Kinetics of Ligand Binding to Aquocobalamin*

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ABSTRACT: The apparent association and dissociation rate constants for the binding of azide, cyanate, and imidazole to aquocobalamin have been determined over a wide pH range. Only for azide does binding occur for the protonated as well as the unprotonated form of the ligand. The apparent association rate constants are smaller for aquocobalamin than for metmyoglobin, but at pH 7 the order azide > cyanate > imidazole is the same for both cases. None of these ligands appears to bind according to a mechanism in which the unimolecular release of the water from aquocobalamin is the rate-limiting step.

The interesting structural features of aquocobalamin which have led to several recent studies of the thermodynamics of ligand binding to the cobalt atom in this compound (Pratt and Thorp, 1966; Hanania and Irvine, 1964; Hayward *et al.*, 1965) also make kinetic studies of ligand binding of interest. A previous study (Randall and Alberty, 1966) yielded rate constants for the binding of thiocyanate to aquocobalamin. In this paper kinetic data will be presented and interpreted for the binding of azide, cyanate, and imidazole by aquocobalamin.

The corrin ring–cobalt(III) structure of aquocobalamin is in many ways analogous to the porphyrin ring–iron(III) structure of metmyoglobin (Hodgkin, 1958; Stryer *et al.*, 1964). There has been some discussion recently about the relative rates of binding of the protonated and unprotonated forms of various ligands to metmyoglobin (Duffey *et al.*, 1966; Goldsack *et al.*, 1965, 1966). For this reason the pH dependence of the binding of azide, cyanate, and imidazole to aquocobalamin has been investigated and compared to the corresponding metmyoglobin results.

It has been suggested that the displacement of the water molecule coordinated to the cobalt(III) in inorganic cobalt complexes by another ligand involves a slow unimolecular dissociation of the water molecule followed by a very fast association of the ligand (Basolo and Pearson, 1958). The kinetic data for the cyanate–, imidazole–, and azide–aquocobalamin reaction are used to check this mechanism.

Experimental Section

All kinetic experiments were performed on a stopped-flow device employing spectrophotometric detection. Dissociation constants for the aquocobalamin–ligand complexes were determined by means of spectrophotometric titrations (Goldsack *et al.*, 1966) using a Cary Model 14 recording spectrophotometer.

The imidazole used was twice recrystallized from benzene. Azide solutions were prepared from practical grade sodium azide and their concentrations were determined by titration with silver nitrate solutions. Stock cyanate solutions were prepared fresh each day from reagent grade potassium cyanate. All cyanate solutions employed in the kinetic experiments were used within 15 min of preparation from the stock solutions, and below pH 5 this time was less than 5 min. The buffer solutions employed for various pH ranges were glycine–HCl, below pH 4; acetate, between pH 4 and 6; phosphate, between pH 6 and 7.8; and Tris, above pH 7.9.

The studies in this paper were done with commercially available hydroxocobalamin solutions sold under the trade name alpha-Redisol by Merck Sharp and Dohme. These solutions contain 1 mg/ml of cobalamin (7.3×10^{-4} M), 0.82% NaCl, 0.02% NaAc, and, as preservative, 0.15% methylparaben and 0.02% propylparaben. Pure hydroxocobalamin obtained from Merck Sharp and Dohme was used to check the results obtained with the solutions of the commercial product.

The kinetic investigations were conducted with solutions containing a buffer contributing 0.01 of the total ionic strength of 0.054. All experiments were conducted at 25°. In each experiment a solution with a cobalamin concentration of about 7×10^{-5} M was rapidly mixed with another solution containing no cobalamin, a total ligand concentration of at least 40 times the cobalamin concentration of the first solution, and the same ionic strength and pH as the first solution. At any pH this was repeated for a series of ligand concentrations.

