCbl (or HOCbl) upon photolysis and conversion to the spectrum of $(CN)_2Cbl$ upon photolysis in excess cyanide, and by migration as a single band during high-performance liquid chromatography.[8,16] The mixture of CF_3Cbl and CF_2HCbl obtained by reductive alkylation of H_2OCbl with CF_3Br[8] was resolved into pure components (as demonstrated by HPLC and ^{19}F NMR) by chromatography on Amberlite XAD[17] by using mixtures of acetonitrile and water to elute, as previously described.[8] All buffer components, inorganic salts and acids, chromatography solvents, alkylating agents, etc., were obtained in the highest purity commercially available and used without further purification. Glass-distilled, deionized water was used throughout.

^{31}P NMR and ^{19}F NMR spectra were obtained at 25 °C on a Nicolet NT-200 wide-bore superconducting spectrometer (4.7 T) operating at 80.988 and 188.239 MHz, respectively. Samples generally contained 5.0 × 10^{-3} to 1.0 × 10^{-2} M cobalamin in water, buffered water, or sulfuric acid/water mixtures, and a deuterium lock signal was provided by a concentric insert (Wilmad) containing D_2O. All chemical shifts were determined on proton-decoupled spectra by Gaussian line fits to the singlet resonances and are reported relative to external 85% H_3PO_4 (negative shifts are upfield from the reference).

Values of $pK_{base\text{-}off}$ for AdoCbl and $NCCH_2Cbl$ were determined by spectrophotometric titration at 25.0 °C in buffered aqueous media, and the ionic strength was adjusted to 1.0 M (KCl) as previously described.[8]

Results and Discussion

The proton-decoupled ^{31}P NMR spectra of H_2OCbl show two resonances at all acidities between 0.15 and 14.0 M H_2SO_4, one of which increases in intensity while the other decreases in intensity with increasing acidity (Figure 1) and both of which shift upfield with increasing acidity due to protonation of the phosphodiester moiety.[2,6] By analogy to CNCbl[2] which showed similar ^{31}P NMR spectra in H_2SO_4/H_2O these resonances may be assigned to the base-off and base-on forms of H_2OCbl, respectively. In the case of CNCbl, these assignments were confirmed by simultaneous observation of the ^{13}C resonance of $^{13}CNCbl$ in the same media. However, there are two significant differences in behavior between CNCbl and H_2OCbl in this regard. First, the concentration of H_2SO_4 needed to obtain equal intensity of the two ^{31}P resonances is significantly higher for H_2OCbl, reflecting the significantly lower value of $pK_{base\text{-}off}$. Second, while the equilibrium between the base-on and base-off forms of CNCbl was establised rapidly on the time scale of sample make-up and observation, the rate of equilibration of the two species is substantially slower for H_2OCbl. This latter observation is in accord with results of Hayward et al.,[12] who studied the base-on–base-off equilibrium of H_2OCbl in H_2SO_4/H_2O mixtures spectrophotometrically and found that equilibration was achieved "only slowly". This had two important consequences for the current measurements. First, in order to obtain accurate estimates of the relative amounts of each species present (for determination of $pK_{base\text{-}off}$) it was necessary to take several sequential sets of scans of each sample where significant amounts of both species were formed until the ratio of the integrals of the two peaks became constant. Second, the relatively slow rate of equilibration allowed observation of both the base-on and base-off species at acidities where one or the other makes a negligible contribution at equilibrium. In order to observe the base-on species at very high acidity, observations were simply made as rapidly as possible after sample make-up prior to the establishment of equilibrium. To make nonequilibrium observations of the base-off species at very low acidity, H_2OCbl was preincubated at a higher acidity until equilibrium was established and the sample was then rapidly diluted to a lower acidity and observed prior to reestablishment of equilibrium. In this manner both the base-on and base-off phosphorus chemical shifts could be observed over the entire range of acidity perturbing both phosphodiester protonations (Figure 2). It should be pointed out that, as was the case with CNCbl,[2] at very high acidities and/or very long scan times significant phosphodiester hydrolysis of H_2OCbl is observed to give, initially, two phosphorus-containing products (i.e., α-ribazole-3′-phosphate and aquocobinamide phosphate) from asymmetric phosphodiester hydrolysis and, finally, phosphoric acid. Fortunately, phosphodiester hydrolysis is sufficiently slow not to interfere with determinations of the relative amounts of base-on and base-off species over the necessary acidity range.

As two sequential protonations of the phosphodiester of both the base-on and base-off species are clearly possible, the complete ionization scheme for H_2OCbl is as given in Scheme I. From

