1090. *The Effect of Co-ordination on Ionization. Part IV.*[1] *Imidazole and its Ferrimyoglobin Complex.*

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Thermodynamic data have been obtained for the acid ionization in dilute aqueous solution of the imino =NH group in imidazole and in its complex with sperm-whale ferrimyoglobin. For the ionization in free imidazole at 25°, $pK_2^0 = 14{\cdot}44 \pm 0{\cdot}03$, $\Delta H_2^0 = 17{\cdot}6 \pm 1{\cdot}6$ kcal. mole^{-1}, and $\Delta S_2^0 = -7 \pm 5$ e.u. In the complex, the corresponding values are: $pK_3^0 = 10{\cdot}34 \pm 0{\cdot}03$, $\Delta H_3^0 = 11{\cdot}4 \pm 1{\cdot}0$ kcal. mole^{-1}, and $\Delta S_3^0 = -9 \pm 4$ e.u. The significance of these results is discussed.

The effect of co-ordination to a metallic cation on the acid strength of a group on a ligand bonded to the metal has been shown[2,3] to be characterized by two features. (1) It is essentially an enthalpy effect, in that the decrease in pK for the group is reflected mainly in a corresponding decrease in ΔH of ionization. (2) It is a composite effect, its magnitude

[1] Part III, G. I. H. Hanania and D. H. Irvine, *J.*, 1965, 1149.
[2] G. I. H. Hanania and D. H. Irvine, *J.*, 1962, 2745.
[3] G. I. H. Hanania and D. H. Irvine, *J.*, 1962, 2750.

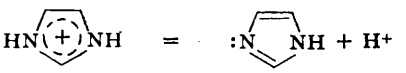
depending upon the electrostatic effect of the metallic cation on the ionizing group, as well as on the extent to which the stability of the conjugate base is enhanced by resonance.

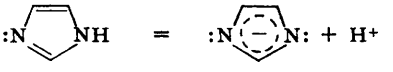
In hæmoproteins, which may be regarded as unsymmetrical octahedral co-ordination complexes of iron, it has also been observed that the acid strengths of certain groups on the protein are affected by the nature of the ligand bonded to iron at the sixth co-ordinating position. Thus, Wyman[4] concluded from analysis of acid–base titration data that the acid strength of a "hæm-linked" group increases from pK 7·8 in ferrohæmoglobin to pK 6·8 in oxyhæmoglobin, the reaction involving replacement of H_2O by O_2 at the sixth co-ordinating position of the iron atom. Coryell and Pauling[5] attributed the effect to the ionization of the imidazolinium =NH$^+$– group of a histidine amino-acid residue to which the iron atom was assumed to be directly bonded. Their interpretation was based on the similarity between the thermodynamic data for this ionization and corresponding data available at the time for several substituted derivatives of imidazole and histidine. Although X-ray studies[6] have substantiated the assumption that the iron atom in hæmoglobin is bonded to the protein through an imidazole nitrogen of a histidine residue, it does not necessarily follow that the hæm-linked group in hæmoglobin is associated with this residue. The argument of Coryell and Pauling is inconclusive since it is based on a comparison of ionizations which are different in nature, the one in hæmoglobin involving the extra effect of the octahedral ligand-field of the iron(II) atom. Our work has shown that this is expected to exert a marked influence on the acid strength of a linked ionizing group, even when charge effects are not significant.

Where charge effects occur, as in the reaction of imidazole with a ferrihæmoprotein, the ionization associated with the imidazole should be affected to a much greater extent, and it might even be possible to detect the acid ionization of the extremely weak imino =NH group in imidazole. In this connection it is worth noting that Russell and Pauling[7] carried out a magnetic study of the ferrihæmoglobin–imidazole equilibrium, and concluded that in the complex there is a hæm-linked group with pK 9·5. Scheler,[8] from a spectral study of the same system, observed an ionization in the complex with p$K \sim 10{\cdot}4$, and suggested that the conjugate base contained an imidazole anion. This was further substantiated by his observation[8] that there is a marked effect of pH on the absorption spectrum of the ferrihæmoglobin–imidazole complex in alkaline solution, but none in the case of the corresponding N-methylimidazole complex.

