1091. *The Effect of Co-ordination on Ionization. Part V.*[1] *The Imidazole Complex of Aquocobalamin.*

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The following thermodynamic data have been obtained at 25° for the acid ionization in dilute aqueous solution of the imino =NH group in aquocobalamin–imidazole: $pK_3^0 = 10{\cdot}25 \pm 0{\cdot}02$, $\Delta H_3^0 = 10{\cdot}5 \pm 1{\cdot}0$ kcal. mole^{-1}, and $\Delta S_3^0 = -12 \pm 3$ e.u. The formation constant of this complex has also been determined at $I = 0{\cdot}042$M over a range of pH and temperature, yielding for the pH-independent equilibrium [equation (1)] the following data at 25° and zero ionic strength: $\Delta G^0 = -6{\cdot}26$ kcal. mole^{-1}, $\Delta H^0 = -7{\cdot}20 \pm 0{\cdot}60$ kcal. mole^{-1}, and $\Delta S^0 = -3{\cdot}2 \pm 2{\cdot}0$ e.u. These results are discussed.

It has been shown[1] that co-ordination has a pronounced effect on the ionization of the imino =NH group in imidazole. Thus, the pK of this group, 14·5 in imidazole itself, drops to 10·3 in the ferrimyoglobin complex, a 10^4-fold increase in acid strength. This effect is accompanied by a decrease in endothermicity, the enthalpy of ionization decreasing by 6 kcal. mole^{-1}, and by a very slight but unfavourable change in the entropy. It was concluded that in this system, although charge is expected to play a part, it is electronic effects that predominate.

Like ferrimyoglobin, aquocobalamin is an unsymmetrical metal complex in which cobalt occupies a position similar to that of iron in myoglobin.[2,3] Thus, the cobalt(III) atom is loosely bonded to a water molecule at the sixth co-ordinating position, as is the iron(III) atom in ferryimyoglobin. Both metal atoms are also joined to four pyrrole nitrogens, but the corrin ring in aquocobalamin differs from the porphyrin ring in ferrimyoglobin in having one less methine bridge. In both cases the metal is attached to an imidazolinium nitrogen, in aquocobalamin to a nucleotide through a benzimidazole residue, and in ferrimyoglobin to

[1] Part IV, P. George, G. I. H. Hanania, D. H. Irvine, and I. Abu-Issa, preceding paper.
[2] E. Lester Smith, "Vitamin B_{12}," Methuen, London, 1960.
[3] P. George *et al.*, *Ann. New York Acad. Sci.*, 1960, **88**, 393.

a protein through a histidine residue. The cobalt(III) atom in aquocobalamin carries a formal charge of +2, but the molecule also contains a phosphate group in the side-chain contributing a charge of −1; in ferrimyoglobin the iron(III) atom carries a formal charge of +1, the effective charge of the molecule depending on pH.

In view of this similarity between aquocobalamin and ferrimyoglobin it was considered of interest to examine the aquocobalamin–imidazole system with particular reference to the ionization of the imino =NH group.

Formation of Aquocobalamin–Imidazole Complex.—Aquocobalamin reacts readily with imidazole in dilute aqueous solution, to give a well-defined reddish purple complex. In acidic solution the absorption spectrum of the parent compound has maxima in the near-ultraviolet and visible regions at 355, 410, 500, and 522 mμ, which are shifted in alkaline solution to 358, 420, 510, and 533 mμ as a result of the ionization of the cobalt-bound water molecule. The complex has maxima at 360, 408, 480, 518, and 546 mμ, which are shifted in strongly alkaline solution to 362, 415, 485, 520, and 552 mμ. This change is reversible and is attributed to the imino =NH ionization in the bound imidazole.

We have determined spectrophotometrically the formation constant of the complex over a range of pH and temperature. The equilibrium is

$$[B\text{–}Co(H_2O)]^+ + ImH = [B\text{–}Co\text{–}ImH]^+ + H_2O \qquad (K) \qquad (1)$$

where $[B\text{–}Co(H_2O)]^+$ represents aquocobalamin in which B, the benzimidazole part of the nucleotide, is attached to cobalt as well as to a side-chain of the corrin ring, and ImH represents a neutral imidazole molecule. K is the pH-independent formation constant, and is defined in terms of concentration. However, the concentrations of the species in equation (1) vary with pH as a result of the following equilibria,

$$[B\text{–}Co(H_2O)]^+ = [B\text{–}Co\text{–}OH]^0 + H^+ \qquad (K_{Co}) \qquad (2)$$