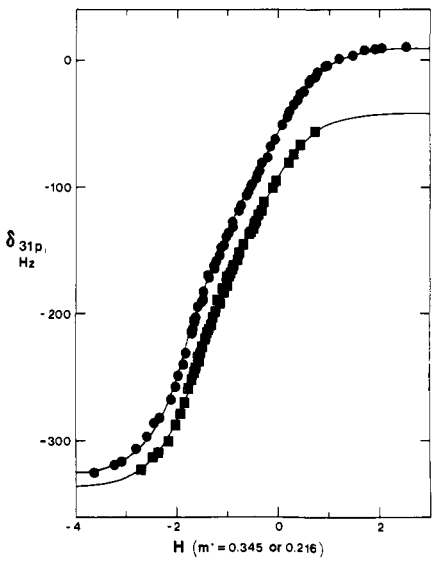
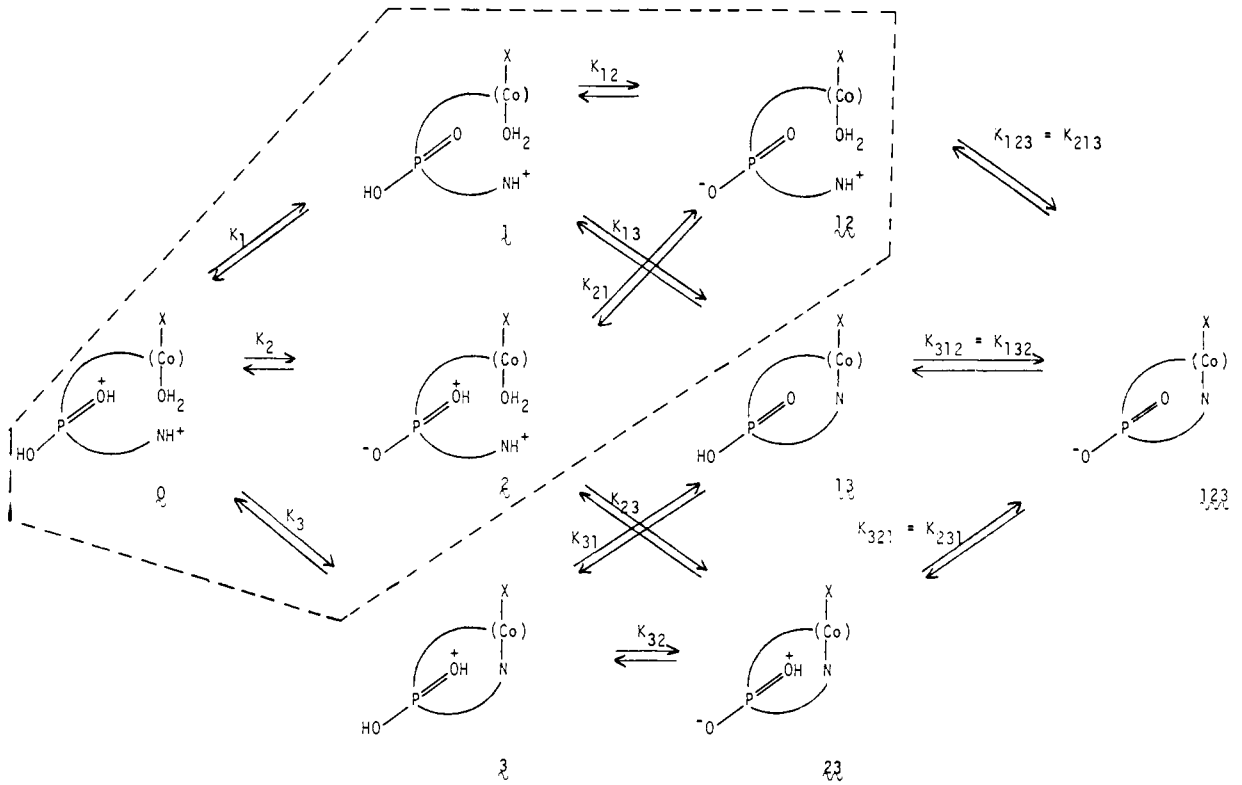


Figure 2. Plots of $\delta_{31P}^{base\text{-}on}$ (●) and $\delta_{31P}^{base\text{-}off}$ (■) for H_2OCbl at 25 ± 1 °C vs. H (eq 22) at m^* = 0.345 and 0.216, respectively. The solid lines are non-linear least-squares fits to eq 19 and 20, respectively. The final fit parameters were as follows: base-off, $\delta^{(0)}$ = −337.10 Hz, $K_1\delta^{(1)} + K_2\delta^{(2)}$ = −5743.21 Hz, $G_4G_5\delta^{(12)}$ = −1969.37 Hz, G_4 = 38.51, and G_4G_5 = 47.29; base-on, $\delta^{(3)}$ = −326.03 Hz, $K_{31}\delta^{(13)} + K_{32}\delta^{(23)}$ = −6385.16 Hz, $G_6G_7\delta^{(123)}$ = 493.31 Hz, G_6 = 54.39, G_6G_7 = 52.68.

(13) Dolphin, D. *Methods Enzymol.* **1971**, *18*, 34–52.
(14) Tortolani, G.; Bianchini, P.; Montovani, V. *J. Chromatogr.* **1970**, *53*, 577–579.
(15) Tortolani, G.; Montovani, V. *J. Chromatogr.* **1974**, *92*, 201–206.
(16) Jacobsen, D. W.; Green, R.; Quadros, E. V.; Montejano, Y. D. *Anal. Biochem.* **1982**, *120*, 394–403.
(17) Vogelman, H.; Wagner, F. *J. Chromatogr.* **1973**, *76*, 359–379.

