Thermochemical Studies of Vitamin B_{12}. Part II.[1] Thermodynamic data for the Interaction of Imidazole and Methylimidazoles with Aquocobalamin (Vitamin B_{12a})

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Thermodynamic data (ΔG, ΔH, and ΔS) have been obtained for the reaction of imidazole, and 1-methyl-, 2-methyl- and 4(5)-methylimidazole with aquocobalamin in aqueous solution at 25 °C and in an ionic background of 0.1 mol dm^{-3} $[NO_3]^-$ ions. In addition, protonation equilibria for both the free and vitamin B_{12}-complexed imidazoles have been investigated by a combination of potentiometric and spectrophotometric methods and direct microcalorimetry. The results are discussed.

In a previous paper [1] it was shown that the dissociation of the cobalt-bound water molecule in aquocobalamin was profoundly influenced by co-ordination to the metal atom and possibly also by hydrogen-bonding interactions with the amide side chains of the corrin ring. The effect is reflected in significant differences in both the enthalpy and entropy contributions to the overall free-energy change for this dissociation. The dissociation of the imino-hydrogen atom of imidazole (imH) and substituted imidazoles is also markedly altered on co-ordination to metal ions, and this work investigates in detail the effect of methyl substitution of imidazole on this dissociation in the vitamin B_{12}-bound ligands.

Aquocobalamin reacts with imH in dilute aqueous solution to give a purple complex. The u.v.–visible spectra of both aquocobalamin and its imidazole complex show a marked variation with pH (see Table 1). The changes are attributed to ionisation of the cobalt-bound water molecule in aquocobalamin and to ionisation of the imino-hydrogen atom of the cobalt-bound imidazole in the complex, respectively. The methylimidazole complexes with aquocobalamin show similar effects. Spectroscopic data indicate that in each case the 5,6-dimethylbenzimidazole moiety of the nucleotide side chain remains bonded to the cobalt atom.[2,3]

TABLE 1

U.v.-visible spectra

		$\lambda_{max.}$/nm			
Aquocobalamin	acid soln. (pH <4)	350	411	500	527
	base soln. (pH >9)	356	420	517	538
Imidazole complex	acid soln. (pH <7)	358	414	517	548
	base soln. (pH >11.5)	360	418	521	558

EXPERIMENTAL

Materials.—Aquocobalamin was a gift from Glaxo Laboratories. The purity was checked by thin-layer

[1] W. J. Eilbeck, M. S. West, and Y. E. Owen, *J.C.S. Dalton*, 1974, 2205.

[2] G. H. Beaven, E. R. Holiday, E. A. Johnson, B. Ellis, P. Mamalis, V. Petrow, and B. Sturgeon, *J. Pharm. Pharmacol.*, 1949, **1**, 957.

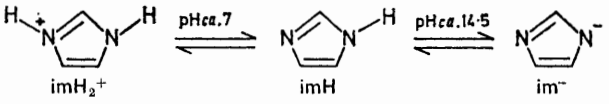
[3] G. H. Beaven, E. R. Holiday, E. A. Johnson, B. Ellis, and V. Petrow, *J. Pharm. Pharmacol.*, 1950, **2**, 944.

chromatography [4] and the material then used without further purification. Solutions of cobalamins were analysed spectrophotometrically after conversion into the dicyanide (ε 3.04 × 10^4 dm^3 mol^{-1} cm^{-1} at 367 nm).[5,6] Imidazoles were obtained from Koch-Light Laboratories Ltd. or from Aldrich Chemical Co. Ltd. Imidazole (imH), 2-methylimidazole (2-MeimH), and 4(5)-methylimidazole (4-MeimH) were purified by vacuum sublimation. 4(5)-Methylimidazole is extremely hygroscopic and was stored in a vacuum desiccator over P_4O_{10}. 1-Methylimidazole was purified by vacuum distillation through a short fractionating column. The purity of all ligands was checked by gas–liquid chromatography (g.l.c.) before use. General reagents were of AnalaR grade. Solutions were prepared from water which had been deionised, distilled, and purged with nitrogen or argon to remove carbon dioxide and oxygen. Solutions were stored in sealed vessels under nitrogen or argon atmospheres.

