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The Association of Imidazole with the Ions of Zinc and Cupric Copper[1a,b,c]

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The interaction of imidazole with the ions of zinc and cupric copper was determined by means of *p*H measurements on solutions containing imidazole, imidazolium ion and either copper or zinc ions, in solutions containing nitrate ion at ionic strength 0.16. In both cases it was found that the coördination number of the ion for imidazole was 4. The successive logarithmic intrinsic association constants at 4.5° were for Cu^{++}: 4.00, 3.63, 3.27 and 2.90; and for Zn^{++}: 2.16, 2.32, 2.53 and 2.80. Association constants at 22.5 or 24° were also determined, and approximate estimates of enthalpy and standard entropy changes in the association processes have been made. A new and rapid method for evaluating the association constants from the data, developed by Professor George Scatchard, is described. Copper–imidazole complexes show marked absorption in the visible between 590 and 690 mμ and in the ultraviolet between 230 and 290 mμ. The latter type of absorption is not shown by complexes between copper and amino groups. Absorption spectra of the copper–histidine complex indicate that the imidazole residues of histidine are involved in the chelate linkages formed.

The imidazole group plays an important role in biochemistry because of its presence in histamine and histidine. The imidazole groups of histidine are indeed generally responsible for most of the buffering power of proteins in the physiological *p*H range. Some work has been reported on the binding of metallic ions by the imidazole groups of histidine, and evidence has been obtained[2,3] that the imidazole groups of histidine residues are the most important binding sites for zinc and other ions in the case of serum albumin. To provide a proper basis for evaluating the binding power of the imidazole group in more complex compounds, a study of the binding of unsubstituted imidazole itself by zinc and by cupric copper ions was undertaken and the results are presented here.

Experimental Materials and Methods

***p*H Measurements.**—The instrument used for *p*H measurements was a Cambridge Instrument Company tubular glass electrode, of the type described by Bjerrum.[4]

Imidazole (m.p. 89.5–90°, lit. 89.5–90°) obtained from the Edcan Laboratories, South Norwalk, Connecticut, was used without further purification, after drying for several days over phosphorus pentoxide. It was prepared as the nitrate by addition to imidazole solution of suitably standardized nitric acid. The subsequent titrations were performed by the addition of sodium hydroxide solution which had been standardized against potassium acid phthalate. All water used was doubly distilled and boiled immediately before addition to the reagents. Since all measurements were made at *p*H values below 7.5—generally well below this value—the absorption of carbon dioxide during the titrations was probably negligible. Copper ion was introduced in the form of a standard solution of the nitrate which was made up approximately, and standardized iodometrically.[5] Zinc ion was also made up in the form of the nitrate. A solution was prepared from the hexahydrate of this salt and the zinc concentration determined by precipitation as zinc ammonium phosphate, filtration on asbestos and ignition at 900–1000° to the pyrophosphate which was weighed.[5] This method gave results reproducible within ±0.2%. Nitrate was chosen as the anion in the solutions used in these studies since the association of nitrate with zinc or cupric copper ions is small compared with that of halogens and other common anions.[6] In the use of solutions containing nitrate ion, and in the value of the ionic strength chosen, these solutions are comparable with those employed in the study of the interaction of serum albumin with zinc ions.[2]

Some of the titrations were carried out at room temperature (22.5–24°) as indicated below; others were carried out in a cold room at 4.5°, the entire glass electrode assembly having been moved into the cold room the day before the titration was to take place.

Interpretation of Experimental Results.—The procedure employed depended on the *p*H shift produced, in a mixture of imidazole and imidazolium ion, by the addition of a small amount of Zn^{++} or Cu^{++} ion. The use of such titrations to determine the association constants between a metallic ion and a ligand—in this case the base imidazole—has already been discussed in detail by Bjerrum.[4] Hence the method need be summarized only briefly here. It is first necessary that the pK' value of the imidazolium ion be determined in a medium of the same ionic strength as that employed in the titration in the presence of metal ions, and as closely similar as possible in general composition. The preliminary *p*H titrations in the absence of Cu^{++} or Zn^{++} were, therefore, carried out in a medium of ionic strength 0.16, the anions present being nitrate ions. The values of pK' determined on the glass electrode were 7.11 at 23° and 7.50 at 4.5°. These may be compared with the value of 7.08 reported by Dedichen[7] and 6.95 reported by Kirby and Neuberger[8] at 25° the latter being an extrapolated value at zero ionic strength. Tanford and Wagner[9] found 7.12 at 25° and ionic strength 0.15. Comparison of a titration curve of imidazole at 0.15 ionic strength with one at 0.01 showed a decrease in pK' by 0.15 *p*H unit when the ionic strength was lowered. The apparent heat of ionization of the imidazolium ion, calculated from our data at 23 and 4.5°, is approximately 6,000 cal./mole. This is not far from the value of 6,900 cal./mole which has been reported for the imidazole dissociation in histidine.[10]