Scheme I[a]



[a] Equilibrium constants subscripted $xx1$ are for P=OH$^+$ ionization, $xx2$ are for P–OH ionization, and $xx3$ are for benzimidazolium ionization and coordination to cobalt. The ionization scheme for the base-off phosphodiester is enclosed in the dashed line.

the scheme and the law of mass action we can define three macroscopic pK_a's for the overall ionization (eq 5–10) as well as two

$$G_1 = ([\mathbf{1}] + [\mathbf{2}] + [\mathbf{3}])a_{H^+}/[\mathbf{0}] \quad (5)$$

$$G_1 = K_1 + K_2 + K_3 \quad (6)$$

$$G_2 = ([\mathbf{12}] + [\mathbf{13}] + [\mathbf{23}])a_{H^+}/([\mathbf{1}] + [\mathbf{2}] + [\mathbf{3}]) \quad (7)$$

$$G_2 = (K_1K_{12} + K_1K_{13} + K_2K_{23})/(K_1 + K_2 + K_3) \quad (8)$$

$$G_3 = [\mathbf{123}]a_{H^+}/([\mathbf{12}] + [\mathbf{13}] + [\mathbf{23}]) \quad (9)$$

$$G_3 = K_1K_{12}K_{23}/(K_1K_{12} + K_1K_{13} + K_2K_{23}) \quad (10)$$

additional macroscopic pK_a's each for the base-off (eq 11–14) and base-on (eq 15–18) phosphodiester ionizations.

$$G_4 = ([\mathbf{1}] + [\mathbf{2}])a_{H^+}/[\mathbf{0}] \quad (11)$$

$$G_4 = K_1 + K_2 \quad (12)$$

$$G_5 = [\mathbf{12}]a_{H^+}/([\mathbf{1}] + [\mathbf{2}]) \quad (13)$$

$$G_5 = K_2K_{21}/(K_1 + K_2) \quad (14)$$

$$G_6 = ([\mathbf{13}] + [\mathbf{23}])a_{H^+}/[\mathbf{3}] \quad (15)$$

$$G_6 = K_{31} + K_{32} \quad (16)$$

$$G_7 = [\mathbf{123}]a_{H^+}/([\mathbf{13}] + [\mathbf{23}]) \quad (17)$$

$$G_7 = K_{31}K_{312}/(K_{31} + K_{32}) \quad (18)$$

From these definitions and the law of mass action we can then derive equations for the dependence of the base-off (eq 19) and base-on (eq 20) ^{31}P chemical shifts on the activity of hydrogen ion, where $\delta^{(0)}$, $\delta^{(1)}$, etc., represent the ^{31}P chemical shifts of species **0**, **1**, etc. (Scheme I). In addition, since we can evaluate the

$$\delta_{^{31}P}{}^{\text{base-off}} = \frac{\delta^{(0)} + (K_1\delta^{(1)} + K_2\delta^{(2)})/a_{H^+} + G_4G_5\delta^{(12)}/a_{H^+}{}^2}{1 + G_4/a_{H^+} + G_4G_5/a_{H^+}{}^2} \quad (19)$$

$$\delta_{^{31}P}{}^{\text{base-on}} = \frac{\delta^{(3)} + (K_{31}\delta^{(13)} + K_{32}\delta^{(23)})/a_{H^+} + G_6G_7\delta^{(123)}/a_{H^+}{}^2}{1 + G_6/a_{H^+} + G_6G_7/a_{H^+}{}^2} \quad (20)$$

fraction of base-on species present at any acidity (i.e., $\alpha_{\text{base-on}}$) from integrations of the two ^{31}P resonances after equilibrium is established, we can also derive eq 21 for the dependence of $\alpha_{\text{base-on}}$ on hydrogen ion activity.

$$\alpha_{\text{base-on}} = \frac{K_3a_{H^+}{}^2 + (K_1K_{13} + K_2K_{23})a_{H^+} + G_1G_2G_3}{a_{H^+}{}^3 + G_1a_{H^+}{}^2 + G_1G_2a_{H^+} + G_1G_2G_3} \quad (21)$$

As was the case with CNCbl[2] attempts to fit the $\delta_{^{31}P}{}^{\text{base-off}}$ and $\delta_{^{31}P}{}^{\text{base-on}}$ data to eq 19 and 20 by using established acidity functions such as H_0[18-20] or H_A[21-24] are unsuccessful, the data clearly demanding an acidity function which rises less steeply with [H_2SO_4] than either of these. Our approach is to use the generalized acidity function treatment of Cox and Yates[25] (eq 22), where C_{H^+} is the

$$-H = m^*X + \log C_{H^+} \quad (22)$$

concentration of hydrogen ion, X is the "excess acidity", and m^* is a parameter reflecting the solvation demands of the protonated species in question. The $\delta_{^{31}P}{}^{\text{base-off}}$ data were fit to eq 19 via a non-linear least-squares routine using a simplex minimization algorithm and an acidity function based on eq 22 with an arbitrary value of m^*. This procedure was repeated with different values of m*. The standard deviation of the fit was found to go through a sharp minimum at $m^* = 0.216$, producing the fit shown in Figure 2 (lower curve). The resulting values of $\delta_{^{31}P}{}^{(0)}$, $\delta_{^{31}P}{}^{(12)}$, pG_4 and pG_5 for H_2OCbl are collected in Table I along with those pre-

(18) Hammett, L. P.; Deyrup, A. J. *J. Am. Chem. Soc.* **1932**, *54*, 2721–2739.
(19) Bascombe, K. N.; Bell, R. P. *J. Chem. Soc.* **1959**, 1096–1104.
(20) Ryabova, R. S.; Medvetskaya, I. M.; Vinnik, M. T. *Zh. Fiz. Khim.* **1966**, *40*, 339–345.
(21) Yates, K.; Stevens, J. B.; Katritsky, A. R. *Can. J. Chem.* **1964**, *42*, 1957–1970.
(22) Yates, K.; Wai, H. *J. Am. Chem. Soc.* **1964**, *86*, 5408–5413.
(23) Yates, K.; Stevens, J. B. *Can. J. Chem.* **1965**, *43*, 529–537.
(24) Edward, J. T.; Wong, S. C. *Can. J. Chem.* **1977**, *55*, 2492–2494.
(25) Cox, R. A.; Yates, K. *J. Am. Chem. Soc.* **1978**, *100*, 3861–3867.