Instrumentation.—Potentiometric measurements were made as described in Part I. U.v.-visible spectra of the complexes were recorded on a Beckman DK2A ratio-recording spectrophotometer. Fixed-wavelength absorbance measurements were made using a Unicam SP 500 manual spectrophotometer fitted with a thermostatically controlled cell compartment. Calorimetric measurements were made with an L.K.B. batch microcalorimeter type 10 700–2C which has been described in detail elsewhere.[7]

RESULTS

Ligand-proton Equilibria.—In aqueous solution the conjugate acids of imidazoles and substituted imidazoles undergo two acid dissociations, one involving the proton attached to the imidazolinium nitrogen atom, occurring in the region of pH 7—8, and the other, involving the



imino-hydrogen atom, occurring at high pH (>14). 1-Substituted imidazoles of course have only one dissociation (*ca.* pH 7). The thermodynamic parameters for the low

TABLE 2

Thermodynamic data for the dissociation of the conjugate acids of imidazole and methylimidazoles

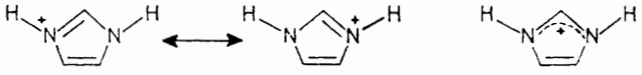
Ligand	pK_1	ΔG / kJ mol^{-1}	ΔH / kJ mol^{-1}	ΔS / J K^{-1} mol^{-1}
Imidazole	7.02 ± 0.01	40.05	33.27 ± 0.05	−22.74
2-Methyl-imidazole	7.88 ± 0.01	44.95	38.76 ± 0.05	−20.80
1-Methyl-imidazole	7.05 ± 0.01	40.22	32.35 ± 0.05	−26.42
4(5)-Methyl-imidazole	7.56 ± 0.01	43.13	40.19 ± 0.05	−9.87

pH (imidazolinium) equilibria were measured at 25 °C in an ionic background of 0.1 mol dm^{-3} $[NO_3]^-$ ions (as K$[NO_3]$), and are presented in Table 2. The data refer to the dissociation of the conjugate acids; pK_1 was determined by potentiometric titration as described elsewhere [8] and is a stoicheiometric constant. The degree of protonation of the imidazole, $\bar{n}_H$, at any titration point was calculated from equation (1), where c_H and c_A are the total concentrations of hydrogen ions and imidazole respectively; K_w is the ionic product of water at 25.0 °C and in 0.1 mol dm^{-3}

$$\bar{n}_H = \{c_H - [H^+] + (K_w/[H]^+)\}/c_A \quad (1)$$

$[NO_3]^-$ ions.[8,9] The constant pK_1 was calculated from *ca.* 20 points in the range $0.8 > \bar{n}_H > 0.2$. The pK_1 values in Table 2 are means of several determinations; the error limits indicate the maximum variation found. The enthalpies of protonation of the ligands were determined by direct microcalorimetry by a method analogous to that described in Part I for aquocobalamin.[1] For each ligand the heat of protonation was determined six times. In Table 2 the heats of deprotonation are given, which are of course equal in magnitude but of opposite sign to the measured protonation enthalpies. Values of ΔS were calculated from the measured enthalpy and free-energy changes.

From the data in Table 2 it is seen that the methylimidazoles are somewhat stronger bases than imidazole itself. This is as one would expect from a consideration of the inductive effect of the methyl group. The pK of 1-methylimidazole (1-Meim) is much lower than might have been expected considering the proximity of the methyl group to the positive charge centre in the conjugate acid, and remembering that a statistical factor of 2 will operate favouring the conjugate acid form of 1-Meim compared with imidazoles where the 1-position carries a hydrogen atom. Both these effects would tend to increase the relative base strength of 1-Meim. The reasons for 1-Meim being a much weaker base than expected are probably (*a*) the much diminished stabilisation of the conjugate acid form by hydrogen bonding compared with the conjugate acids of imidazoles where the 1-position carries an acidic proton, and (*b*) resonance stabilisation of these imidazoles due to the existence of equivalent canonical forms. Methylation at the 1-position of course destroys this equivalence.