$$ImH_2^+ = ImH + H^+ \qquad (K_1) \qquad (3)$$

$$[B\text{–}Co\text{–}ImH]^+ = [B\text{–}Co\text{–}Im]^0 + H^+ \qquad (K_3) \qquad (4)$$

where the ionization constants are given subscripts which conform with the definitions already used.[1] The observed formation constant, K_{obs}, therefore varies with pH, and it can be shown that K_{obs} is related to K by the expression

$$K = K_{obs}(K_{Co} + H)(K_1 + H)/K_1(K_3 + H) \qquad (5)$$

Values of K_{obs} at 25·0° and $I = 0{\cdot}042$M are given in the Table, which also lists the calculated values of K. In the calculation of K we have taken $pK_{Co} = 7{\cdot}72$ (Hanania, Irvine, and Irvine, unpublished results), $pK_3 = 10{\cdot}31$ (this paper, see below), and $pK_1 = 7{\cdot}06$, estimated

Variation with pH of the measured formation constant, K_{obs}, for the aquocobalamin–imidazole complex at 25·0° and $I = 0{\cdot}042$M. Values of the pH-independent formation constant, K, were calculated by use of equation (5). The mean deviations are about ±3% for K_{obs} and ±4% for K.

pH	6·65	7·20	7·52	8·56	9·44
$10^{-3}K_{obs}$	10·5	17·0	17·0	4·84	0·96
$10^{-4}K$	4·11	3·84	3·76	4·06	3·62

from the listed values of this ionization.[4] The values of K are seen to be constant within experimental error. We have also measured K_{obs} at three other temperatures over a wide pH range at $I = 0{\cdot}042$M, with the following results: $10^{-4}K = 7{\cdot}60$ (10·7°), 5·62 (18·5°), 3·90 (25·0°), and 3·39 (29·8°), the mean deviation being ±4%. The plot of $\log K$ against $1/T$ is linear and yields $\Delta H = -7{\cdot}20 \pm 0{\cdot}60$ kcal.mole^{-1}, which is also assumed to be the value of

[4] "Stability Constants," Part I, *Chem. Soc. Special Publ.* No. 6, 1957.

ΔH^0. Since the reaction in equation (1) involves no change in charge, it is assumed that K is independent of I at low ionic strength. Hence, at 25°, $\Delta G^0 = \Delta G = -6{\cdot}26$ kcal. mole^{-1}, and $\Delta S^0 = -3{\cdot}2 \pm 2{\cdot}0$ e.u.

A comparison of the data for this system with data for the corresponding ferrimyoglobin system[1] shows that the formation constant for the aquocobalamin–imidazole complex is about 250 times that of the ferrimyoglobin complex, and that this difference is reflected largely in enthalpy (ΔH^0 $-7{\cdot}2$ kcal. mole^{-1}, compared with $-4{\cdot}05$ kcal. mole^{-1} for the ferrimyoglobin system). Thus, the greater stability of the aquocobalamin–imidazole complex is due to stronger metal–nitrogen bonding. Stronger metal–nitrogen bonding is observed in inorganic octahedral complexes of cobalt(III) relative to those of iron(III) [cf. ammines of cobalt(III) and iron(III)], and this is attributed to greater ligand-field stabilization in the former case. A similar explanation probably holds for the larger octahedral chelates, aquocobalamin and ferrimyoglobin.

The entropy of formation for both complexes is small and negative, $-3{\cdot}2$ and $-3{\cdot}5$ e.u., respectively. It is interesting to consider this entropy change from the point of view of standard molar entropies. For a reaction of the type

$$M_{aq} + L_{aq} = ML_{aq} \quad (6)$$

the entropy change, following the usual convention, is

$$\Delta S = S_{ML} - S_M - S_L \quad (7)$$

where S_{ML}, S_M, and S_L are the standard molar entropies of the product complex, the reactant, and the ligand, respectively. By use of Cobble's equation,[5] and by taking the density of imidazole to be about unity, the standard molar entropy of imidazole is estimated at approximately 35 e.u. Then, from equation (7), $(S_{ML} - S_M)$ is approximately 32 e.u. for both the aquocobalamin and ferrimyoglobin systems. These large positive values of $(S_{ML} - S_M)$ suggest that complex-formation brings about a greater degree of disorder equally in both systems. In this connection it may be noted that a large value, $(S_{ML} - S_M) = 21$ e.u., was obtained for the reaction of ferrimyoglobin with phenol, whereas with small anions such as OH^-, F^-, and CN^- the corresponding value is about -3 e.u.[6]