Table I. ^{31}P Chemical Shifts and Phosphodiester Macroscopic pK_a's for Cobalamins, 25 ± 1 °C

	base-off species					base-on species				
cobalamin	$\delta_{^{31}P}^{(0)}$,[a] Hz	$\delta_{^{31}P}^{(12)}$,[a] Hz	m^*[b]	pG_4[c]	pG_5[d]	$\delta_{^{31}P}^{(3)}$,[a] Hz	$\delta_{^{31}P}^{(123)}$,[a] Hz	m^*[b]	pG_6[e]	pG_7[f]
H_2OCbl	−337.10	−41.64	0.216	−1.59	−0.09	−326.03	+9.37	0.345	−1.74	+0.01
$CNCbl$[g]	−335.81	−37.40	0.217	−1.57	−0.04					
CH_3Cbl[g]	−335.43	−38.47	0.225	−1.62	−0.02					
$CH_3(CH_2)_2Cbl$	−337.76	−38.18	0.215	−1.58	−0.05					
$NC(CH_2)_3Cbl$	−342.14	−36.63	0.204	−1.60	−0.02					
CF_3CH_2Cbl	−345.50	−33.51	0.197	−1.56	−0.02					
CF_2HCbl	−342.31	−33.47	0.213	−1.61	−0.03					
$NCCH_2Cbl$	−342.14	−34.33	0.204	−1.60	−0.02					
average	−339.88 ± 3.8	−36.70 ± 2.8	0.212 ± 0.009	−1.59 ± 0.02	−0.04 ± 0.02					

[a] From external 85% H_3PO_4. Negative shifts are upfield from the reference. [b] Equation 22. [c] Defined in eq 11 and 12. [d] Defined in eq 13 and 14. [e] Defined in eq 15 and 16. [f] Defined in eq 17 and 18. [g] Reference 2.

viously determined for CNCbl and CH_3Cbl from which it is clear that the limiting chemical shifts, the phosphodiester pK_a's, and the acidity function followed (i.e., m^*) are virtually identical for all three base-off cobalamins.

A similar treatment of the $\delta_{^{31}P}^{\text{base-on}}$ data for H_2OCbl via eq 20 produced the fit shown in Figure 2 (upper line) and the limiting chemical shifts and base-on phosphodiester pK_a's listed in Table I. These data show that the acid–base behavior of the base-on phosphodiester and the protonated and deprotonated base-on phosphodiester chemical shifts are clearly different than those of the base-off species. In addition, we may now calculate the change in chemical shift upon displacement of the axial base from the phosphodiester deprotonated species, $\Delta\delta_{^{31}P}$ (eq 23), to be +51.01 Hz, significantly larger than the value of $\Delta\delta_{^{31}P}$ of +35.68 Hz previously obtained for CNCbl.[2]

$$\Delta\delta_{^{31}P} = \delta^{(123)} - \delta^{(12)} \tag{23}$$

We have also attempted to analyze our data for $\alpha_{\text{base-on}}$ for H_2OCbl according to eq 21 but with considerably less success. Unlike the case of CNCbl, the standard deviation of the fit showed significant variation with m^*, had a minimum at $m^* = 1.005$, and produced the values p$K_3 = -2.13$, p$G_1 = -2.14$, p$G_2 = 0.06$, and p$G_3 = -0.52$. However, all of the equation parameters except K_3 were significantly sensitive to initial guesses and hence must be considered to be poorly determined. As for CNCbl, however, these data may be successfully treated as a single protonation (i.e., eq 1) by fitting to eq 24. The standard deviation of this fit (Figure 3) was minimized at $m^* = 0.992$ (compared to 0.919 for CNCbl[2]) and gave a value of −2.13 for p$K_{\text{base-off}}$, in quite good agreement with the value of −2.4 previously obtained by Hayward et al.[12] from spectrophotometric titrations of H_2OCbl in H_2SO_4/H_2O mixtures using the Hammett acidity function, H_0 (i.e., $m^* = 1.02$[25]).

$$\alpha_{\text{base-on}} = \frac{K_{\text{base-off}}}{K_{\text{base-off}} + a_{H_2O}} \tag{24}$$

In order to determine accurate values of $\delta_{^{31}P}^{(12)}$ (Scheme I) for additional cobalamins, five alkylcobalamins ($CH_3(CH_2)_2Cbl$, $NC(CH_2)_3Cbl$, CF_3CH_2Cbl, CF_2HCbl, and $NCCH_2Cbl$) were also subjected to base-off phosphodiester titration in H_2SO_4/H_2O mixtures by ^{31}P NMR. In all cases the lowest acidity used was at least two acidity function units below the measured value of p$K_{\text{base-off}}$[8] to avoid perturbation of the observed ^{31}P resonance by the base-on–base-off equilibrium. Unfortunately, this precluded the use of CF_3Cbl whose p$K_{\text{base-off}}$ value (1.44[8]) was too low to permit accurate determination of the upper end point of the titration curve. AdoCbl could not be titrated in this manner due to its known acid lability.[26] These data were fit to eq 19 by using the Cox and Yates acidity function (eq 22) as described above. The limiting chemical shifts, phosphodiester macroscopic pK_a's, and minimized m^* values for these five base-off cobalamins are listed in Table I along with those of CN^-, H_2O–, and CH_3Cbl.