Imidazole and Methylimidazole Complexes of Aquocobalamin.—In an aqueous solution containing both imidazole and aquocobalamin, equilibria (2)—(5) are established,

$$[B\text{-}Co\text{-}OH_2]^+ + imH \overset{K}{\rightleftharpoons} [B\text{-}Co\text{-}imH]^+ + H_2O \quad (2)$$

$$imH_2^+ \overset{K_1}{\rightleftharpoons} imH + H^+ \quad (3)$$

$$[B\text{-}Co\text{-}OH_2]^+ \overset{K_{Co}}{\rightleftharpoons} [B - Co\text{-}OH] + H^+ \quad (4)$$

$$[B\text{-}Co\text{-}imH]^+ \overset{K_3}{\rightleftharpoons} [B\text{-}Co\text{-}im] + H^+ \quad (5)$$

where K, K_1, K_{Co}, and K_3 are the stoicheiometric equilibrium

[4] R. A. Firth, H. A. O. Hill, J. M. Pratt, and T. G. Thorpe, *J. Chem. Soc.* (*A*), 1968, 453.
[5] H. A. Barker, R. D. Smyth, H. Weissbach, A. Munch-Petersen, J. I. Toohey, J. N. Ladd, B. E. Volcani, and R. M. Wilson, *J. Biol. Chem.*, 1960, **235**, 181.
[6] J. A. Hill, J. M. Pratt, and R. J. P. Williams, *J. Chem. Soc.*, 1964, 5149.
[7] I. Wadsö, *Acta Chem. Scand.*, 1968, **22**, 927.
[8] W. J. Eilbeck, F. Holmes, G. C. Philips, and A. E. Underhill, *J. Chem. Soc.* (*A*), 1967, 1161.
[9] R. F. Jameson and M. F. Wilson, *J.C.S. Dalton*, 1972, 2607.

constants. In any solution containing a fixed total amount of aquocobalamin and imidazole the concentrations of the species involved in the formation of the new complex [equation (2)] will vary with pH due to equilibria (3)—(5). However, at constant pH the quotients (6)—(8) are all

$$[\text{B–Co–OH}_2^+]/[\text{B–Co–OH}] = [\text{H}^+]/K_{\text{Co}} \quad (6)$$

$$[\text{B–Co–imH}^+]/[\text{B–Co–im}] = [\text{H}^+]/K_3 \quad (7)$$

$$[\text{imH}_2^+]/[\text{imH}] = [\text{H}^+]/K_1 \quad (8)$$

constant and therefore a pH-dependent formation constant, $K_{\text{obs.}}$, can be defined as in equation (9); $[\text{im}^-]$ does not need

$$K_{\text{obs.}} = \frac{\Sigma[\text{Product complexes}]}{\Sigma[\text{Reagent complexes}]\Sigma[\text{ligand}]} = \frac{[\text{B–Co–imH}^+] + [\text{B–Co–im}]}{([\text{B–Co–OH}_2^+] + [\text{B–Co–OH}])([\text{imH}_2^+] + [\text{imH}])} \quad (9)$$

to be considered in the pH range investigated. It can readily be shown that $K_{\text{obs.}}$ is related to the required K by equation (10). In the case of 1-methylimidazole, where there is no ionisation of the new complex equivalent to

$$K = \frac{K_{\text{obs.}}(K_{\text{Co}} + [\text{H}^+])(K_1 + [\text{H}^+])}{K_1(K_3 + [\text{H}^+])} \quad (10)$$

equation (5), equation (10) simplifies accordingly with $K_3 = 0$. In the system aquocobalamin–4(5)-methylimidazole it should be noted that in principle two distinct pairs of product complexes are possible since co-ordination of one nitrogen atom to cobalt will prevent tautomeric exchange of hydrogen between the 1- and 3-positions on the ligand. The 4-methyl- and 5-methyl-imidazole complexes will therefore be distinct species. 2-Methylimidazole did not form a complex with aquocobalamin. This is expected due to the steric effect of the methyl group adjacent to the co-ordinating nitrogen atom. This suggests that with 4(5)-methylimidazole only the 5-methylimidazole tautomer will complex since the steric effect of the 4-methyl group is identical to the 2-methyl with respect to the co-ordinating nitrogen atom.