Results

In all cases the observed concentration *vs.* time traces were simple exponential curves. For any ligand at a definite pH the plot of the reciprocal of the relaxation time *vs.* total ligand concentration was linear within experimental error. The apparent association

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TABLE I: Apparent Rate and Equilibrium Constants for the Azide–Aquocobalamin Reaction.

pH	$k_{+1\text{app}} \times 10^2$ (M^{-1} sec^{-1})	$k_{-1\text{app}}{}^a$ (sec^{-1})	$K_{\text{app}} \times 10^5$ (M)	$k_{-1\text{app}}{}^b$ (sec^{-1})
2.0	1.13 ± 0.03	0.5 ± 0.1	610 ± 30	0.66 ± 0.04
2.4	1.20 ± 0.04	0.25 ± 0.1	207 ± 13	0.23 ± 0.02
2.4	1.13 ± 0.03	0.30 ± 0.1		
3.0	1.35 ± 0.04	0.05 ± 0.01	58 ± 3	0.080 ± 0.005
3.4	1.82 ± 0.06	0.02 ± 0.05		
4.0	3.57 ± 0.08	0.1 ± 0.1	11 ± 1	0.042 ± 0.003
4.5	7.69 ± 0.07	−0.1 ± 0.1		
5.0	11.6 ± 0.4	0.4 ± 0.3	3.8 ± 1.0	0.045 ± 0.01
5.5	14.8 ± 0.3	−0.3 ± 0.2		
6.0	16.2 ± 0.4	−0.2 ± 0.3		
6.0	17.6 ± 0.4	0.2 ± 0.2	2.7 ± 0.5	0.044 ± 0.008
6.4	14.8 ± 0.5	1.0 ± 0.5	2.1 ± 0.3	0.034 ± 0.005
6.4	15.9 ± 0.1	0.5 ± 0.5		
7.0	14.2 ± 0.4	0.3 ± 0.3	2.4 ± 0.5	0.033 ± 0.007
7.4			2.4 ± 0.2	0.025 ± 0.003
7.6	7.9 ± 0.1	0.5 ± 0.2		
8.0	5.65 ± 0.07	0.04 ± 0.05	5.1 ± 0.2	0.027 ± 0.002
8.0	5.9 ± 0.1	0.0 ± 0.1		
8.5	2.4 ± 0.1	−0.05 ± 0.07		
9.0	0.80 ± 0.03	0.03 ± 0.02		
9.6	0.16 ± 0.01	0.02 ± 0.01		

[a] Determined from the intercepts of the $1/\tau$ *vs.* (azide)$_0$ plots. [b] Determined from eq 14 using the K_{dis} values and corresponding values of $k_{+1\text{app}}$ calculated from eq 12.

rate constants, $k_{+1\text{app}}$, apparent dissociation rate constants, $k_{-1\text{app}}$, and apparent equilibrium dissociation constants, K_{app}, for each ligand at several pH values are given in Tables I–III. The apparent association rate constants are also plotted *vs.* pH in Figures 1 and 2 for the three ligands.

Discussion

The kinetic results are consistent with the general bimolecular mechanism

$$\text{CBM} + \text{L} \underset{k_{-1\text{app}}}{\overset{k_{+1\text{app}}}{\rightleftharpoons}} \text{CBM-L} \tag{1}$$

where CBM and L refer to all of the rapidly equilibrating protonic forms of cobalamin and the ligand, respectively. For the above mechanism one obtains

$$1/\tau = k_{-1\text{app}} + k_{+1\text{app}}[(\overline{\text{CBM}}) + (\overline{\text{L}})] \tag{2}$$

where τ is the relaxation time and $(\overline{\text{CBM}})$ and $(\overline{\text{L}})$ are the equilibrium concentrations of all of the protolytic species of cobalamin and ligand, respectively. In general $k_{-1\text{app}}$ and $k_{+1\text{app}}$ are pH dependent. In all of the experiments reported here $(\overline{\text{L}}) \gg (\overline{\text{CBM}})$ and, therefore, $[(\overline{\text{CBM}}) + (\overline{\text{L}})]$ may be replaced by $(\text{L})_0$, the total ligand concentration, in eq 2.