We have determined the thermodynamics of the acid ionization of the imino =NH group in imidazole, and have examined the effect of co-ordination on this ionization by obtaining thermodynamic data for the corresponding ionization in the ferrimyoglobin–imidazole complex.

Ionization of the Imino =NH *Group in Imidazole.*—In imidazole, two acid ionizations occur:





For the imidazolinium ionization [equation (1)], the best thermodynamic data available[9] at 25° are: p$K_1^0 = 6{\cdot}98$, $\Delta H_1^0 = 7750$ cal. mole^{-1}, $\Delta S_1^0 = -6$ e.u. For the second ionization,

[4] J. Wyman, *J. Biol. Chem.*, 1939, **127**, 581.
[5] C. D. Coryell and L. Pauling, *J. Biol. Chem.*, 1940, **132**, 769.
[6] A. F. Cullis *et al.*, *Proc. Roy. Soc.*, 1962, *A*, **265**, 161.
[7] C. D. Russell and L. Pauling, *Proc. Nat. Acad. Sci. U.S.A.*, 1939, **25**, 517.
[8] W. Scheler, *Acta Biol. Med. Ger.*, 1959, **2**, No. 5, 468.
[9] "Stability Constants," Part I, *Chem. Soc. Special Publ.* No. 6, 1957.

which is that of the extremely weak imino-group [equation (2)], Walba and Isensee[10] obtained pK_2^0 based on measurements of the hydrolysis constant K_h for the equilibrium

$$Im^- + H_2O = ImH + OH^- \qquad (K_h) \qquad (3)$$

at ionic strength $I = 0{\cdot}50$M and 25°. They used an empirical relationship $pK_h^0 = pK_h + 0{\cdot}10I^{1/2}$ to obtain the value at zero ionic strength, pK_h^0, and from the value of K_w^0 they computed the thermodynamic acid ionization constant $pK_2^0 = 14{\cdot}52$ at 25°.

Following the same procedure, we have measured K_h at three temperatures and $I = 0{\cdot}50$M. We have corrected these values to zero ionic strength by use of Walba and Isensee's empirical relationship, and by use of the appropriate values[11] of K_w^0 we have calculated pK_2^0 at the three temperatures. The data (Table 1) yield pK_2^0 (25°) = 14·44 (cf. above value of 14·52), $\Delta H_2^0 = 17{\cdot}6 \pm 1{\cdot}6$ kcal. mole^{-1}, and $\Delta S_2^0 = -7 \pm 5$ e.u.

TABLE 1.

Determination of acid ionization constant for the imino =NH group in imidazole at three temperatures. K_h = hydrolysis constant at $I = 0{\cdot}50$M [equation (3)]; K_h^0 = hydrolysis constant at $I = 0$, calculated by use of the empirical relationship:[10] $pK_h^0 = pK_h + 0{\cdot}10I^{1/2}$; K_w^0 = ionic product for water;[11] K_2^0 ($= K_w^0/K_h^0$) the imino =NH acid ionization constant at $I = 0$ [equation (2)].

Temperature (°c)	K_h	$-pK_h^0$	pK_w^0	pK_2^0
15·3	4·30 ± 0·30	0·56 ± 0·03	14·330	14·89 ± 0·03
25·3	3·20 ± 0·30	0·44 ± 0·04	13·990	14·43 ± 0·04
35·2	2·60 ± 0·30	0·34 ± 0·05	13·675	14·02 ± 0·05

The large positive value of ΔH is characteristic of the ionization of very weak acids. However, the value of ΔS is small for an ionization of the type 0 to −1, normal values being of the order −20 to −30 e.u. It appears that in this case a simple interpretation of entropy change in terms of electrorestriction of water molecules is not adequate.