The pH-dependent formation constants of the complexes were determined by spectrophotometric measurements over a range of pH. For each determination a series of solutions was prepared containing a constant concentration of aquocobalamin (*ca.* 4×10^{-5} mol dm^{-3}) and increasing concentrations of ligand (10^{-5}—10^{-3} mol dm^{-3}) at constant pH. All solutions contained 0.1 mol dm^{-3} $[\text{NO}_3]^-$. Spectrophotometric measurements were made at 358 nm at 25 °C and the results are given in Table 3 together with the calculated values for the pH-independent constant, K. The values obtained for K for the imidazole and 1-methylimidazole complexes are constant within acceptable limits, but in the case of the tautomeric 4(5)-methylimidazole K decreases steadily with increasing pH. This anomaly is discussed later.

The enthalpies of formation of the complexes were determined by direct microcalorimetry. Solutions of aquocobalamin (3.0 cm^3, *ca.* 4×10^{-4} mol dm^{-3}) and ligand (1.50 cm^3, *ca.* 4×10^{-2} mol dm^{-3}) were mixed in the reaction cell and solutions of ligand (1.50 cm^3, *ca.* 4×10^{-2} mol dm^{-3}) and potassium nitrate (3.0 cm^3, 0.1 mol dm^{-3}) were simultaneously mixed in the reference cell. All aquocobalamin and ligand solutions contained 0.1 mol dm^{-3} $[\text{NO}_3]^-$. The initial and final pH of the solutions in the reaction cell were measured and from the change in pH and the corresponding enthalpies of reaction the corrections necessary for the enthalpies of protonation of the free ligand and hydroxide ion were calculated. The heat of dilution of aquocobalamin was measured in a separate experiment. Each enthalpy of formation was determined six times. The results are given in Table 4.

TABLE 3

Formation constants

(*a*) The aquocobalamin–imidazole complex						
$-\log_{10}[\text{H}^+]$	6.40	6.80	7.40	8.00	8.60	9.41
$10^{-3}K_{\text{obs.}}$	7.06	11.2	19.4	10.9	4.46	1.14
$\log_{10}K$	4.59	4.53	4.64	4.60	4.64	4.64
(*b*) The aquocobalamin–1-methylimidazole complex						
$-\log_{10}[\text{H}^+]$	6.17	6.55	7.08	7.49	8.33	9.15
$10^{-3}K_{\text{obs.}}$	2.96	4.79	8.15	9.05	4.45	0.99
$\log_{10}K$	4.46	4.38	4.33	4.32	4.46	4.45
(*c*) The aquocobalamin–4(5)-methylimidazole complex						
$-\log_{10}[\text{H}^+]$	6.03	6.43	6.92	7.29	7.52	8.20	8.94
$10^{-3}K_{\text{obs.}}$	1.36	2.27	4.41	6.16	5.87	3.52	0.81
$\log_{10}K$*	4.68	4.54	4.45	4.41	4.34	4.29	4.18

* See text.

TABLE 4

Thermodynamic data for the formation of the imidazole and methylimidazole complexes of aquocobalamin

Ligand	10^4K / dm^3 mol^{-1}	ΔG / kJ mol^{-1}	ΔH / kJ mol^{-1}	ΔS / J K^{-1} mol^{-1}
Imidazole	4.06 ± 0.5	−26.29	−29.34 ± 0.05	−10.22
1-Methylimidazole	2.53 ± 0.5	−25.12	−23.08 ± 0.05	+6.85
2-Methylimidazole [a]				
4(5)-Methylimidazole	*b*	*b*	−22.12 ± 0.05	

[a] No complex formation with aquocobalamin. [b] See text.

The imidazole and 4(5)-methylimidazole complexes of vitamin B_{12a} exist in different forms in acid and basic solutions as evidenced by changes in their u.v.–visible spectra. The difference is attributed to ionisation of the imino-hydrogen atom of the imidazole ligand. The pK of this ionisation was measured both potentiometrically and spectrophotometrically. In the potentiometric determination, aquocobalamin (*ca.* 2×10^{-5} mol) plus imidazole (*ca.* 2×10^{-3} mol) in acidified (HNO_3) solution (10 cm^3) were titrated with aqueous potassium hydroxide (*ca.* 0.1 mol dm^{-3}). The large excess of imidazole required to ensure complete complex formation before ionisation means that potentiometric measurements must be made with great care, since even at pH 9 about ten times as many hydrogen ions are bound to uncomplexed imidazole (as the imidazolinium ion, pK *ca.* 7) as is present in the complex as potentially ionisable imino-hydrogen. The degree of protonation of the complex was calculated from expression (11), where c_{H} is the total hydrogen-ion concentration both