The binding of imidazole by Zn^{++} and Cu^{++} was immediately apparent from the decrease of *p*H associated with the addition of zinc or copper nitrate to imidazole buffer solutions. The *p*H decrease was much greater for copper than for zinc, showing clearly that the binding constants for the former must be considerably larger than for the latter. For quantitative determination of the binding, the concentration of metallic ion in the solution was maintained constant and the concentration of free imidazole available for reaction with the metallic ion was adjusted by adding variable amounts of sodium hydroxide to solutions of imidazolium nitrate.

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(2) F. R. N. Gurd and D. S. Goodman, THIS JOURNAL, **74**, 670 (1952).

(3) C. Tanford, *ibid.*, **74**, 211 (1952).

(4) J. Bjerrum, "Metal Ammine Formation in Aqueous Solution," P. Haase and Son, Copenhagen, 1941, p. 116.

(5) I. M. Kolthoff and E. B. Sandell, "Textbook of Quantitative Analysis," The Macmillan Co., New York, N. Y., 1948; copper analysis, p. 630; zinc, p. 703.

(6) J. Bjerrum, *Chem. Revs.*, **46**, 381 (1950).

(7) G. Dedichen, *Ber.*, **39**, 1831 (1906).

(8) A. H. M. Kirby and A. Neuberger, *Biochem. J.*, **32**, 1146 (1938).

(9) C. Tanford and M. L. Wagner, THIS JOURNAL, **75**, 431 (1953).

(10) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, N. Y., 1943, p. 89.

In order to calculate the amount of imidazole bound to the copper or zinc ions, it is necessary to know the total stoichiometric concentration, T_{Im}, of imidazole present in all forms, bound and unbound, and to subtract from this the concentration of unbound basic imidazole (Im) and of imidazolium ion (ImH^+). It is readily demonstrated from the requirement of electroneutrality that the concentration of the latter is given by the equation

$$(ImH^+) = T_{Im} - (Na^+) + (OH^-) - (H^+) \quad (1)$$

The concentrations of both hydrogen and hydroxyl ions were generally small in our experiments compared with T_{Im} and (Na^+); the (OH^-) term generally was completely negligible, since the maximum value of hydroxyl ion concentration in these experiments was about 10^{-7}, while the imidazolium ion concentration was always of the order of 10^{-1}. The (H^+) term was similarly negligible in nearly all cases. It was thus found, in nearly all the solutions studied, that the concentration of imidazolium ion (ImH^+) could be taken as the total imidazole concentration, minus the amount of sodium hydroxide added to the original imidazolium nitrate solution; the amount of imidazole formed by dissociation of the imidazolium ion could be neglected except in the most acid solutions.

Since the value of pK' has been previously determined, and (ImH^+) is determined from eq. 1, the concentration (Im) of free basic imidazole is directly determined from the pH measurements.

$$pH = pK' + \log \frac{(Im)}{(ImH^+)} \quad (2)$$

The total imidazole concentration in the solution, T_{Im}, is given by the relation

$$T_{Im} = (Im) + (ImH^+) + \sum_{i=1}^{N} i(MIm_i^{++})$$

Here M^{++} stands for the metallic ion, which is assumed to be capable of forming with imidazole a series of complexes denoted by the symbol MIm_i^{++}, with the number (i) of bound imidazole molecules varying from 1 to the maximum coordination number, N. It will be seen later that N is equal to 4 for both Zn^{++} and Cu^{++}. The average number, $\bar{\nu}$, of imidazole molecules bound per metallic ion present is then given by the equation

$$\bar{\nu} = \frac{T_{Im} - [(Im) + (ImH^+)]}{C_M} \quad (3)$$

where C_M is the total concentration of metallic ions of all species, from M^{++} to MIm_N^{++} inclusive.