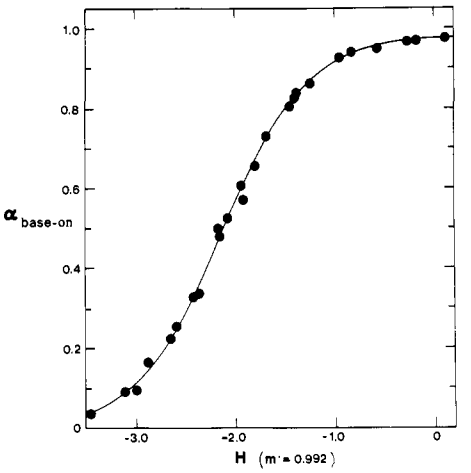


Figure 3. Plot of $\alpha_{\text{base-on}}$ for H_2OCbl (25 ± 1 °C) vs. H (eq 22) at $m^* = 0.992$. The solid line is a least-squares fit to eq 24 from which the value p$K_{\text{base-off}} = -2.13$ was obtained.

It is readily apparent that all five of these parameters describing the base-off phosphodiester ($\delta_{^{31}P}^{(0)}$, $\delta_{^{31}P}^{(12)}$, m^*, pG_4, and pG_5) are independent of the nature of the upper axial ligand. The average values and standard deviations of each parameter are given in Table I from which it can be seen that all eight values for each parameter fall within 95% confidence limits of the mean. It seems reasonable to conclude that *all* base-off cobalamins will have the same phosphodiester macroscopic pK_a's, follow the same acidity function, and have the same limiting values of ^{31}P NMR chemical shifts (i.e., $\delta_{^{31}P}^{(0)}$ and $\delta_{^{31}P}^{(12)}$).

In order to calculate values of $\Delta\delta_{^{31}P}$ (eq 23) for these five alkylcobalamins and estimate them for two others (AdoCbl and CF_3Cbl), values for the base-on, phosphodiester-deprotonated ^{31}P chemical shifts (i.e., $\delta_{^{31}P}^{(12)}$, Scheme I) were measured in pure water at 25 °C. Calculations based on the measured values of p$K_{\text{base-off}}$ (1.44 to 4.10) showed that "hydrolysis" to form the base-off species in water was negligible at the concentration of cobalamin used (4.0×10^{-3} M). These values of $\delta_{^{31}P}^{(12)}$ along with those for CNCbl and H_2OCbl are listed in Table II. For values of $\Delta\delta_{^{31}P}$ for AdoCbl and CF_3Cbl to be estimated, their $\delta_{^{31}P}^{(12)}$ values were assumed to be equal to the average $\delta_{^{31}P}^{(12)}$ for the eight cobalamins listed in Table I (i.e., −36.70 Hz).

In order to check these values of $\Delta\delta_{^{31}P}$ and to determine the effects of ionic strength on the ^{31}P chemical shifts, which are sometimes very large for phosphate compounds,[27] we have determined the values of $\delta_{^{31}P}^{(123)}$ and $\delta_{^{31}P}^{(12)}$ for seven of the alkylcobalamins by ^{31}P NMR titration through the p$K_{\text{base-off}}$ regions in buffered aqueous solution and ionic strength 1.0 M (adjusted with KCl). For all the alkylcobalamins only a single ^{31}P resonance is seen at all pH's indicating that the equilibration of the base-on and base-off forms is fast on the NMR time scale for these

(26) Hogenkamp, H. P. C.; Barker, H. A. *J. Biol. Chem.* **1961**, *236*, 3097–3101.

(27) Costello, A. J.; Glorek, T.; VanWazer, J. R. *Inorg. Chem.* **1976**, *15*, 972–974.

Table II. ^{31}P NMR Chemical Shifts of Base-On and Base-Off Phosphodiester Deprotonated Cobalamins, RCbl, 25 ± 1 °C[a]

R	$pK_{base\text{-}off}$[d]	K_{Co}[e]	ΔG_{Co},[e] kcal/mol^{-1}	I = 0 M[b] $\delta_{31P}^{(123)}$, Hz	$\delta_{31P}^{(12)}$, Hz	$\Delta\delta_{31P}$,[f] Hz	I = 1.0 M[c] $\delta_{31P}^{(123)}$, Hz	$\delta_{31P}^{(12)}$, Hz	$\Delta\delta_{31P}$,[f] Hz
$CH_3(CH_2)_2$-	4.10	28.0	-1.97	-50.16	-38.18	-11.98	-50.36	-40.45	-9.91
Ado-	3.67[g]	76.6	-2.57	-47.15	-36.70[h]	-10.45	-50.42	-39.98	-10.44
$NC(CH_2)_3$-	3.50	1.15×10^2	-2.81	-43.66	-36.63	-7.03	-45.84	-39.82	-6.02
CH_3-	2.89	4.67×10^2	-3.64	-35.66	-38.47	2.81	-37.93	-40.79	2.86
CF_3CH_2-	2.60	9.23×10^2	-4.04	-32.91	-33.51	0.60	-34.30	-37.29	2.99
CF_2H-	2.15	2.60×10^3	-4.66	-25.58	-33.47	7.89	-28.04	-37.75	9.71
$NCCH_2$-	1.81[g]	6.40×10^3	-5.19	-25.91	-34.33	8.42	-26.97	-40.16	13.19
CF_3-	1.44	1.32×10^4	-5.62	-11.99	-36.70[h]	24.71			
CN-	0.10[i]	2.88×10^5	-7.44	-1.72[i]	-37.40[i]	35.68			
H_2O-	-2.13[g]	4.90×10^7	-10.48	+9.37	-41.64	51.01			