$$\bar{n}_{\text{H}} = \{c_{\text{H}} - [\text{H}^+] + (K_{\text{w}}/[\text{H}^+]) - [\text{imH}_2^+]\}/c_{\text{A}} \quad (11)$$

ionised and potentially ionisable, c_{A} is the total concentration of complex, and $[\text{imH}_2^+]$ is the concentration of protonated uncomplexed imidazole. Because of the magnitude of $[\text{imH}_2^+]$, small errors in its calculation will result in

much larger errors in pK_3. Also the total concentration of imidazole approaches the concentration of the nitrate ionic background; however, over the pH range of the experiment less than 1% was in the protonated form, so the activity coefficients will not change significantly. Over six titrations the mean value of pK_3 was 9.60 ± 0.1, a result which agrees favourably with that (pK_3 9.59 ± 0.04) obtained spectrophotometrically by absorbance measurements at 358 nm in the pH range 7—12. The enthalpies of protonation of the complexes were measured by direct calorimetry, the necessary corrections for the protonation of uncomplexed imidazole and hydroxide ions being calculated from the measured pH changes and the known enthalpies of protonation. The thermodynamic parameters are given in Table 5.

TABLE 5

Thermodynamic data for the acid dissociation of the imidazole and 4(5)-methylimidazole complexes of aquocobalamin

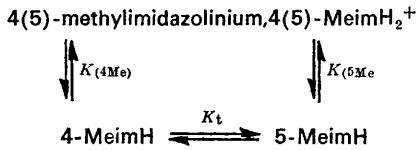
Ligand	pK_3	ΔG_3 / kJ mol^{-1}	ΔH_3 / kJ mol^{-1}	ΔS_3 / J K^{-1} mol^{-1}
Imidazole	9.59 ± 0.04	54.72	43.46 ± 0.05	−37.78
5-Methyl-imidazole	9.61 ± 0.04	54.82	47.61 ± 0.05	−24.22

DISCUSSION

Ionisation of the Imino-hydrogen Atom.—The effect of co-ordination of imidazole to the cobalt atom of vitamin B_{12} is very similar to that on water [1] in that the acidity of the potentially ionisable protons is greatly increased (the pK of the imino-hydrogen ionisation in uncomplexed imidazole is *ca.* 14.5). Hanania and Irvine [10] carried out an earlier study on the effect of co-ordination on ionisation of the imino-hydrogen atom in B_{12}- and ferrimyoglobin-bound imidazole and concluded that the effect is predominantly reflected in a much decreased enthalpy of ionisation. Their value (10.3) of pK_3 for the B_{12}-imidazole complex seems unexpectedly high by comparison both with our present result and with their result (10.25) for the ferrimyoglobin–imidazole complex in which the metal atom carried a formal charge of +1 compared with +2 on the cobalt atom of aquocobalamin. Our present value of 9.6 for pK_3 is much more in line with the predicted trend. The thermodynamic parameters for the ionisation of the imidazole and 4(5)-methylimidazole complexes are very similar. This is in spite of the fact that the methyl group in the latter complex is almost certainly at the 5-position and thus placed, adjacent to the ionising hydrogen atom, might be expected to have a more marked effect.