TABLE I

TITRATION OF ZINC NITRATE–IMIDAZOLIUM NITRATE MIXTURES

Total $Zn(NO_3)_2$, 0.0110 M; total imidazolium nitrate, 0.1260 M

(NaOH)	pH	(A) = (Im)	$\bar{\nu}$
Temp., 4.5°; pK' of imidazole = 7.50			
0.001716	4.72	0.000207	0.14
.003420	5.09	.000471	.27
.00685	5.40	.000950	.54
.01369	5.73	.00191	1.07
.02397	6.03	.00347	1.86
.03424	6.29	.00568	2.68
.04451	6.57	.00950	3.18
.0582	6.93	.0183	3.63
.0685	7.17	.0271	3.76
.0856	7.53	.0435	3.83
Temp., 24°; pK' of imidazole = 7.12			
0.001725	4.44	0.000263	0.12
.003450	4.82	.000620	.26
.006900	5.16	.00132	.51
.01380	5.52	.00275	.95
.02415	5.75	.00435	1.78
.0345	5.98	.00660	2.51
.0448	6.24	.0105	3.07
.0586	6.58	.0209	3.43

Titrations of zinc imidazole solutions were carried out at 4.5 and at 24° and the results are recorded in Table I. Titrations of cupric ion with imidazole were made at 22.5 and 23°; the results are recorded in Table II. From the data in these tables, what Bjerrum has denoted as the "formation function" of the system—that is, a plot of $\bar{\nu}$ against log (Im)—can immediately be constructed. It is obvious from the form of the curves, which are nearly symmetrical about $\bar{\nu} = 2$, that $\bar{\nu}$ is approaching a limiting value of 4 at high concentrations of free imidazole.

TABLE II[a]

TITRATION OF CUPRIC NITRATE–IMIDAZOLIUM NITRATE MIXTURES

Total $Cu(NO_3)_2$, 0.0100 M; total imidazolium nitrate, 0.1260 M

(NaOH)	pH	(A) = (Im)	$\bar{\nu}$
Temp., 4.5°; pK' of imidazole = 7.50			
0.0028	3.47	0.0000115	0.31
.0056	3.79	.0000235	0.58
.0084	4.07	.0000437	0.83
.0112	4.33	.0000776	1.11
.0140	4.57	.000132	1.38
.0168	4.73	.000186	1.65
.0224	5.13	.000442	2.19
.0280	5.56	.00112	2.68
.0364	6.08	.00339	3.28
Temp., 22.5°; pK' of imidazole = 7.11			
0.00312	3.34	0.0000210	0.36
.00624	3.70	.0000470	0.64
.00936	4.02	.0000960	0.93
.0125	4.30	.000178	1.23
.0156	4.52	.000282	1.53
.0312	5.58	.00282	2.83
.0406	6.03	.00710	3.33
.0530	6.46	.0162	3.65

DETERMINATIONS WITH VARIED CONCENTRATIONS OF TOTAL COPPER AND TOTAL IMIDAZOLE

t = 23°; pK' = 7.10

$NaNO_3$ added to bring ionic strength to 0.16

$T_{Cu^{++}}$	T_{Im}	(NaOH)	pH	pIm	$\bar{\nu}$
0.01191	0.0299	0.0075	4.50	4.25	0.62
.01191	.0299	.0150	5.22	3.71	1.24
.02382	.0598	.0299	4.92	3.70	1.25
.02382	.0598	.0448	5.64	3.28	1.86
.02382	.0598	.0523	6.10	3.12	2.16
.01191	.0598	.0299	5.72	2.90	2.40

[a] The values recorded in this last section of Table II were obtained with a different sample of imidazole about two years after the earlier measurements to provide an additional check on the validity of the method of calculating $\bar{\nu}$.