[a] All chemical shifts in Hz from external 85% H_3PO_4. Negative shifts are upfield from the reference. [b] $\delta_{31P}^{(123)}$ from Table I, $\delta_{31P}^{(12)}$ measured in plain water. [c] From fits of δ_{31P}^{obsd} vs. $\alpha_{base\text{-}on}$ (Figure 4) to eq 25. [d] Reference 8, unless otherwise noted. [e] Calculated from eq 4, assuming pK_{Bz} = 5.56.[8] [f] Equation 23. [g] This work. [h] Assumed to be equal to the average value of $\delta_{31P}^{(12)}$ for the eight cobalamins listed in Table I. [i] Reference 2.

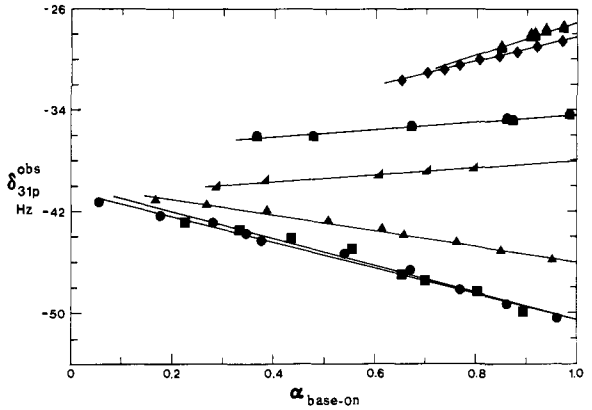


Figure 4. Plots of δ_{31P}^{obsd} vs. $\alpha_{base\text{-}on}$ (eq 24) at 25 ± 1 °C for seven alkylcobalamins. The solid lines are linear least-squares fits to eq 25: (●) $CH_3(CH_2)_2$Cbl, slope = -9.91 Hz, intercept = -40.45 Hz; (■) AdoCbl, slope = -10.44 Hz, intercept = -39.98 Hz; (▲) $NC(CH_2)_3$Cbl, slope = -6.02 Hz, intercept = -39.82 Hz; (◢) CH_3Cbl, slope = 2.86 Hz, intercept = -40.79 Hz; (⬟), CF_3CH_2Cbl, slope = 2.99 Hz, intercept = -37.29 Hz; (◆) CF_2HCbl, slope = 9.71 Hz, intercept = -37.75 Hz; (⬟) $NCCH_2$Cbl, slope = 13.19 Hz, intercept = -40.16 Hz.

cobalamins.[28,29] Data were collected only down to pH 2.20 in order to avoid perturbation of the upper phosphodiester macroscopic pK_a (Table I) and the resultant large changes in δ_{31P}. Unfortunately, this precludes the use of CF_3Cbl whose $pK_{base\text{-}off}$ value (1.44) is too low to permit a reasonable extrapolation to $\delta_{31P}^{(12)}$. No decomposition of AdoCbl was detected under these conditions as evidenced by the lack of a second ^{31}P resonance at +9.37 Hz (for base-on H_2OCbl).

Data for each alkylcobalamin were fit to eq 25 by the least-squares method, where $\alpha_{base\text{-}on}$ was calculated from eq 24 and the measured values of $pK_{base\text{-}off}$. The data are shown in Figure 4,

$$\delta_{31P}^{obsd} = \delta_{31P}^{(12)} + (\delta_{31P}^{(123)} - \delta_{31P}^{(12)})\alpha_{base\text{-}on} \quad (25)$$

and the values of $\delta_{31P}^{(12)}$, $\delta_{31P}^{(123)}$, and $\Delta\delta_{31P}$ (eq 23) thus determined are listed in Table II. The data show that the effect of ionic strength on both $\delta_{31P}^{(12)}$ and $\delta_{31P}^{(123)}$ for these cobalamins is extremely small, the average value of $\delta_{31P}^{(12)}$ for all seven RCbl's at ionic strength 1.0 M (-39.43 ± 1.37 Hz) differing by only a few hertz from that at zero ionic strength (-36.70 ± 2.8 Hz). In addition, the agreement between the values of $\Delta\delta_{31P}$ obtained by the two methods is for the most part quite good, although somewhat less good for those RCbl's (CF_2HCbl and $NCCH_2$Cbl) requiring a long extrapolation in the application of eq 25 (Figure 4).