Formation of the Complexes.—Both the pH-dependent and pH-independent formation constants obtained for the imidazole complex of vitamin B_{12a} are in good agreement with those obtained by Hanania and Irvine.[10] The formation constants for the methyl-substituted imidazole complexes might be expected to vary in parallel with the basicity of the ligand. The 2-methylimidazole complex is not formed due to the steric hindrance of the methyl group adjacent to the co-ordinating nitrogen atom. In this context it is interesting to note that, contrary to earlier reports, benzimidazole did not form a complex with vitamin B_{12a}.[11] The colour change on mixing solutions of B_{12a} and benzimidazole can be entirely attributed to a change in the relative concentrations of aquo- and hydroxo-cobalamin due to the pH change. In buffered solutions no change in the spectrum is observed. It follows therefore that in the case of 4(5)-methylimidazole only the 5-methyl tautomer will complex. 1-Methylimidazole forms a weaker complex than imidazole and not (as predicted from its basicity) a complex of equal or slightly greater stability. Model studies indicate that steric effects are small and it is quite possible that the imidazole complex is stabilised to some extent by hydrogen bonding to the amide side chains of the corrin ring. This is precluded in the case of 1-Meim. It is noteworthy that the entropy of formation is positive (favourable) in the 1-Meim complex, whereas formation of the imH complex occurs with a negative entropy change. Direct comparison of the thermodynamic parameters for the 4(5)-methylimidazole complex is complicated for the reasons outlined below.

4(5)-Methylimidazole–Aquocobalamin System.—In this system equilibria corresponding to equations (2), (4), and (5) are established. The equilibrium for the protonation of free ligand, (3), must however be replaced by a more complex set of equilibria which take into account the tautomerism between the 4-methyl- and 5-methylimidazoles. The experimentally determined dissoci-



ation constant, K_1, in this case is an empirical constant and is related to the dissociation constants of the tautomers as in equations (12)—(15) since the protonated forms

$$K_1 = \frac{[H^+]([4\text{-MeimH}] + [5\text{-MeimH}])}{[4(5)\text{-MeimH}_2^+]} \quad (12)$$

of the two tautomers are identical. The individual dissociation constants are given by equations (13) and (14) and related by (15). The constant K_t is given by

$$K_{(4Me)} = \frac{[H^+][4\text{-MeimH}]}{[4(5)\text{-MeimH}_2^+]} \quad (13)$$

$$K_{(5Me)} = \frac{[H^+][5\text{-MeimH}]}{[4(5)\text{-MeimH}_2^+]} \quad (14)$$

$$K_{(4Me)} + K_{(5Me)} = K_1 \quad (15)$$

equation (16). All these equilibria (except K_t) are pH

[10] G. I. H. Hanania and D. H. Irvine, *J. Chem. Soc.*, 1964, 5694.

[11] J. A. Hill, J. M. Pratt, and R. J. P. Williams, *J. Theor. Biol.*, 1962 **3**, 423.

dependent. However, at constant pH the quotient ([4-MeimH] + [5-MeimH])/[4(5)-MeimH$_2$] = $K_1/[H^+]$ is

$$K_t = \frac{[4\text{-MeimH}]}{[5\text{-MeimH}]} = \frac{K_{(4Me)}}{K_{(5Me)}} \quad (16)$$

a constant and so a pH-dependent formation constant for the aquocobalamin complex can be defined. In a solution of 4(5)-methylimidazole, relations (17) and (18)

$$\Sigma[\text{ligand}] = [5\text{-MeimH}] + [4\text{-MeimH}] + [4(5)\text{-MeimH}_2^+] \quad (17)$$

$$[4\text{-MeimH}] = K_t[5\text{-MeimH}] \quad (18)$$

hold. From equations (12), (17), and (18) we obtain (19).

$$\Sigma[\text{ligand}] = [5\text{-MeimH}](1 + K_t + \{[H^+](K_t + 1)/K_1\}) \quad (19)$$

The true formation constant for the 5-methylimidazole complex [*cf.* equation (2)] is therefore related to the observed constant $K_{obs.}$ as in (20). This expression differs from that of the non-tautomeric ligands only by the (unknown) factor (K_t + 1). This does not therefore

$$K = \frac{K_{obs.}([H^+] + K_{Co})([H^+] + K_1)(K_t + 1)}{K_1([H^+] + K_3)} \quad (20)$$

account for the variation of the ' pH-independent ' formation constant with pH in the 4(5)-methylimidazole system, and this is still puzzling.

The enthalpy change for the formation of the 5-methylimidazole complex should not be compared with the enthalpies of formation of the other complexes since it will include a contribution due to re-establishing the tautomeric equilibrium as one tautomer is preferentially removed by co-ordination. The contribution is, however, likely to be small and probably negligible.

One of us (M. S. W.) thanks the S.R.C. for a research grant.

[5/1147 *Received*, 12*th June*, 1975]