Consider the mechanism in which one protonated form of the ligand binds to the aquocobalamin, but not to hydroxocobalamin

$$\begin{tikzcd} \text{L} + \text{CBM-OH}_2 \arrow[r, shift left, "k_{+1}"] \arrow[d, shift left, "K_0"'] & \text{CBM-L} \arrow[l, shift left, "k_{-1}"] \\ \text{CBM-OH} + \text{H}^+ \arrow[u, shift left] & \end{tikzcd} \tag{3}$$

When the above mechanism is followed the apparent association and dissociation rate constants, obtained from the slopes and intercepts of the $1/\tau$ *vs.* $(\text{L})_0$ plots, have the following hydrogen ion dependence when the protolytic steps are rapidly equilibrated and the solution is buffered.

$$k_{+1\text{app}} = \frac{k_{+1}}{1 + K_0/(\text{H}^+)} \tag{4}$$

$$k_{-1\text{app}} = k_{-1} \tag{5}$$

where

$$K_0 = \frac{(\overline{\text{CBM-OH}})(\overline{\text{H}^+})}{(\overline{\text{CBM-OH}_2})} \tag{6}$$

Equation 4 did predict the correct hydrogen ion dependence of the apparent association rate constant for the binding of thiocyanate (Randall and Alberty,

TABLE II: Apparent Rate and Equilibrium Constants for the Cyanate–Aquocobalamin Reaction.

pH	$k_{+1\text{app}} \times 10^2$ (M^{-1} sec^{-1})	$k_{-1\text{app}}{}^a$ (sec^{-1})	$K_{\text{app}} \times 10^3$ (M)	$k_{-1\text{app}}{}^b$ (sec^{-1})
3.4	2.1 ± 0.2	1.0 ± 0.2		
4.0	4.2 ± 0.1	1.1 ± 0.1		
4.5	5.9 ± 0.07	1.2 ± 0.1		
5.0	6.6 ± 0.1	1.2 ± 0.1		
5.6	7.3 ± 0.1	0.8 ± 0.2		
6.0	7.4 ± 0.2	0.8 ± 0.1		
6.1	7.0 ± 0.2	0.64 ± 0.06		
6.5			1.3 ± 0.1	0.9 ± 0.1
6.6	6.7 ± 0.2	1.1 ± 0.2		
7.0			1.4 ± 0.1	0.8 ± 0.1
7.1	5.45 ± 0.3	1.0 ± 0.1		
7.5			1.7 ± 0.2	0.7 ± 0.1
7.6	4.1 ± 0.1	0.88 ± 0.08		
8.0	2.1 ± 0.04	0.91 ± 0.04	3.0 ± 0.5	0.7 ± 0.1
8.5	0.80 ± 0.03	0.84 ± 0.03		

[a] Determined from the intercepts of the $1/\tau$ *vs.* $(\text{cyanate})_0$ plots. [b] Determined from eq 14 using the K_{dis} values and corresponding values of $k_{+1\text{app}}$ calculated from eq 12.

1966), but fails to do so for the azide, cyanate, and imidazole results.

TABLE III: Apparent Rate and Equilibrium Constants for the Imidazole–Aquocobalamin Reaction.

pH	$k_{+1\text{app}}$ (M^{-1} sec^{-1})	$K_{\text{app}} \times 10^4$ (M)	$k_{-1\text{app}}{}^b \times 10^4$ (sec^{-1})
5.8	1.15 ± 0.05		
6.0	1.6 ± 0.05		
6.0	2.1 ± 0.1	2.6 ± 0.5	5.0 ± 0.9
6.2	3.0 ± 0.1		
6.4	4.1 ± 0.1		
6.5		1.09 ± 0.08	5.5 ± 0.4
6.6	5.8 ± 0.1		
6.65		0.95[a]	6.0
6.8	7.9 ± 0.1		
7.0	10.1 ± 0.6		
7.2	10.3 ± 0.4	0.59[a]	6.4
7.4	11.1 ± 0.3		
7.52		0.59[a]	6.4
7.6	9.9 ± 0.1		
7.6	10.7 ± 1.1		
7.8	10.1 ± 0.9		
8.0	6.6 ± 0.2		
8.4	3.3 ± 0.1		
8.56		2.06[a]	6.2
8.8	1.5 ± 0.05		

[a] Hanania and Irvine (1964). [b] Determined by means of eq 14.

