The Coordination of Imidazole and Its Derivatives by Aquocobalamin

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Abstract

Formation constants have been determined for the substitution of coordinated water in aquocobalamin (vitamin B_{12a}) by imidazole and a number of its derivatives at 25 °C, $\mu = 1.0$ M (KCl) in the pH range 5.5 to 10.5. The observed formation constants are all strongly pH dependent. Since the binding constant for 2-methylimidazole ($\log K = 1.15$) is low compared to that for imidazole ($\log K = 4.59$) because of steric repulsion between the annular substituent and the corrin ring, only the five-substituted tautomers of asymmetrically substituted imidazoles are considered to be significantly coordinated by Co(III). Taking this and the state of ionisation of the pendant amino group of histamine and histidine into account, equations were derived to explain the pH dependence of the observed formation constants. From these the following pH independent formation constants ($\log K$) were deduced: imidazole, 4.59; 2-methylimidazole, 1.15; 5-methylimidazole, 4.90; *N*-methylimidazole, 4.90; 1,5-dimethylimidazole, 4.82; imidazole lactic acid, 4.86; cationic histamine, 4.43; neutral histamine, 4.71; neutral histidine, 4.30; and anionic histidine, 4.60. The magnitude of the formation constant is virtually independent of the basicity of the ligand. HPLC evidence is presented to show that at sufficiently high concentrations, these imidazoles displace 5,6-dimethylbenzimidazole (dbzm) from the fifth coordination position of Co(III) with $\log K = 1.4$ to < -1. At high pH, ionisation of bound imidazole labilises the *trans* 5,6-dimethylbenzimidazole ligand which is substituted by an imidazole from solution.

From fits of the relevant equations to the experimental data, and from direct spectrophotometric titrations, it is shown that coordination decreases the pK_a of the imino group of the imidazoles from >14 to *c.* 10 due to polarisation by the metal ion. Furthermore, the pK_as of the pendant amino groups of histamine and histidine decrease from 9.96 and 9.18 to 8.89 and 7.99, respectively, on coordination. This is ascribed to destabilisation of the conjugate acids by coulombic repulsion with the residual positive charge at the metal centre. The possible biological significance of these results are discussed.

Introduction**

Aquocobalamin (Vitamin B_{12a}) contains a water molecule in the upper (β) axial position which is readily displaced by a wide variety of ligands (see for example refs. 1–8). Of particular interest are imidazole and its derivatives [8]. The imidazole side chain of histidine is a potential ligand for metal complexes in proteins (for example, for B_{12a} in bovine and human serum albumin [9, 10]). We have developed bis(substituted-imidazole)iron(III) porphyrin complexes as models for such diverse effects as the pH dependence of the midpoint potential of a number of cytochromes, and the proton-pumping action of cytochrome oxidase and the cytochrome b component of Complex III of the mitochondrial respiratory chain [11–15]. Cyclic voltammetry and spectroscopic studies of these complexes show that reduction of Fe(II) is accompanied by proton uptake on a pendent functional group (RNH_2 in histidine and histamine; RO^- in pilocarpate) whose pK_a in the Fe(III) complexes is lower than in the Fe(II) complexes due to coulombic interaction with the residual positive charge at the metal centre.

We recently investigated the kinetics of the substitution of H_2O in aquocobalamin by imidazole and its derivatives [8]. From this study, values for the equilibrium constant, K_T, for interconversion between the 5- and 4-tautomers of asymmetrically substituted imidazoles in solution were deduced. We have extended our studies and have determined the binding constants for coordination of a number of imidazole derivatives by B_{12a}.

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**Abbreviations used in this paper: dbzm, 5,6-dimethylbenzimidazole, the nucleotide base of the cobalamins; Cbl, cobalamin; MP8, microperoxidase-8, the hemeoctapeptide from cytochrome c. For other abbreviations, see Schemes 1–7.

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Binding constants for the reaction

$$dbzm{\cdot}Co{\cdot}H_2O + L \rightleftharpoons dbzm{\cdot}Co{\cdot}L + H_2O \quad (1)$$

(only the axial ligands are shown and the overall charge is ignored) are available for a number of imidazole derivatives: imidazole ($\log K = 4.6 \pm 0.1$ [16]; 4.61 ± 0.04 [18]); histidine (5.8 [17]); *N*-methylimidazole (4.40 ± 0.07 [18]); and 4(5)-methylimidazole (4.4 [18]). Furthermore, benzimidazole [19], but not 2-methylimidazole [18] was qualitatively reported to bind to B_{12a}. These results have been obtained under a variety of experimental conditions. Some are of dubious validity because of failure to take tautomerisation into account. We have determined binding constants (25 °C, $\mu = 1.0$ M) for imidazole, 2-methylimidazole, 4(5)-methylimidazole, *N*-methylimidazole, 1,5-dimethylimidazole, imidazole 4(5)-lactic acid, histidine and histamine, to aquocobalamin utilising the recently delineated behaviour of these imidazoles in aqueous solution [8], and wish to report these here.

Experimental

The materials used have been described previously [8]. Buffers (phosphate, Tris/HCl and bicarbonate) were all 0.1 M; ionic strength was maintained at 1.0 M with KCl. All pH measurements were made with a Metrohm Series 6.0216 combination glass microelectrode and a Metrohm 605 ion meter operating in the pH mode. Spectra were recorded on a Cary 2300 spectrophotometer fitted with a constant temperature cell block connected to an external thermostatted bath (25.0 ± 0.2 °C). HPLC work was done on a Brownlee 100 × 4.6 mm RP18 (5 μm) analytical column using a Spectra-Physics 8800 ternary gradient pump connected to a Rheodyne sampling valve, a Linear UVis 200 detector, and a Varian 4290 integrator.

The spectroscopic pK_as of the Cbl complexes of the various imidazoles were determined by titrating in a thermostatted cell (25 °C) 50 ml of 50 μM B_{12a} + 10 mM of the relevant imidazole in 1 M KCl from pH 6 to *c.* 14 by addition of small quantities of conc. HCl or saturated NaOH. At each pH, a sample was withdrawn, placed in a 1 cm pathlength cuvette and the absorbance at the applicable wavelength was measured before returning the sample to the reaction mixture.