Analysis of the Experimental Data in Terms of the Association Constants.—To interpret the experimental data, we must evaluate the four successive association constants for the complexes MIm^{++}, MIm_2^{++}, MIm_3^{++} and MIm_4^{++}

$$k_1 = \frac{(MIm^{++})}{(M^{++})(Im)}; \quad k_2 = \frac{(MIm_2^{++})}{(MIm^{++})(Im)}; \quad k_3 = \frac{(MIm_3^{++})}{(MIm_2^{++})(Im)}; \quad k_4 = \frac{(MIm_4^{++})}{(MIm_3^{++})(Im)}$$

Alternatively we may express the results in terms of the "intrinsic constants" κ_1, κ_2, κ_3 and κ_4 which give the association constant for a single bonding locus, on the ion MIm_i^{++} for an imidazole molecule. The k's and κ's are related by the equations

$$\kappa_1 = k_1/4; \quad \kappa_2 = 2k_2/3; \quad \kappa_3 = 3k_3/2; \quad \kappa_4 = 4k_4$$

In our first calculations, the evaluation of the constants from the experimental data was carried out by the method of Bjerrum.[4] However, the procedure can be considerably shortened and simplified, without loss of accuracy, by the use of a method suggested to us by Professor George Scatchard. We define a function Q by the relation $Q = \bar{\nu}/(N - \bar{\nu})(\mathrm{A})$, where (A) denotes the concentration of free ligand molecules—in this case the concentration of uncharged imidazole. In terms of the association constants defined above, $\bar{\nu}$ for a metallic ion capable of binding four imidazole molecules is given by the equation

$$\bar{\nu} = \frac{k_1(\mathrm{A}) + 2k_1k_2(\mathrm{A})^2 + 3k_1k_2k_3(\mathrm{A})^3 + 4k_1k_2k_3k_4(\mathrm{A})^4}{1 + k_1(\mathrm{A}) + k_1k_2(\mathrm{A})^2 + k_1k_2k_3(\mathrm{A})^3 + k_1k_2k_3k_4(\mathrm{A})^4} \quad (4a)$$

or by the alternative expression written in terms of the κ's:

$$\bar{\nu} = \frac{4[\kappa_1(\mathrm{A}) + 3\kappa_1\kappa_2(\mathrm{A})^2 + 3\kappa_1\kappa_2\kappa_3(\mathrm{A})^3 + \kappa_1\kappa_2\kappa_3\kappa_4(\mathrm{A})^4]}{1 + 4\kappa_1(\mathrm{A}) + 6\kappa_1\kappa_2(\mathrm{A})^2 + 4\kappa_1\kappa_2\kappa_3(\mathrm{A})^3 + \kappa_1\kappa_2\kappa_3\kappa_4(\mathrm{A})^4} \quad (4b)$$

From eq. 4b, and the definition of Q, we obtain

$$Q = \frac{\bar{\nu}}{(4 - \bar{\nu})(\mathrm{A})} = \frac{\kappa_1[1 + 3\kappa_2(\mathrm{A}) + 3\kappa_2\kappa_3(\mathrm{A})^2 + \kappa_2\kappa_3\kappa_4(\mathrm{A})^3]}{1 + 3\kappa_1(\mathrm{A}) + 3\kappa_1\kappa_2(\mathrm{A})^2 + \kappa_1\kappa_2\kappa_3(\mathrm{A})^3} \quad (5)$$

The limiting value of Q as (A) approaches zero is given by the relation

$$\lim_{(\mathrm{A})\to 0} Q = \frac{k_1}{4} = \kappa_1 \quad (6a)$$

On the other hand, as (A) approaches infinity and $\bar{\nu}$ approaches N, Q assumes the limiting form

$$\lim_{(\mathrm{A})\to\infty} Q = 4k_4 = \kappa_4 \quad (6b)$$

Figures 1 and 2 show the plot of log Q as a function of $\bar{\nu}$ for the interaction of imidazole with zinc and with cupric ions, respectively, derived from the data of Tables I and II. The extrapolations of log Q to $\bar{\nu} = 0$ and $\bar{\nu} = 4$, although attended by some uncertainty, give the values of log κ_1 and log κ_4 to a fairly close approximation, probably within ± 0.05 for the data considered here.