If the ligand has two protonated forms, however, and if it is again assumed that the protolytic steps are rapidly adjusted, the solution is buffered, and only aquocobalamin binds the ligand species, the mechanism becomes

$$\begin{tikzcd}
\text{CBM-OH} + \text{H}^+ \arrow[d, shift left] & \\
\text{L} + \text{CBM-OH}_2 \arrow[u, shift left, "K_0"] \arrow[r, shift left, "k_{+1}"] \arrow[d, shift left] & \text{CBM-L} \arrow[l, shift left, "k_{-1}"] \arrow[d, shift left] \\
\text{HL} + \text{CBM-OH}_2 \arrow[u, shift left, "K_1"'] \arrow[r, shift left, "k_{+2}"] \arrow[d, shift left, "K_0"'] & \text{CBM-LH} \arrow[u, shift left, "K_2"'] \arrow[l, shift left, "k_{-2}"] \\
\text{CBM-OH} + \text{H}^+ \arrow[u, shift left] &
\end{tikzcd} \qquad (7)$$

For this mechanism the apparent association and dissociation rate constants are given by

$$k_{+1\text{app}} = \frac{k_{+1}}{(1 + K_0/(\text{H}^+))(1 + (\text{H}^+)/K_1)} + \frac{k_{+2}}{(1 + K_0/(\text{H}^+))(1 + K_1/(\text{H}^+))} \qquad (8)$$

$$k_{-1\text{app}} = \frac{k_{-1} + k_{-2}(\text{H}^+)/K_2}{1 + (\text{H}^+)/K_2} \qquad (9)$$

where

$$K_1 = \frac{(\overline{\text{L}})(\overline{\text{H}}^+)}{(\overline{\text{HL}})} \qquad (10)$$

$$K_2 = \frac{(\overline{\text{CBM-L}})(\overline{\text{H}}^+)}{(\overline{\text{CBM-LH}})} \qquad (11)$$

Equation 8 predicts that $k_{+1\text{app}}$ should approach a

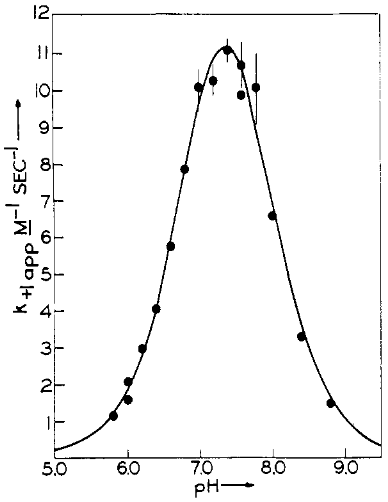


FIGURE 1: Plot of $k_{+1\mathrm{app}}$ *vs.* pH for the binding of imidazole to aquocobalamin. The solid line indicates the best fit to eq 12. Vertical lines through some circles indicate standard deviations in $k_{+1\mathrm{app}}$ greater than 0.1 M^{-1} sec^{-1}.

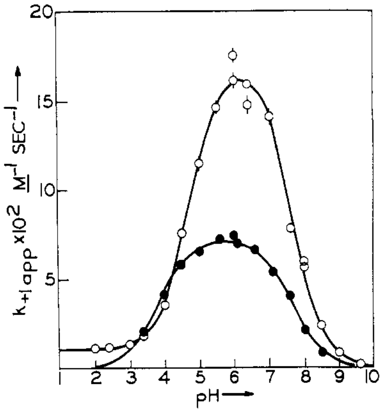


FIGURE 2: Plots of $k_{+1\mathrm{app}}$ *vs.* pH for the binding of azide (○) and cyanate (●) to aquocobalamin. The solid line through the azide data was computed from the best fit to eq 8. The solid line through the cyanate data was computed from the best fit to eq 12. Vertical lines indicate standard deviations in $k_{+1\mathrm{app}}$ greater than 0.1 × 10^2 M^{-1} sec^{-1}.

limiting value of k_{+2} at low pH and a limiting value of 0 at high pH. If $k_{+1\mathrm{app}}$ were to approach a finite value at high pH this would indicate that the hydroxocobalamin was also binding the ligand.