Because of the relatively slow formation of the complexes [8], the binding constants were determined as follows. At each pH, a series of between 10 and 15 five millilitre solutions containing a constant amount of B_{12a} (*c.* 50 μM), buffer, KCl and the relevant imidazole (between 0.1 mM and 0.1 M) were prepared and allowed to equilibrate overnight in a thermostatted bath (25 °C). Using the known pK_as of the imidazoles under these experimental conditions [8], sufficient acid or base was added to the mixture to ensure that the imidazole solution was at the same pH as the buffer. After equilibration, the pH was measured, a sample from each was introduced into a cuvette and the absorbance at the γ-band maximum of the complex (see below) was recorded. The pH values were usually between ±0.008 pH units between samples. The data were fitted to a binding isotherm of the form

$$(A_0 - A)/(A - A_\infty) = K_{obs}[L] \quad (2)$$

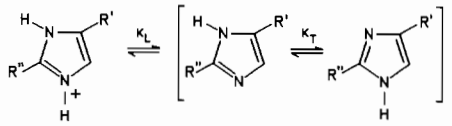
where L = ligand, using a Newton-Raphson procedure with Marquardt's algorithm, and in which A_0, A_∞ and K were allowed to vary. This method was found to be more reliable than the usual plot of $\log(A_0 - A/A - A_\infty)$ vs. $\log[L]$ as no assumptions are made about A_0 and A_∞. This is particularly important in the present system since at high imidazole concentrations, the bis(imidazole) species is formed in solution (see below).

The effect of high (i.e. ≥0.1 M) concentrations of imidazole was investigated by HPLC. One millilitre containing 50 μM B_{12a}, ≥0.1 M of the relevant imidazole, Tris/HCl (pH 7.5), a calculated amount of acid or base to adjust the pH of the imidazole solution to the same pH as the buffer, and KCl (to $\mu = 1.0$ M) was prepared and allowed to equilibrate at 25 °C overnight. The solution was filtered through a Millipore 0.2 μm filter and 20 μl of the solution injected onto the column. The following elution profile (1.5 ml/min flow rate) was used. Solvent A: 0.1 M phosphate, pH 6.5; solvent B: MeCN; solvent C: deionised H_2O. Between $T = 0$ and 1 min, 95% A and 5% B, increased linearly to reach 70% A and 30% B at $T = 10$ min. Between $T = 10$ min and $T = 10.1$ min, A was replaced with C and held at this composition to $T = 12$ min. Between $T = 12$ min and 14 min, B was increased linearly to 80%, then decreased linearly back to 30% before returning to starting conditions at $T =$ 15 min. This purge with a high concentration of MeCN was to prevent any possible build-up of imidazoles or B_{12} derivatives on the column. Detection was at 355 nm, 0–0.1 AUFS.

Results

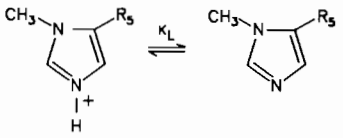
The solution chemistry of substituted imidazoles, as determined potentiometrically and deduced from a study of the kinetics of their reaction with aquocobalamin [8] is summarised in Schemes 1–4.

The observed binding constants, K_{obs}, are dependent upon pH (Table 1). In contrast to an earlier report [18] we found that although 2-methylimidazole has a substantially smaller binding constant



R' = R'' = H	Im^+	Im^o	
R' = H; R'' = CH_3	$2MeIm^+$	$2MeIm^o$	
R' = CH_3; R'' = H	$MeIm^+$	$5T\text{–}MeIm^o$	$4T\text{–}MeIm^o$
		pK_L	K_T
Imidazole :		7·24	
2–methyl imidazole :		7·21	
4(5)–methyl imidazole :		7·76	1·4

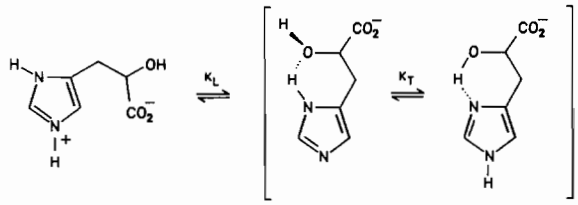
Scheme 1.



R_5 = H :	$NMeIm^+$	$NMeIm^o$
R_5 = CH_3 :	$1,5\ MeIm^+$	$1,5\ MeIm^o$
		pK_L
N–methyl imidazole (R_5=H) :		7·21
1,5–dimethyl imidazole (R_5=CH_3) :		7·97

Scheme 2.

than the other imidazoles, it is coordinated by B_{12a}. We could find no evidence, however, for complex formation between B_{12a} and benzimidazole, even in the presence of 1 M ligand in 40% (vol./vol.) ethanol–water mixtures at pH 7 (see ref. 19). If it is assumed that 50% complex formation is detectable, then $K_{obs} < 0.05$ for benzimidazole.



ILA^+	$5T\text{–}ILA^-$	$4T\text{–}ILA^-$
	pK_L	K_T
Imidazole lactic acid :	7·39	4·3

Scheme 3.

Based on the results in Table 1, it is assumed that for an asymmetrically substituted imidazole, the 4-tautomer in which the side chain is α to the donor N atom (cf. 2-methylimidazole), will coordinate negligibly weakly, and any observed complexation will occur through the 5-tautomer. The pH dependence of K_{obs} can therefore be accounted for by Schemes 5–7. Scheme 5 is shown in detail; Schemes 6 and 7 are abbreviated and readily understood by reference to Scheme 5.