Ionization of the Imino =NH *Group in the Ferrimyoglobin–Imidazole Complex.*—Direct spectrophotometric measurement of the acid ionization constant for the =NH group in this complex is difficult to carry out with precision. This is because a large excess of imidazole is required to effect full formation of the complex at high pH, and this tends to cause protein denaturation. Accordingly, we adopted an alternative procedure in which the formation constant of the complex was measured over a range of pH, and the ionization constant obtained from analysis of the data.

Measurements were carried out in the pH range 9—12. In this region the following equilibria are assumed:

$$Fe^+(H_2O) + \underset{(ImH)}{:N\!\!\diagup\!\!\diagdown NH} = \underset{(Fe^+\text{—}ImH)}{Fe\text{—}N\!\!\diagup\!\!\diagdown\overset{+}{N}H} + H_2O \qquad (K) \qquad (4)$$

$$Fe^+(H_2O) = FeOH + H^+ \qquad (K_{Fe}) \qquad (5)$$

$$\underset{(Fe^+\text{—}ImH)}{Fe\text{—}N\!\!\diagup\!\!\diagdown\overset{+}{N}H} = \underset{(Fe\text{—}Im)}{Fe\text{—}N\!\!\diagup\!\!\diagdown N:} + H^+ \qquad (K_3) \qquad (6)$$

where $Fe^+(H_2O)$ represents ferrimyoglobin in which the iron(III) atom has a formal charge of +1. The participation of the imidazolinium ionization of ImH_2^+ [equation (1); pK_1 7·0] and of the imino ionization of ImH [equation (2); pK_2 14·5] may be neglected. It is easily deduced

[10] H. Walba and R. W. Isensee, *J. Org. Chem.*, 1956, **21**, 702.
[11] H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolytic Solutions," 3rd edn., Rheinhold, New York, 1958, p. 754.

that the relationship between the measured formation constant, K_{obs}, and the pH-independent constant K [equation (4)] is then

$$K_{obs}(K_{Fe}+h) = KK_3+Kh \quad (7)$$

where $K_{obs} = \Sigma(\text{complex})/\Sigma(\text{myoglobin}).(\text{imidazole}) = ([FeImH^+]+[FeIm])/\{([Fe^+H_2O]+[FeOH])[ImH]\}$, h is the hydrogen-ion activity obtained from pH measurements assuming $pH = -\log h$, and the square brackets indicate molar concentrations. The ratio (complex)/(myoglobin) is obtained spectrophotometrically. Values of K_{obs} obtained at $I = 0{\cdot}20$ over the pH range 9—12, and at four temperatures, are given in Table 2. In each case the plot

TABLE 2.

Variation of the formation constant, K_{obs}, for the ferrimyoglobin–imidazole complex with pH, at $I = 0{\cdot}20$M and four temperatures.

15·3°		20·0°		25·0°		35·0°	
pH	K_{obs}	pH	K_{obs}	pH	K_{obs}	pH	K_{obs}
9·87	37·4±0·4	10·32	16·8±0·5	9·61	39·3±0·7	9·70	25·0±0·8
10·22	24·7±0·7	10·52	14·2±0·9	9·97	22·2±0·6	9·94	18·9±0·5
10·47	18·1±0·4	10·85	10·3±0·3	10·20	17·3±0·6	10·13	16·0±0·8
10·68	14·1±0·7	11·11	8·1±0·3	10·40	14·2±0·6	10·46	12·3±0·4
10·99	9·5±0·4			10·74	9·1±0·9	10·71	9·7±0·1
11·28	9·0±0·3			11·00	8·3±0·3		

of $K_{obs}(K_{Fe}+h)$ against h is linear. From the slope and intercept, values of K and K_3 are obtained. The results are shown in Table 3, which also contains the values of K_{Fe}. From the linear plot of $\log K$ against $1/T$, $\Delta H = -4{\cdot}05\pm0{\cdot}70$ kcal. mole^{-1} for the reaction in equation (4), and, from the corresponding plot for K_3, $\Delta H_3 = 11{\cdot}4\pm1{\cdot}0$ kcal. mole^{-1} for the imino ionization in equation (6).