Also collected in Table II are the measured values of $pK_{base\text{-}off}$ for all ten cobalamins, the values of K_{Co} (eq 3) calculated from eq 4 (assuming pK_{Bz} = 5.56[8]), and the values of ΔG_{Co}, the apparent free energy of coordination of the pendent 5,6-dimethylbenzimidazole ligand in the base-on cobalamins. The obvious trend of $\Delta\delta_{31P}$ with increasing strength of axial ligand coordination is shown graphically in Figure 5. The correlation has two striking features: it passes through zero on the $\Delta\delta_{31P}$ axis, and it is surprisingly linear. In addition, Figure 6 shows an apparently significant correlation (r^2 = 0.882 for all six points, but r^2 = 0.971 if the point for "dry" CNCbl is omitted) between $\delta_{31P}^{(123)}$, which is solely responsible for the variations in $\Delta\delta_{31P}$, and the existing values of the Co–N(Bz) axial bond distances of base-on cobalamins from X-ray crystal structure determinations.[30-33] These effects

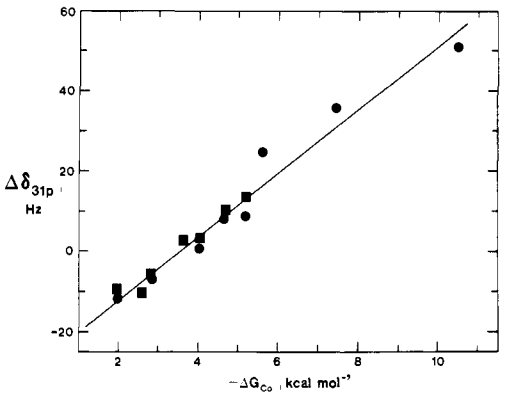


Figure 5. Plot of $\Delta\delta_{31P}$ (eq 23) at ionic strength 0.0 (●) and 1.0 M (■) for 10 cobalamins vs. $-\Delta G_{Co}$ (eq 3 and 4) at 25 ± 1 °C. The solid line is a linear least-squares line with slope = 7.93 ± 0.40 Hz kcal^{-1} mol, intercept = -28.06 ± 1.90 Hz, and correlation coefficient r^2 = 0.964.

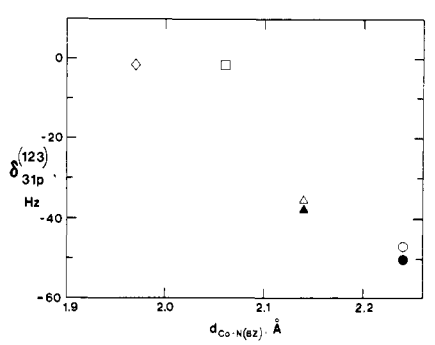


Figure 6. Plot of $\delta_{31P}^{(123)}$ (Scheme I) at ionic strength 1.0 (solid symbols) and 0.0 M (open symbols) vs. the axial Co–N bond distance ($d_{Co\text{-}N(Bz)}$) for the base-on cobalamin: (○, ●) AdoCbl, ref 30; (△, ▲) CH_3Cbl, ref 31; (◇) "wet" CNCbl, ref 32; (□) "dry" CNCbl, ref 33.

(28) Milton, P. A.; Brown, T. L. *J. Am. Chem. Soc.* **1977**, *99*, 1390–1396.
(29) Brown, K. L.; Awtrey, A. W.; Chock, P. B.; Rhee, S. G. In "Vitamin B_{12}"; Zagalak, B., Friedrich, W., Eds.; deGruyter: Berlin, 1977; pp 199–202.
(30) Lenhart, P. G. *Proc. R. Soc. London, Ser. A* **1968**, *303*, 45–84.
(31) Marzilli, L. G.; Toscano, P. J.; Summers, M.; Randaccio, L.; Bresciani-Pahor, N.; Glusker, J. P.; Rossi, M. *Inorg. Chim. Acta* **1983**, *79*, 29, as presented at the 1st International Conference on Bioinorganic Chemistry, Florence, Italy, June 13–17, 1983.
(32) Brink-Shoemaker, C.; Cruickshank, D. W. J.; Hodgkin, D. C.; Kamper, M. J.; Pilling, D. *Proc. R. Soc. London, Ser. A* **1964**, *228*, 1–26.