K_{obs} is related to K_I by eqn. (3).

$$K_{obs} = \frac{K_I \alpha}{(1 + K_{CO}/[H^+])\beta\gamma} \qquad (3)$$

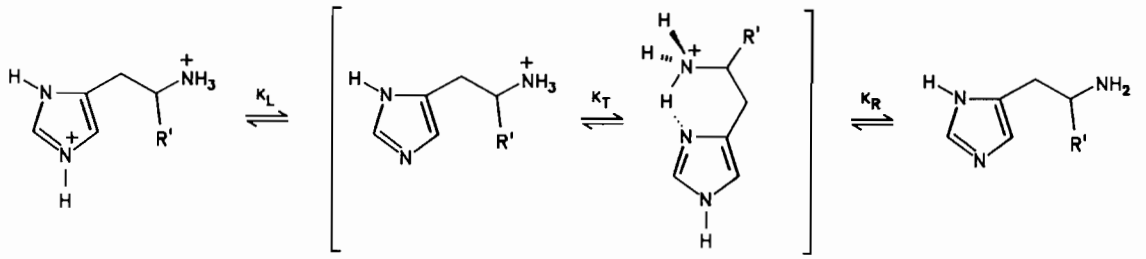
where pK_{CO}, the pK_a of coordinated H_2O in B_{12a}, is 8.10 at 25 °C, μ = 1.0 M [7]. The terms α, β and γ of eqn. (3) are given in Table 2.

In deriving these equations the following factors were taken into account

(i) Only an imidazole with a deprotonated endocyclic N atom can bind to the metal ion

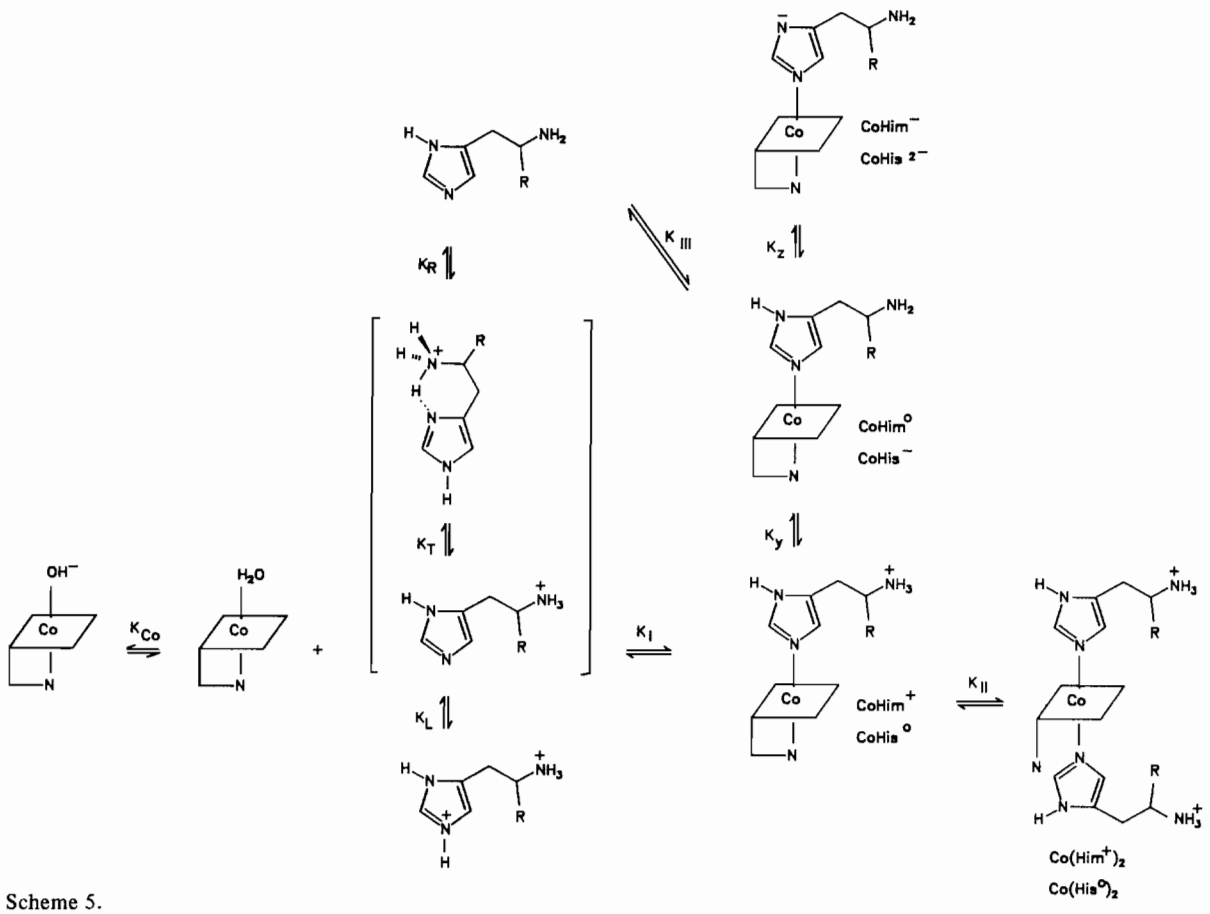
(ii) Coordination by 4-tautomers is negligible

(iii) In the case of histamine and histidine, which have amino groups in the side chain, two possible forms of the ligands can bind to the metal, viz. with



R' = H :	Him^{2+}	$5T\text{–}Him^+$	$4T\text{–}Him^+$	Him^o
R' = CO_2 :	His^+	$5T\text{–}His^o$	$4T\text{–}His^o$	His^-
		pK_L	K_T	pK_R
Histamine (R=H)		6·41	5·2	9·96
Histidine (R=CO_2)		6·23	6·9	9·18

Scheme 4.



Scheme 5.