TABLE 3.

Determination of the pH-independent formation constant, K, and the imino acid ionization constant, K_3, of the ferrimyoglobin–imidazole complex [eqn. (6)] by use of eqn. (7) and the data in Table 2, at $I = 0{\cdot}20$M and four temperatures.

t (°c)	$10^{10}K_{Fe}$*	K	$10^{11}K_3$	pK_3
15·3	6·21	208±10	2·12±0·18	10·67±0·04
20·0	7·41	182±12	2·75±0·24	10·56±0·03
25·0	9·12	158±10	3·99±0·31	10·40±0·03
35·0	13·4	138±15	7·83±0·55	10·11±0·03

* G. I. H. Hanania, D. H. Irvine, and A. Yeghiayan, unpublished results.

It is assumed that the pH-independent formation constant, K, is independent of ionic strength up to $I = 0{\cdot}20$, since reaction (4) involves the replacement of the neutral water molecule by the neutral imidazole, and that K_3 may be corrected to zero ionic strength by use of the equation

$$\log K_3 = \log K_3^0 - 0{\cdot}5I^{1/2}/(1+BaI^{1/2}) \quad (8)$$

where B is the Debye–Hückel constant, and a, the distance of closest approach, is taken as 18 Å for ferrimyoglobin–imidazole (making $Ba \sim 6$). This relationship is suggested by the work of Beetlestone and Irvine,[12] but is strictly valid only at low ionic strengths. In this connection it is worth noting that in the ionization of the iron-bound water in ferrimyoglobin[13] there is a sharp reversal of ionic strength effect at $I \sim 0{\cdot}01$M. Application of equation (8) to the ionization data[13] at $I < 0{\cdot}01$M indicates that the effective charge on ferryimyoglobin in

[12] J. G. Beetlestone and D. H. Irvine, *Proc. Roy. Soc.*, 1964, *A*, **277**, 401.
[13] P. George and G. I. H. Hanania, *Biochem. J.*, 1957, **65**, 756.

the pH region of our present measurements (pH 9—12) is negative, and that the negative charge increases with increasing pH. The small formation constant of ferrimyoglobin–imidazole makes it impractical to obtain data for the equilibrium in equation (6) at ionic strengths low enough for valid extrapolation. Accordingly, we have applied equation (8) to our data at $I = 0{\cdot}2$M on the assumption that at this ionic strength considerable screening of charge occurs, and hence that the charges involved are +1 to 0, as appear in equation (6). On this basis the resulting value of pK_3^0 is $10{\cdot}34 \pm 0{\cdot}03$ at 25·0°.

Thus, the thermodynamic data at 25·0° for both the formation and the ionization of ferrimyoglobin–imidazole are as follows:

for equation (4), $K^0 = 158 \pm 10$ M^{-1}; $\Delta H^0 \sim \Delta H = -4{\cdot}05 \pm 0{\cdot}70$ kcal. mole^{-1};

$$\Delta S^0 = -3{\cdot}5 \pm 2{\cdot}5 \text{ e.u.}$$

for equation (6), $pK_3^0 = 10{\cdot}34 \pm 0{\cdot}03$; $\Delta H_3^0 \sim \Delta H_3 = 11{\cdot}4 \pm 1{\cdot}0$ kcal. mole^{-1};