can be understood by reference to the work of Gorenstein and co-workers on the ^{31}P chemical shifts of phosphate compounds.[9-11] These workers have shown that the chemical shifts of phosphate compounds are virtually totally controlled by O–P–O bond angles and the coupled R–O–P–O torsion angles. From Gorenstein's empirical correlation of ^{31}P chemical shifts vs. O–P–O bond angles, phosphodiesters with chemical shifts in the range of approximately +0.1 to −0.7 ppm (i.e., the base-on cobalamins) would be expected to have RO–P–OR bond angles of approximately 102 to 103°. Decreases in the RO–P–OR bond angle would be expected to shift the ^{31}P resonance upfield, while increases in the bond angle would shift the resonance downfield. In the existing X-ray structures of base-on cobalamins[30,32,33] the RO–P–OR bond angles run from 100° to 102°, although it is not at all clear that the solid-state phosphodiester conformations are not significantly perturbed by crystal lattice forces. From the Gorenstein work and our data the following explanation of trends in $\delta_{^{31}P}$ in cobalamins seems most likely. When the axial ligand is deteched (base-off forms) the phosphodiester moiety assumes a "relaxed" conformation with an RO–P–OR bond angle of about 102° to 103°, regardless of the nature of the upper axial ligand. When the axial ligand is bound moderately weakly in a base-on cobalamin such as CH_3Cbl ($K_{Co} = 4.67 \times 10^2$, $d_{Co\text{-}N(Bz)} = 2.14$ Å[31] or CF_3CH_2Cbl ($K_{Co} = 9.23 \times 10^2$), the phosphodiester moiety evidently assumes a solution conformation which results in a nearly identical RO–P–OR bond angle and hence a negligible value of $\Delta\delta_{^{31}P}$ results (Table II). In those base-on cobalamins where the axial ligand is bound significantly more weakly, such as AdoCbl ($K_{Co} = 76.6$), the axial Co–N bond is lengthened ($d_{Co\text{-}N(Bz)} = 2.24$ Å[30]), the RO–P–OR bond angle is decreased, and the ^{31}P resonance is shifted upfield, producing a negative $\Delta\delta_{^{31}P}$. In those base-on cobalamins where the axial ligand is bound significantly more tightly, such as CNCbl ($K_{Co} = 2.88 \times 10^5$), the axial Co–N bond distance is shortened ($d_{Co\text{-}N(Bz)} = 2.06$ Å in "dry" CNCbl[33] or 1.97 Å in "wet" CNCbl[32]), the RO–P–OR bond angle is increased, and the ^{31}P resonance is shifted downfield producing a positive $\Delta\delta_{^{31}P}$. It should be pointed out that Gorenstein's empirical correlation of $\delta_{^{31}P}$ with O–P–O bond angles is exceedingly steep in the area of current interest having an instantaneous slope of about 5.9 ppm per degree of change in O–P–O bond angle. Consequently, the changes in phosphodiester conformation and RO–P–OR bond angle throughout the series of base-on cobalamins listed in Table II is quite small. Clearly this very small expected excursion of the RO–P–OR bond angle is responsible for the surprising linearity of the dependence of $\Delta\delta_{^{31}P}$ on ΔG_{Co} (Figure 5) since the Gorenstein correlation is distinctly nonlinear in the whole, and the changes in RO–P–OR bond angle with changes in $d_{Co\text{-}N(Bz)}$ would also not be expected to be linear over large excursions. Nontheless, we are currently attempting to obtain further evidence for changes in conformatin of the nucleotide loop through the series of cobalamins listed in Table II.

Finally, Satterlee, who first observed the relatively large value of $\Delta\delta_{^{31}P}$ for CNCbl, suggested that ^{31}P NMR might serve as an ideal probe for the possible displacement of the axial ligand of cobalamins upon binding to proteins. It now appears that ^{31}P NMR may be a much more useful probe of nucleotide loop conformation and Co–N(Bz) bond distances of protein-bound cobalamins. These may be important factors in the activation of the coenzymes in AdoCbl- and CH_3Cbl-dependent enzyme systems.

Acknowledgment. This work was supported by the Robert A. Welch Foundation, Grant No. Y-749 (K.L.B.), the Organized Research Fund of The University of Texas at Arlington, and the National Institutes of Health, Grant No. AM25406 (D.W.J.). The authors are grateful to Professor Dennis S. Marynick (Chemistry Department, UTA) for his invaluable assistance with curve fitting and to Professor L. G. Marzilli and M. Summers (Chemistry Department, Emory University) for supplying a sample of $NCCH_2Cbl$ for initial evaluation.

Registry No. H_2OCbl (base-on), 13422-52-1; CNCbl (base-on), 68-19-9; CH_3Cbl (base-on), 13422-55-4; $CH_3(CH_2)_2Cbl$ (base-on), 13985-72-3; $NC(CH_2)_3Cbl$ (base-on), 92670-10-5; CF_3CH_2Cbl (base-on), 21180-98-3; CF_2HCbl (base-on), 69496-07-7; $NCCH_2Cbl$ (base-on), 92670-11-6; AdoCbl (base-on), 13870-90-1; CF_3Cbl (base-on), 31532-05-5; H_2OCbl (base-off), 59980-64-2; CNCbl (base-off), 92670-12-7; CH_3Cbl (base-off), 92760-82-2; $CH_3(CH_2)_2Cbl$ (base-off), 92670-13-8; $NC(CH_2)_3Cbl$ (base-off), 92670-14-9; CF_3CH_2Cbl (base-off), 92670-15-0; CF_2HCbl (base-off), 92760-83-3; $NCCH_2Cbl$ (base-off), 92670-16-1; AdoCbl (base-off), 73827-23-3; CF_3Cbl (base-off), 92694-69-4.

(33) Hodgkin, D. C.; Lindsey, J.; Sparks, R. A.; Trueblood, K. N.; White, J. G. *Proc. R. Soc. London, Ser. A* **1962**, *266*, 494–517.

(34) **Note Added in Proof**: Further refinement of the X-ray crystal structure of CH_3Cbl (Rossi, M.; Glusker, J. P.; Randaccio, L.; Summers, M. F.; Toscano, P. J.; Marzilli, L. G. *J. Am. Chem. Soc.*, in press) has led to a revised estimate of the axial Co–N bond distance of 2.19 Å. When this distance is used with the data in Figure 6 the correlation coefficient improves to 0.928 for all six points and to 0.986 if the point for "dry" CNCbl is omitted. The authors are grateful to Drs. J. P. Glusker and L. G. Marzilli and their co-workers for communicating this information prior to its publication.