TABLE 1. Binding constants for the reaction of imidazole and its derivatives with aquocobalamin, $T = 25$ °C, $\mu = 1.0$ M (KCl)

Ligand, L	$CoH_2O + L \rightleftharpoons CoL + H_2O$	
	pH	log K_{obs}
Imidazole	5.50	2.85
	6.01	3.33
	6.50	3.78
	7.00	4.13
	7.48	4.31
	8.01	4.28
	8.50	4.04
	9.00	3.68
	9.48	3.30
	10.00	2.99
	10.52	2.92
2-Methylimidazole	7.00	0.16
	8.03	0.64
4(5)-Methylimidazole	5.40	2.14
	5.92	2.62
	6.43	3.19
	7.00	3.62
	7.52	3.97
	8.14	4.05

(continued)

TABLE 1. *(continued)*

Ligand, L	$CoH_2O + L \rightleftharpoons CoL + H_2O$	
	pH	log K_{obs}
4(5)-Methylimidazole	8.62	3.88
	9.13	3.40
	9.62	3.03
	10.00	2.77
	10.52	2.45
N-Methylimidazole	5.52	2.91
	6.03	3.39
	7.04	4.15
	7.50	4.35
	8.11	4.22
	8.50	4.06
	9.13	3.48
	10.02	2.70
	10.51	2.23
1,5-Dimethylimidazole	5.63	2.48
	6.04	2.83
	6.51	3.34
	7.06	3.84
	7.50	4.13
	8.05	4.36
	8.48	4.18

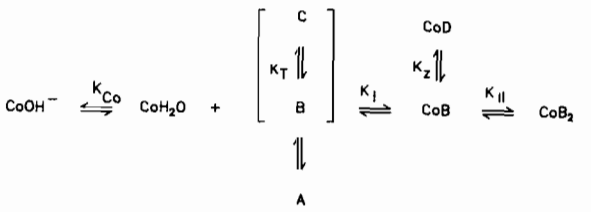
(continued)

TABLE 1. *(continued)*

Ligand, L	$CoH_2O + L \rightleftharpoons CoL + H_2O$	
	pH	log K_{obs}
1,5-Dimethylimidazole	9.02	3.87
	9.55	3.35
	10.07	2.75
	10.52	2.40
Imidazole lactic acid	5.48	2.22
	6.02	2.80
	6.51	3.21
	7.02	3.59
	7.50	3.82
	8.11	3.77
	8.48	3.57
	9.02	3.10
	9.36	2.96
	9.59	2.70
	10.05	2.35
	10.51	2.23
Histamine	5.48	2.63
	5.98	3.07
	6.25	3.26
	6.50	3.36
	7.07	3.52
	7.26	3.54
	7.68	3.50
	8.11	3.38
	8.54	3.20
	8.99	3.02
	9.47	2.95
	9.99	2.90
	10.53	2.90
Histidine	5.55	2.66
	6.03	3.05
	6.52	3.18
	7.10	3.31
	7.46	3.41
	8.07	3.39
	8.38	3.33
	8.52	3.34
	8.94	3.31
	9.21	3.33
	9.26	3.27
	9.80	2.97
	10.54	2.71

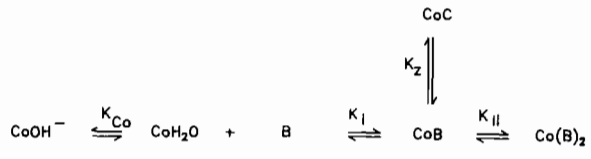
protonated or deprotonated amino groups; these will not necessarily have the same binding constant because of coulombic interaction between RNH_3^+ and the residual +2 charge at the metal centre. (In the cobalamins, the +3 charge of the metal is balanced by a −1 charge on a corrin N atom; the −1 charge of the phosphate is probably too far away to influence matters significantly.)

(iv) Although the pK_as for formation of the imidazolates of the free ligands which are unsubstituted at the N-1 position are high and beyond



A = $MeIm^+$; ILA^0
B = 5T–$MeIm^0$; 5T–ILA^-
C = 4T–$MeIm^0$; 4T–ILA^-
D = $MeIm^-$; ILA

Scheme 6.



A = Im^+; $2MeIm^+$; 1,5 $MeIm^+$; $NMeIm^+$
B = Im^0; $2MeIm^0$; 1,5 $MeIm^0$; $NMeIm^0$
C = Im^-; $2MeIm^-$; 1,5 $MeIm^-$; $NMeIm^-$

Scheme 7.

the pH range of this study (5.5–10.5) (e.g. pK_a = 14.44 for imidazole itself [20]), once the ligand is coordinated, this ionisation (pK_z) may well occur in the operating pH range

(v) In the case of histamine and histidine, the pK_a of the side chain amino group of the coordinated ligand (pK_y) may influence the value of K_{obs}.

The experimental data (Table 1) were fitted to the relevant form of eqn. (3) by non-linear least-squares methods, with K_I, K_z and, where applicable, K_y, as adjustable variables. The values of K_L and K_T used are those in Schemes 1–4. For histamine and histidine, K_{III} was then determined from eqn. (4).

$$K_{III} = \frac{K_I K_y}{K_R(1 + K_T)} \tag{4}$$

The results are shown in Fig. 1 and summarised in Table 3.

The values for pK_y and pK_z deduced from Fig. 1 were verified spectroscopically (see 'Experimental'). In Figs. 2 and 3 are shown the variation in A_{358} and A_{550}, respectively, for the histamine complex of B_{12a}. The data were fitted to eqn. (5)

$$A = A_1 f_1 + A_2 f_2 + A_3 f_3 + A_4 f_4 \tag{5}$$

where f_1 through f_4 are the fraction of $CoHim^+$, $CoHim^0$, $CoHim^-$ (see Scheme 5) and a fourth species present at high pH, respectively, such that

TABLE 2. Equations used to fit experimental data for the binding of imidazole and its derivatives to aquocobalamin[a]

Ligand	$K_{obs} = \frac{K_I\alpha}{(1 + K_{CO}/[H^+])\beta\gamma}$		
	α	β	γ
Imidazole	$1 + K_z/[H^+]$	$1 + [H^+]/K_L$	1
2-Methylimidazole	$1 + K_z/[H^+]$	$1 + [H^+]/K_L$	1
4(5)-Methylimidazole	$1 + K_z/[H^+]$	$1 + [H^+]/K_L$	$1 + K_T$
N-Methylimidazole	1	$1 + [H^+]/K_L$	1
1,5-Dimethylimidazole	1	$1 + [H^+]/K_L$	1
Imidazole lactic acid	$1 + K_z/[H^+]$	$1 + [H^+]/K_L$	$1 + K_T$
Histamine	$1 + K_y/[H^+] + K_yK_z/[H^+]^2$	$1 + [H^+]/K_L + K_R/[H^+]$	$1 + K_T$
Histidine	$1 + K_y/[H^+] + K_yK_z/[H^+]^2$	$1 + [H^+]/K_L + K_R/[H^+]$	$1 + K_T$

[a]See Schemes 5–7 for definition of equilibrium and acid dissociation constants.