$$\Delta S_3^0 = -9 \pm 4 \text{ e.u.}$$

These results show that the acid strength of the imino =NH group has increased from pK_2^0 (25°) = 14·44 in free imidazole to pK_3^0 (25°) = 10·34 in the complex, which is clearly the result of co-ordination to the charged metal ion. Further, the change in the free-energy of ionization is predominantly the result of a change in enthalpy, from 17·6 to 11·4 kcal. mole^{-1}. The entropy of ionization hardly changes. It is interesting to note in this connection that in their study of the ionization of various mammalian hæmoglobins Beetlestone and Irvine[12] observed that ΔH and $T\Delta S$ acted in opposite directions so as nearly to compensate each other, and attributed this to the operation of predominantly electrostatic effects in these systems. Our study of the 2-2′-pyridylimidazoline–iron(II) system,[3] where charge effects operate, also suggests that ΔH and ΔS act in opposite directions.* A similar opposing effect of ΔH and ΔS might have been anticipated in the present system as a result of the influence of the positively charged iron atom. It is difficult to say whether this obtains or not because of the limits of uncertainty in the results. However, in this system, imidazole is directly conjugated to the metal ion, and electronic effects are also expected to play some part in affecting the acid strength of the imino =NH group in the complex. We have shown[2] that where electronic effects predominate the effect of co-ordination on ionization is reflected almost entirely in enthalpy. It is therefore perhaps not too surprising that in the present system where electronic and electrostatic effects are both expected to operate we observe a large decrease in endothermicity (6·2 kcal. mole^{-1}) but a considerably smaller, if indeed any, effect on entropy.

Experimental

Reagents and Materials.—The ferrimyoglobin was a sample of sperm-whale skeletal ferrimyoglobin purchased as a lyophilized salt-free solid from Seravac Laboratories, Maidenhead, England. Dried samples of pure imidazole (British Drug Houses) were used without further purification. The buffers were made from pure B.D.H. glycine and AnalaR sodium hydroxide, and ionic strength adjusted with AnalaR sodium chloride. Conductivity water was used in making all solutions.

Determination of the Imino =NH Acid Ionization Constant, pK_2, in Imidazole.—The method followed is that of Walba and Isensee[10] and involves the measurement of the hydrolysis constant (K_h) for the imidazole anion [equation (3)]. This is obtained spectrophotometrically from optical-density measurements in the range 230—260 mμ on dilute aqueous solutions of imidazole (0·01—0·3M) in the presence of varying sodium hydroxide concentration (pH 9·7—10). The calculation involves a series of successive approximations which is described in detail by the authors.[10] K_2 is computed from the K_h values and the ionic product of water using the relationship $K_2 = K_w/K_h$.

Determination of the Imino =NH Acid Ionization Constant, pK_3, in the Complex.—This is obtained from measurements of the formation constant (K_{obs}) for the complex over a range of

* In the paper referred to the value of ΔS_2^0 appears incorrectly as −5 e.u. instead of +5 e.u.

pH, using equation (7). K_{obs} was measured spectrophotometrically. Solutions of ferrimyoglobin (5×10^{-6}M) containing different concentrations of imidazole (0·001—0·10M) were made in glycine buffers covering the pH range 9·6—11·3 and adjusted to constant total ionic strength, $I = 0{\cdot}20$M. Under these conditions between 30% and 60% formation of complex was attained and no denaturation was detected. For each experiment, at a given pH and temperature, about eight mixtures were used. Optical densities were read at 417 mμ. K_{obs} is related to the optical densities of ferrimyoglobin, D_0, the complex, D_{100}, and the optical density of each mixture, D, by the relationship:

$$K_{obs} = (D - D_0)/(D_{100} - D)\,(\mathrm{Im}) \qquad (9)$$

where (Im) is the concentration of free imidazole in the equilibrium mixture. Optical density, D, is plotted against the function $(D_0 - D)/(\mathrm{Im})$ giving a straight line of slope $1/K_{obs}$ and intercept D_{100}. The method gives K_{obs} values with a precision of about ±4%.

Measurement of Optical Density and of pH.—The equipment and procedure used in making these measurements have already been described.[3]

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