If $k_{+2} \ll k_{+1}$ the second term of eq 8 is much smaller than the first and the plot of $k_{+1\mathrm{app}}$ *vs.* pH will yield a bell-shaped curve. Then eq 8 and 9 become, to a first approximation

$$k_{+1\mathrm{app}} = \frac{k_{+1}}{(1 + K_0/(\mathrm{H}^+))(1 + (\mathrm{H}^+)/K_1)} \qquad (12)$$

$$k_{-1\mathrm{app}} = k_{-1} \qquad (13)$$

Figure 1 contains the plot of $k_{+1\mathrm{app}}$ *vs.* pH for imidazole binding to aquocobalamin. These data may be fit to eq 12 and, therefore, no appreciable rate of binding is observed between the protonated, positively charged imidazolium ion and aquocobalamin. The best fit of eq 12 to these data yields k_{+1} = 27 ± 6 M^{-1} sec^{-1}, pK_0 = 7.6 ± 0.3, and pK_1 = 7.1 ± 0.3 (compared to a literature value of 7.06 (Martell, 1964)). The dissociation rate constant, k_{-1}, was too small to be determined from the intercepts of the $1/\tau$ *vs.* $(\mathrm{Im})_0$ plots, but was calculated from

$$k_{-1} = k_{+1\mathrm{app}}K_{\mathrm{app}} \qquad (14)$$

where K_{app} is the apparent equilibrium dissociation constant of the complex, to have the approximate value 6 ± 1 × 10^{-4} sec^{-1}.

The plot of $k_{+1\mathrm{app}}$ *vs.* pH for the cyanate data is given in Figure 2. Here again there is no evidence of the protonated form of the ligand, in this case the neutral HOCN molecule, binding to aquocobalamin. The increase in the rate of decomposition of cyanate at low pH values (Lister, 1955), however, puts a lower limit on the pH at which one may study cyanate binding to aquocobalamin. A least-squares fit to the cyanate data indicates that the upper limit on k_{+2} would be about 100 M^{-1} sec^{-1}. The best fit to eq 12 for these data yields k_{+1} = 7.3 ± 0.2 × 10^2 M^{-1} sec^{-1}, pK_0 = 7.64 ± 0.07, and pK_1 = 3.85 ± 0.1 (compared to a range of 3.46–3.70 reported in the literature (Sillen, 1964)). The apparent dissociation rate constant was found from the intercepts of the $1/\tau$ *vs.* $(\mathrm{cyanate})_0$ plots to have an average value of 0.95 ± 0.1 sec^{-1} independent of pH. Equilibrium constant determinations, combined with experimental values of the association rate constants, yield an average dissociation rate constant of 0.8 ± 0.2 sec^{-1} which is in satisfactory agreement with the value determined kinetically.

The data for the binding of azide to aquocobalamin, on the other hand, do indicate that both the neutral HN_3 molecule as well as the N_3^- anion bind to aquocobalamin. The $k_{+1\mathrm{app}}$ values given in Figure 2 may be fit to eq 8 to yield k_{+1} = 1.7 ± 0.1 × 10^3 M^{-1} sec^{-1}, k_{+2} = 1.0 ± 0.5 × 10^2 M^{-1} sec^{-1}, pK_0 = 7.6 ± 0.1, and pK_1 = 4.7 ± 0.1 (compared to equilibrium determinations of 4.72 (Sillen, 1964)).