TABLE 3. Equilibrium and acid dissociation constants for the binding of imidazole and its derivatives by aquocobalamin at 25 °C, μ = 1.0 M[a]

Ligand	log K_I	log K_{II}	log K_{III}	pK_y	pK_z
Imidazole	4.59 ± 0.01	0.6			9.85 ± 0.04
2-Methylimidazole	1.15 ± 0.06				
4(5)-Methylimidazole	4.90 ± 0.03	1.3			10.43 ± 0.18
N-Methylimidazole	4.63 ± 0.05	0.2			
1,5-Dimethylimidazole	4.82 ± 0.02	1.4			
Imidazole lactic acid	4.86 ± 0.04	0.9			10.20 ± 0.08
Histamine	4.43 ± 0.02	−0.9	4.71 ± 0.03	8.89 ± 0.05	9.89 ± 0.09
Histidine	4.30 ± 0.03	< −1	4.60 ± 0.06	7.99 ± 0.05	10.06 ± 0.09

[a]For definitions, see Schemes 5–7.

$$f_1 = 1/(1 + K_y/[H^+] + K_yK_z/[H^+]^2 + K_yK_zK_x/[H^+]^3)$$

$$f_2 = 1/(1 + [H^+]/K_y + K_z/[H^+] + K_yK_x/[H^+]^2)$$

$$f_3 = 1/(1 + [H^+]^2/K_yK_z + [H^+]/K_z + K_x/[H^+])$$

$$f_4 = 1/(1 + [H^+]/K_x + [H^+]^2/K_yK_x + [H^+]^3/K_yK_zK_x)$$

where pK_x is the apparent pK_a for conversion of $CoHim^-$ to the high pH species. In the case of imidazoles without an ionisable side chain, an analogous equation to eqn. (5), but without the K_y terms, was used. For *N*-methylimidazole and 1,5-dimethylimidazole, there were no significant spectral changes with pH.

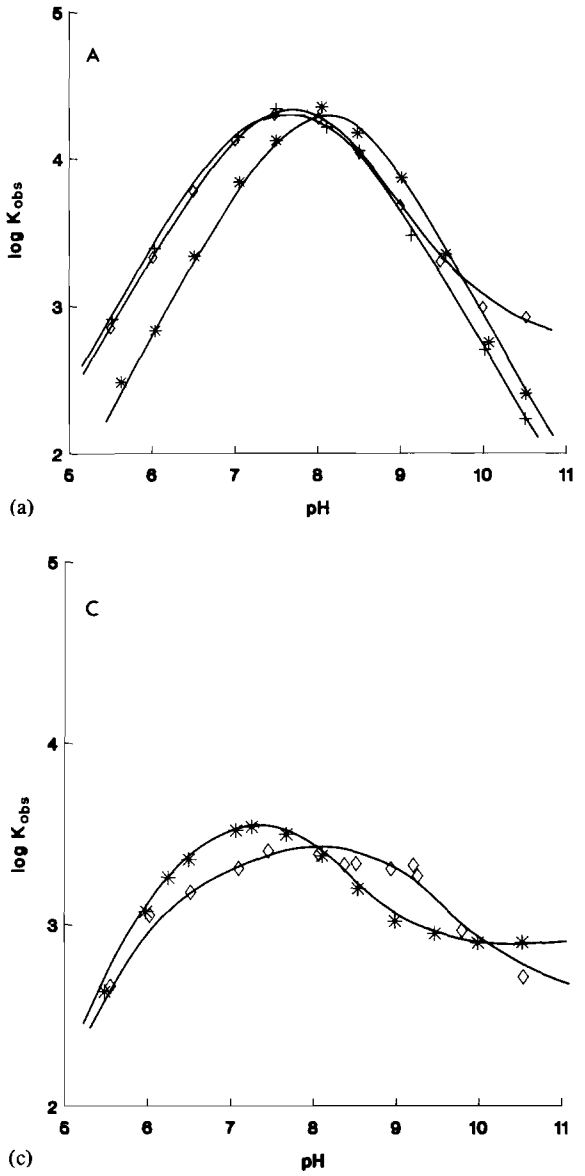
All spectral changes were reversible; they were established rapidly (i.e. within the sampling time) except for those observed at high pH, where up to 10 min was required for equilibrium to be reached.

The spectroscopic pK_as are summarised in Table 4. The γ-band positions of the various imidazole–Cbl species are shown in Table 5.

TABLE 4. Spectroscopic pK_as of complexes of aquocobalamin with imidazole and its derivatives[a]

Ligand	Observed pK_as	
Imidazole	9.9 ± 0.2	12.5 ± 0.3
4(5)-Methylimidazole	10.2 ± 0.2	12.3 ± 0.3
N-Methylimidazole		⩾13
1,5-Dimethylimidazole		12.1 ± 0.2
Imidazole lactic acid	10.1 ± 0.3	11.6 ± 0.4
Histamine	8.4 ± 0.3	10.0 ± 0.4	12.1 ± 0.4
Histidine	8.3 ± 0.3	10.1 ± 0.1	11.9 ± 0.2

[a]The results are averages of pK_as determined by monitoring changes in the γ-band (*c.* 358 nm) and the α-band (*c.* 550 nm).