Ionization of the Imino =NH *Group in the Aquocobalamin–Imidazole Complex.*—Complete formation of the aquocobalamin–imidazole complex, unlike the case of ferrimyoglobin, is possible even in strongly alkaline solution because of the large favourable free-energy of formation (*loc. cit.*). It was therefore possible to determine, by direct spectrophotometric measurement, the acid ionization constant for the =NH group in this complex. The equation for this ionization is (4), with ionization constant K_3. The results of the measurements at $I = 0{\cdot}042$M give pK_3 values of 10·44 and 10·27 at 20·0° and 26·5°, respectively, the mean deviation being $\pm 0{\cdot}01$ in each case. $\Delta H_3 \sim \Delta H_3^0$ is thus 10·5 kcal. mole^{-1}, and since this value is obtained from measurements at two temperatures only we estimate the uncertainty to be $\pm 1{\cdot}0$ kcal. mole^{-1}. From this, pK_3 (25°) = 10·31. The value of pK_3 at zero ionic strength, pK_3^0, is obtained by applying the same ionic strength equation as in Part IV, except that in this case the distance of closest approach is taken as 9Å, giving $Ba \sim 3$. Then, pK_3^0 (25°) = $10{\cdot}25 \pm 0{\cdot}02$, and consequently ΔS_3^0 is -12 ± 3 e.u.

It is interesting to note that the thermodynamic data for this ionization are similar to those for the corresponding ionization in ferrimyoglobin–imidazole. In view of the fact that the metal carries a formal charge of +2 in aquocobalamin and only +1 in ferrimyoglobin, a considerably lower pK_3^0 value might have been expected in the former case. The results suggest that some additional factor exists which stabilises the conjugate acid in aquocobalamin. *X*-Ray studies have shown that in the analogous compound, Factor V_{1A} of vitamin B_{12}, an amide group in one of the side-chains is hydrogen-bonded to the cobalt-bound

[5] J. Cobble, *J. Chem. Phys.*, 1953, **21**, 1443.
[6] P. George, "Currents in Biochemical Research," ed. Green, Interscience, New York, 1956, p. 338.

water molecule.* Thus, an amide group is structurally in the vicinity of the cobalt atom, and the possibility cannot be excluded that in aquocobalamin–imidazole a carbonyl group from the side-chain is hydrogen-bonded to the imino =NH group of the bound imidazole.

Experimental

Reagents and Materials.—The aquocobalamin was a gift from Dr. E. Lester Smith (Glaxo Laboratories, Greenford, Middlesex). Dried samples of pure imidazole (British Drug Houses) were used without further purification. Buffers were made from AnalaR reagents, phosphate for pH 6—8, borate for pH 8—10, and bicarbonate and phosphate for the more strongly alkaline solutions. Ionic strengths were adjusted with AnalaR sodium chloride. Conductivity water was used throughout.

Determination of the Formation Constant, K_{obs}, of the Aquocobalamin–Imidazole Complex.—K_{obs} was obtained spectrophotometrically. For each determination, a series of solutions were prepared containing different concentrations of imidazole (usually between 10^{-4} and 10^{-3}M) and constant total aquocobalamin concentration ($\sim 10^{-4}$M), adjusted with buffer and salt to the required pH at $I = 0{\cdot}042$M. K_{obs} is calculated from the equation

$$K_{obs} = (\text{product})/(\text{reactant})\,(\text{imidazole}),$$

the concentration ratio (product)/(reactant) being obtained directly from optical densities measured at 360 mμ in the acid range, and at 552 mμ in alkaline solutions. Each K_{obs} value reported in the Table is the mean of about five determinations, and has a mean deviation of ±3%.

Determination of the Imino =NH *Acid Ionization Constant,* pK_3, *in the Complex.*—pK_3 was obtained spectrophotometrically from the relationship

$$pK_3 = pH + \log(\text{acid})/\text{base}),$$

where the concentration ratio (acid)/(base) was obtained from optical-density measurements at 552 mμ. For each determination, a series of solutions were prepared containing constant total concentration of the complex, with pH varying from 9·5 to 12·0 and ionic strength 0·042M. The optical density of the corresponding solution at pH 7·0 was taken to represent 100% conjugate acid. Because of irreversible changes in strongly alkaline solution, the optical density corresponding to 100% conjugate base had to be corrected by a small factor to obtain the best fit of the data. In every case pH was measured simultaneously with optical density. The reported pK_3 values are the means of six determinations and have a mean deviation of ±2%.

Optical Density and pH *Measurements.*—The equipment and procedure have already been described. (See Part III of this series.)

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