The $k_{-1\mathrm{app}}$ values found from the intercepts of the $1/\tau$ *vs.* $(\mathrm{azide})_0$ plots scatter too much to yield useful data, but $k_{-1\mathrm{app}}$ values computed from eq 14 demonstrate a large increase at low pH values. This is in agreement with eq 9 when $k_{-2} \gg k_{-1}$. Fitting the azide data to eq 9 yields k_{-1} = 0.03 ± 0.01 sec^{-1}, $k_{-2} > 0.7$ sec^{-1}, and p$K_2 < 2.0$. It is interesting to note that the binding of the neutral hydrazoic acid species to aquocobalamin is not detectable by means of equilibrium dissociation constant determinations

TABLE IV: Rate and Equilibrium Constants for Mechanism 7.

Ligand	k_{+1} (M^{-1} sec^{-1})	k_{+2} (M^{-1} sec^{-1})	k_{-1} (sec^{-1})	k_{-2} (sec^{-1})	pK_0	pK_1	pK_2	K_{dis} (M)
Thiocyanate[a]	$7.1 \pm 0.3 \times 10^3$		1.8 ± 0.6		7.55 ± 0.2			$4.4 \pm 0.4 \times 10^{-4}$
Azide	$1.7 \pm 0.1 \times 10^3$	$1.0 \pm 0.5 \times 10^2$	0.03 ± 0.01	0.7	7.6 ± 0.1	4.7 ± 0.1	2.0	$1.8 \pm 0.3 \times 10^{-5}$
Cyanate	$7.3 \pm 0.2 \times 10^2$		0.95 ± 0.1		7.64 ± 0.07	3.85 ± 0.1		$1.0 \pm 0.3 \times 10^{-3}$
Imidazole	27 ± 6		$6 \pm 1 \times 10^{-4}$		7.6 ± 0.1	7.1 ± 0.3		$2.6^{b} \times 10^{-5}$
Cyanide	$1.5^{c} \pm 0.4 \times 10^3$		10^{-9}					10^{-12d}

[a] Randall and Alberty (1966). [b] Hanania and Irvine (1964). [c] Conn and Wartman (1952). [d] George *et al.* (1960).

carried out in the same pH range as the kinetic studies. If only the ligand and aquocobalamin exist in two protonic forms the apparent equilibrium dissociation constant, K_{app}, may be written

$$K_{\text{app}} = K_{\text{dis}}(1 + K_0/(\text{H}^+))(1 + (\text{H}^+)/K_1) \quad (15)$$

where

$$K_{\text{dis}} = \frac{(\bar{\text{L}})(\overline{\text{CBM-OH}_2})}{(\overline{\text{CBM-L}})} \quad (16)$$

If the complex may also exist in two protonic forms eq 15 becomes

$$K_{\text{app}} = \frac{K_{\text{dis}}(1 + K_0/(\text{H}^+))(1 + (\text{H}^+)/K_1)}{(1 + (\text{H}^+)/K_2)} \quad (17)$$

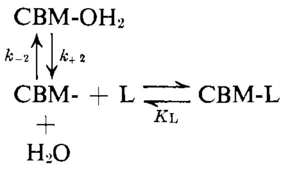
At very low pH values K_{app} will approach the limiting value of $K_{\text{dis}}K_2/K_1$. The apparent equilibrium dissociation constants for the azide–cobalamin complex can be satisfactorily fit by eq 15. This is due to the fact that the highest hydrogen ion concentration attained in these experiments, 0.01 M, is smaller than the value of K_2.

Data reported earlier on the kinetics of cyanide binding to aquocobalamin (Conn and Wartman, 1951) can be used to obtain an approximate association rate constant for the binding of the cyanide anion $k_{+1} = 1.5 \times 10^3$ M^{-1} sec^{-1}. The association constant for the cyanide–aquocobalamin complex has been estimated to be 10^{12} M^{-1} (George *et al.*, 1960). The dissociation rate constant for this complex will, therefore, be of the order of 10^{-9} sec^{-1}. The kinetic experiments were not conducted at a low enough pH to determine if the HCN species also binds at an appreciable rate.