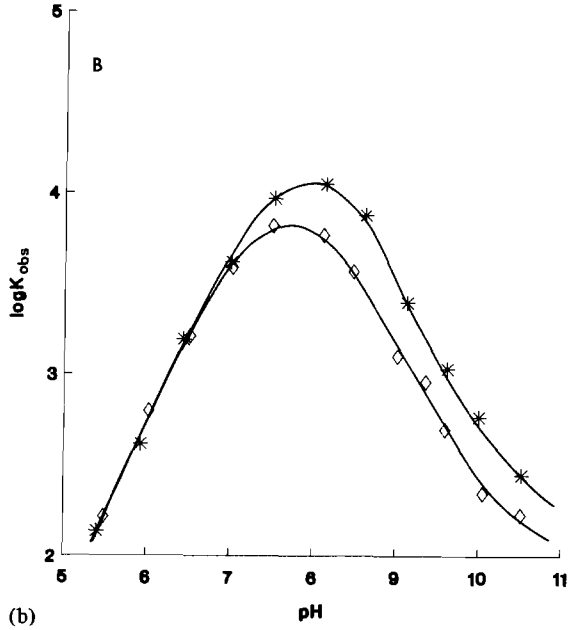


Fig. 1. The pH dependence of log K_{obs} for coordination by aquocobalamin of imidazole and its derivatives at 25 °C, μ = 1.0 M (KCl). The solid lines were obtained from the equations of Table 2 and the values of Table 3. (a) Imidazole (◇); *N*-methylimidazole (+); 1,5-dimethylimidazole (*). (b) Imidazole lactic acid (◇); 5-methylimidazole (*). (c) Histidine (◇); histamine (*).

At sufficiently high concentrations, an imidazole in solution might be expected to compete with dbzm for the fifth (α) coordination position of Co(III). Perhaps the easiest way of establishing unambiguously the formation of a bis(imidazole)-Cbl species is by HPLC. As shown in Fig. 4 for imidazole itself as an example, the peak for B_{12a} obtained in the absence of imidazole is replaced by a peak with a longer retention time due to imidazolecobalamin. At [imidazole] $\geqslant$ 10 mM, a second peak, at even longer retention times, is obtained, and the area of the second peak increases as [imidazole] increases; this is clearly due to the bis(imidazole)Cbl complex. From the variation in the ratios of the peak areas with [imidazole] (typically between 10 mM and 0.2 M), and assuming

TABLE 5. The γ-band positions of characterised complexes of aquocobalamin with imidazole and its derivatives

Ligand	Complex[a]	γ (nm)[b]
Imidazole	dbzm·Co·Im^0	358.3
	dbzm·Co·Im^-	359.8
4(5)-Methylimidazole	dbzm·Co·$MeIm^0$	359.2
	dbzm·Co·$MeIm^-$	359.9
2-Methylimidazole	dbzm·Co·$2MeIm^0$	355.8
N-Methylimidazole	dbzm·Co·$NMeIm^0$	358.5
1,5-Dimethylimidazole	dbzm·Co·$1,5MeIm^0$	359.0
Imidazole lactic acid	dbzm·Co·ILA^-	359.1
	dbzm·Co·ILA^{2-}	359.8

(continued)

TABLE 5. *(continued)*

Ligand	Complex[a]	γ (nm)[b]
Histamine	dbzm·Co·Him$^+$	358.0
	dbzm·Co·Him^0	358.3
	dbzm·Co·Him$^-$	358.9
Histidine	dbzm·Co·His^0	358.1
	dbzm·Co·His$^-$	358.3
	dbzm·Co·His^{2-}	359.0

[a]dbzm = 5,6-Dimethylbenzimidazole, the nucleotide base of the cobalamins; for definitions see Schemes 5–7. [b]The error on the band position is estimated to be ±0.2 nm.

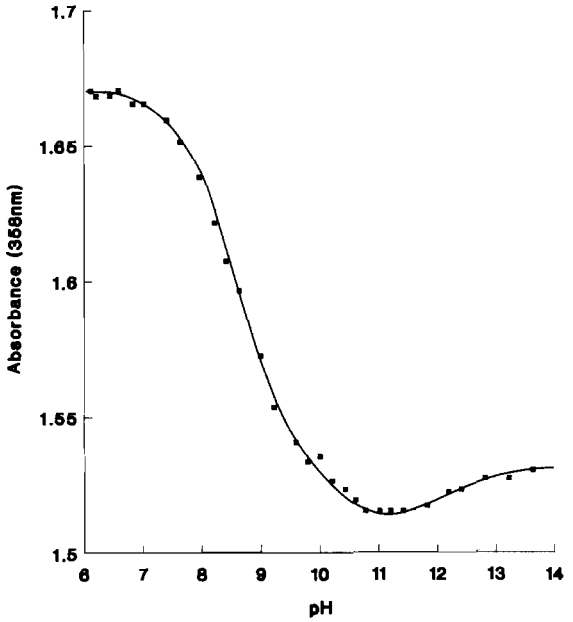


Fig. 2. Variation in A_{358} with pH for histaminecobalamin. The solid line was generated from eqn. (5) using pK_y = 8.4 ± 0.3; pK_z = 10.0 ± 0.4; pK_x = 12.1 ± 0.4; A_1 = 1.671; A_2 = 1.550; A_3 = 1.508; A_4 = 1.531.

identical response factors, K_{II} could be estimated. The values are listed in Table 2.

The conversion of the mono- to the bis-imidazole complexes can be seen spectroscopically as well, as shown in Fig. 5 for imidazole.

Discussion

Imidazoles that are unsubstituted at N-1 undergo tautomerisation in solution (see ref. 8 and references therein). This is of no consequence in symmetric molecules (e.g. imidazole and 2-methylimidazole); in the case of asymmetric imidazoles (e.g. 4(5)-methylimidazole, imidazole 4(5)-lactic acid, histidine, histamine), the predominant tautomer in

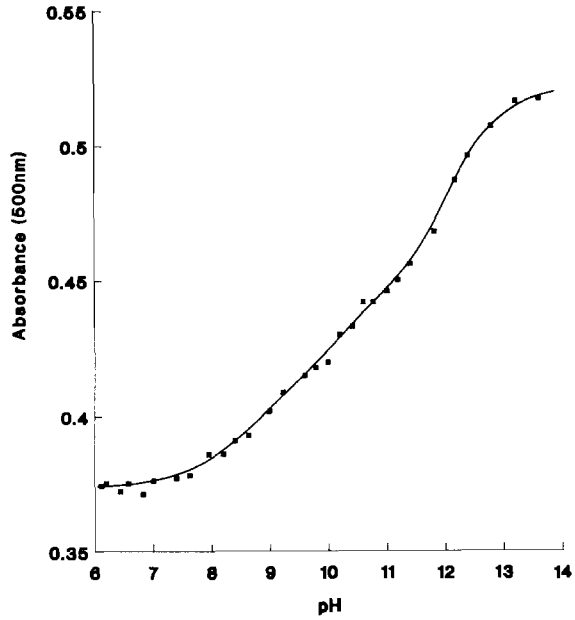


Fig. 3. Variation in A_{550} with pH for histaminecobalamin. The solid line was generated from eqn. (5) using the pK_as listed in Table 4, and A_1 = 0.374; A_2 = 0.405; A_3 = 0.445; A_4 = 0.520.

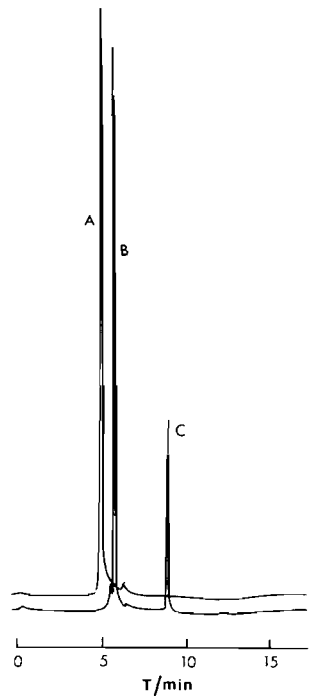


Fig. 4. HPLC chromatogram for: A, aquocobalamin; B and C, aquocobalamin (50 μM) + imidazole (0.100 M). See text for details.

solution is determined by the nature of the side chain. Hence for 4(5)-methylimidazole, K_T = [4-tautomer]/[5-tautomer] = 1.4 [8]. For Him$^+$ and

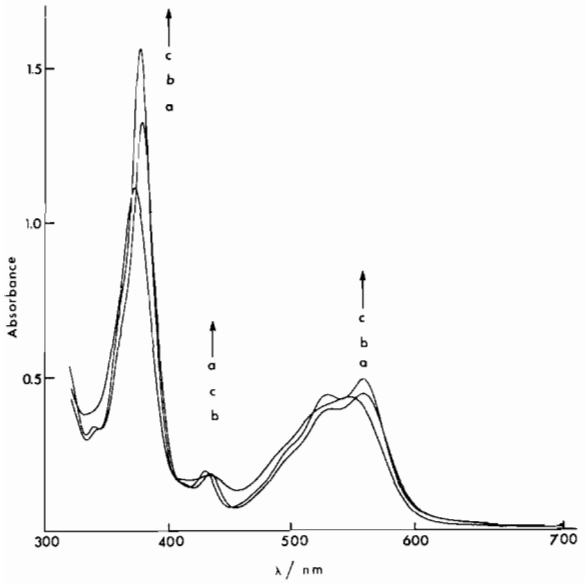


Fig. 5. UV–Vis spectrum of: (a) aquocobalamin, 50 μM, pH 7.5; (b) a + 0.40 mM imidazole, pH 7.5; (c) a + 0.40 M imidazole, pH 7.5. From the binding constants of Table 3, (b) consists of 44 μM $CoIm^0$ + 65 nM $Co(Im^0)_2$ + 5.5 μM CoH_2O; (c) consists of 20 μM $CoIm^0$ + 30 μM $Co(Im^0)_2$. See Scheme 7 for abbreviations used.

His^0 (see Schemes 1–4 for definitions), the 4-tautomer is heavily favoured (K_T = 5.2 and 6.9, respectively) because of intramolecular hydrogen bonding; when the exocyclic amino group of these is deprotonated, the 5-tautomer is the exclusive species in solution. Preferential hydrogen bonding with O as hydrogen donor and the endocyclic N atom as acceptor favours the 4-tautomer of imidazole lactic acid (K_T = 4.3).

Reference to Table 3 shows that the binding constant for 2-methylimidazole is low compared to that for imidazole itself, undoubtedly due to steric repulsion between the annular substituent and the macrocycle. The 4-tautomers of imidazoles in solution are therefore virtually excluded as candidates for coordination by the metal ion, and experimentally observed binding constants have to be corrected for this effect.

We have previously demonstrated [11–13] that coordination of histidine and pilocarpate by ferric protoporphyrin IX decreases the pK_a of the pendent amino and hydroxyl groups to <6.5 and 9.7, respectively, due, in the first case, to repulsion and, in the second case, to attraction between the metal centre and the conjugate base. Therefore, as in the case of histamine and histidine, if the ligand has an ionisable functional group with a pK_a in the operating pH range, then the possible effect of the state of ionisation of this group on the binding constant has to be taken into account.

Consideration of these factors together with the requirement that the donor atom be unprotonated, leads to Schemes 5–7, and the associated equations (Table 2). The generally good fits to the experimental data (Fig. 1) is evidence for the correctness of these Schemes and provides, to our knowledge, the first complete interpretation of data for the coordination of imidazoles by B_{12a}. Supporting evidence comes from the direct determination of the relevant acid dissociations by spectrophotometry (Figs. 2 and 3, Table 4). Although some of the spectral changes are very small, there is reasonable agreement between the spectroscopically determined pK_as (Table 4) and those deduced from the binding studies (Table 3).

Log K values for binding of various ligands by B_{12a} can vary enormously, from 0.7 for acetate [21] to 14.1 for CN^- [22], and it has proved difficult to delineate factors which control the magnitude of these equilibrium constants [1]. For the imidazoles there is only a relatively small variation evident in log K_I values (between 4.30 and 4.90), if the value for the sterically hindered 2-methylimidazole is excluded. The position of the γ-band of cobalamin complexes shifts to longer wavelength as the donor strength of the ligand increases [1]. The very small differences in the γ-band positions of the imidazole complexes (Table 5) are therefore consistent with the small differences found in the binding constants. The significantly lower band position of the 2-methylimidazole complex is a consequence of significantly smaller donation of electron density onto the corrin ring (i.e. a smaller *cis* effect).