No detectable rate of binding to hydroxocobalamin was observed for any of the above ligands. The collected k_{+1}, k_{+2}, k_{-1}, k_{-2}, pK_0, pK_1, pK_2, and K_{dis} values for the above ligands are given in Table IV. One can see from Table IV that the magnitudes of the association rate constants for the various ligand species are in the order $\text{SCN}^- > \text{N}_3^-, \text{CN}^- > \text{CNO}^- > \text{HN}_3 >$ imidazole. The corresponding dissociation rate constants are in the order $\text{SCN}^- > \text{CNO}^- > \text{N}_3^- >$ imidazole $> \text{CN}^-$. It should be noted that the rate constants for dissociation of the cyanate–cobalamin and thiocyanate–cobalamin complexes are much larger than the dissociation rate constants observed for the simpler cobalt(III) complexes (Basolo and Pearson, 1958). The magnitudes of the corresponding imidazole and cyanide complex dissociation rate constants are in the general region observed for the inorganic cobalt(III) complexes.

A comparison of rate constants for the reaction of azide, cyanate, and imidazole with aquocobalamin and metmyoglobin reveals that the apparent association rate constants are larger for the metmyoglobin for each ligand and at pH 7 the order azide > cyanate > imidazole is the same for both. Azide is the only ligand for which both the neutral and anionic forms clearly bind to aquocobalamin. In this case the association rate constant for the anion is approximately 17 times greater than the corresponding rate constant for the neutral form. The pH dependence of the binding of azide to metmyoglobin has also been interpreted in terms of binding of the neutral HN_3 species (Duffey *et al.*, 1966), but in this case it was necessary to assume that the association rate constant was 300 times greater for the neutral form than for the anionic form. The kinetics of azide binding to metmyoglobin have also been successfully treated, however, using the assumption that the anionic form binds much more rapidly than the neutral form (Goldsack *et al.*, 1965, 1966).

It should be noted that the kinetic data for the binding of azide, cyanate, and imidazole by aquocobalamin cannot be interpreted in terms of a rate-limiting, unimolecular release of the water molecule coordinated to the cobalt(III) atom followed by a very fast binding of the ligand. This mechanism may be written



$$\begin{array}{l} \mathrm{CBM\text{-}OH_2} \\ k_{-2}\uparrow\downarrow k_{+2} \\ \mathrm{CBM\text{-}} + \mathrm{L} \underset{K_\mathrm{L}}{\rightleftharpoons} \mathrm{CBM\text{-}L} \\ + \\ \mathrm{H_2O} \end{array} \qquad (18)$$

where

$$K_\mathrm{L} = \frac{(\overline{\mathrm{CBM\text{-}}})(\overline{\mathrm{L}})}{(\overline{\mathrm{CBM\text{-}L}})} \qquad (19)$$

It was previously stated (Randall and Alberty, 1966) that the reciprocal of the observed relaxation time for a system employing this mechanism would be given by

$$1/\tau = k_{-2} + \frac{k_{+2}}{1 + (\overline{\mathrm{L}})/K_\mathrm{L}} \qquad (20)$$

It should be noted, however, that the above equation is based on the assumption, which is valid for all of the experiments reported here, that $(\overline{\mathrm{L}}) \gg (\overline{\mathrm{CBM\text{-}L}})$. For very small concentrations of ligand

$$1/\tau = k_{-2} + \frac{k_{+2}}{1 + \dfrac{(\overline{\mathrm{L}})/K_\mathrm{L}}{[1 + (\overline{\mathrm{CBM\text{-}L}})/(\overline{\mathrm{L}})]}} \qquad (21)$$

Neither eq 20 or 21, however, is consistent with the kinetic data. As indicated in the Results section, in all cases a plot of $1/\tau$ *vs.* $(\overline{\mathrm{L}})$ was linearly increasing within experimental error.

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