There appears to be a linear relationship between the magnitude of the formation constants and ligand basicity (curve A of Fig. 6). On coordination, the pK_as of the amino groups of histamine and histidine decrease by about 1.1 units presumably due to destabilisation of the conjugate acids by the residual positive charge on the metal ion. Deprotonation of the side chain amino group causes the binding constants for histamine and histidine to increase from log K_I = 4.43 and 4.30, respectively, to log K_{III} = 4.71 and 4.60, respectively (Table 3), and is accompanied by a red shift in the γ-band position. Both the band positions and the formation constants are, within experimental error, identical for those of the imidazole and *N*-methylimidazole complexes, and it becomes evident that, provided the complication of the charged side chain is taken into account, the formation constants are virtually independent of the base strength of the ligand. The donor power of the ligand therefore appears to play only a small role in determining the complex stability and other factors, such as hydrogen bonding between the bound ligand and the corrin side chains may be important. This is in distinct contrast to

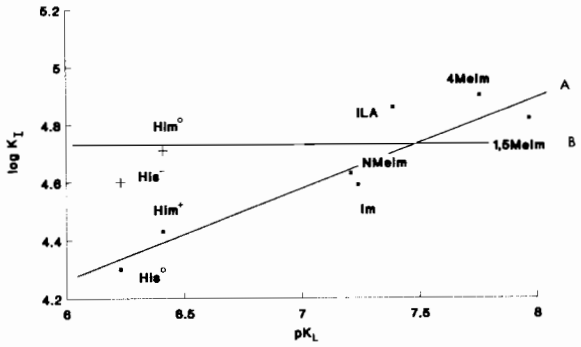


Fig. 6. Dependence of $\log K_I$ on pK_L. The apparent linear relationship (A) disappears when the binding constants, $\log K_{III}$, for histamine and histidine with neutral amino side chain, are taken into account (B).

the rate of formation of these complexes where a clear dependence on pK_L exists [8].

Obviously, many factors may contribute to the observed stability constant for a complex; even with a series of closely related ligands such as the imidazoles, delineation of these factors is difficult. We plan to investigate possible interactions between the corrin side chains and bound imidazoles by examining the effect of substitution on the side chains of histamine and histidine.

At sufficiently high concentrations, the imidazoles displace dbzm, as shown by our HPLC results (see Table 3; Fig. 4 shows the case of imidazole itself as an example), and K_{II} appears to increase with the donor power of the ligand. The spectra (Fig. 5) show that conversion of the mono-imidazole to the bis-imidazole complex results in an increase in the intensities of the γ-band (together with a small blue shift), and the α- and β-bands. This provides a possible explanation for the high pH species observed in the pH titrations (e.g. Figs. 2 and 3, Table 4). As the pH is increased (Fig. 2), the absorbance at 358 nm decreases as the γ-band shifts to longer wavelength (see Table 5), and then increases as the γ-band undergoes a blue shift and an increase in intensity; at 550 nm, there is a continuous increase in intensity with pH (Fig. 3); the high pH equilibrium, unlike the others, is established relatively slowly; we found that pK_x decreases as the concentration of the imidazole increases; there were no analogous spectral changes in the case of the *N*-methyl- and 1,5-dimethylimidazole complexes; and the spectra are not consistent with the high pH complexes being hydroxocobalamin. These observations are consistent with the high pH species being the bis(imidazole) complex, with the substitution of dbzm driven presumably by the labilising effect of the *trans* imidazolate (pK_z = *c.* 10). This effect is not without precedent. When studying the kinetics of the addition of CN^- to the hemeoctapeptide, microperoxidase-8 (MP8), which contains histidine as proximal ligand, we found that OH^- *trans* to neutral histidine is inert, but labile when histidine is ionised [23].

There is a marked decrease in the pK_a of the endocyclic N atom on coordination by Co(III). The mean pK_z (Table 3) is 10.0, whereas the pK_a for formation of free imidazolate is 14.44 [20]. The increase in the acidity of the endocyclic N atom is a consequence of polarisation of the heterocyclic electron density by the metal ion and is accompanied by a shift in the γ-band to longer wavelength by *c.* 1 nm (Table 4); this is consistent with an increase in the donor power of the axial ligand. This effect is well-known in hemepeptides and in hemoproteins. For example, coordinated imidazole in imidazole MP8 ionises with pK_a = 13.03 [24]; this pK_a is 10.34 in imidazolemetmyoglobin [25]; 10.45 in imidazolemethemoglobin from *Chironomous plumosus* [26]; and as low as 6.5–7.0 in imidazolelegoglobin *a* [27]. It has been suggested [28–30], in fact, that the strength of the Fe–N bond of proximal histidines in hemoproteins may be controlled by hydrogen bonding of the endocyclic NH to the protein, leading to partial formation of an imidazolate.

The cobalamins provide a second example of protein-free complexes in which there is a perturbation of the pK_a of a proximal functional group by the charge at the metal centre. These effects, undoubtedly important in the chemistry of the iron porphyrins, may be equally important in the biological chemistry of vitamin B_{12}. Methionine synthase catalyses the transfer of a methyl group from N^5-methyltetrahydrofolate to homocysteine to produce methionine [31]. The enzyme contains protein-bound methylcobalamin, which can be regarded as a methyl carbonium ion coordinated to cob(I)alamin [1]. Oxidation to Co(II) inactivates the enzyme, and catalytic activity is maintained by a reducing system consisting of two flavoproteins [32, 33]. The Co(II)/Co(I) couple (−610 mV), at least in aqueous solution, is beyond the potential range of biological reductants [34]. Hence the protein must facilitate the reduction back to the active Co(I) state either by stabilising Co(I) relative to Co(II), or conversely by destabilising Co(II) relative to Co(I). Since the presumably five-coordinate Co(II) form has a net +1 charge at the metal centre, while the Co(I) form is neutral, one way of achieving preference for Co(I) could be by coulombic destabilisation of Co(II) by proximity of a positively charged group such as the side chain of Lys or Arg.

Delineation of the solution behaviour of imidazole and its derivatives has enabled us to determine reliable formation constants for their complexes with aquocobalamin and to provide a further model showing how coulombic interaction between the

residual positive charge at the metal centre and a proximal ionisable functionality can perturb the latter's pK_a. We have shown [11–15] that this is one way in which proteins may control the potential of the Fe(II)/Fe(III) couple; it may be equally important in controlling the potential of the Co(II)/Co(I) couple as well.

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