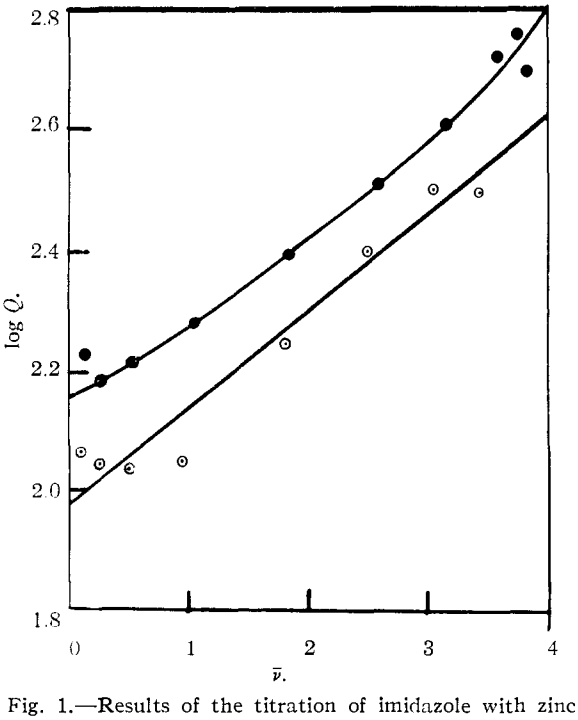

Fig. 1.—Results of the titration of imidazole with zinc ion at two different temperatures (●, 4.5°; ○, 24.0°) plotted by the method of Scatchard discussed in the text.

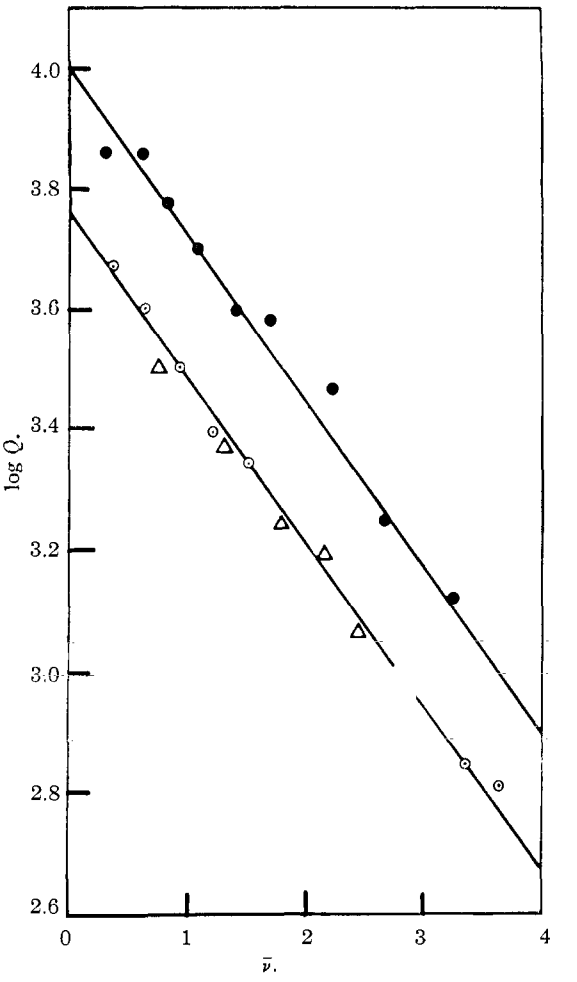

Fig. 2.—Titration data for copper with imidazole at two different temperatures (●, 4.5°; ⊙, 22.5°) plotted by the method of Scatchard. Lower curve was drawn to fit the points represented by circles (see data of Table II). The points represented by triangles, corresponding to the data listed in the last portion of Table II, were added subsequently to the curve and show the consistency of the measurements under different conditions.

Estimates of κ_2 and κ_3 may be derived from the functions Q, if κ_1 and κ_4 are known, by considering the limiting slopes of the curves for log Q as $\bar{\nu}$ approaches zero or 4, respectively. As $\bar{\nu}$ approaches zero, the derivative of ln Q with respect to $\bar{\nu}$ is readily shown to be

$$\lim_{\bar{\nu}\to 0} \frac{\mathrm{d} \ln Q}{\mathrm{d}\nu} = 3(\kappa_2 - \kappa_1)/4\kappa_1 \quad (7)$$

and, as $\bar{\nu}$ approaches 4, and 1/(A) approaches zero

$$\lim_{\bar{\nu}\to 4} \frac{\mathrm{d} \ln Q}{\mathrm{d}\nu} = 3(\kappa_4 - \kappa_3)/4\kappa_3 \quad (8)$$

Equation 7 is most readily derived by taking the limit of $\mathrm{d} \ln Q/\mathrm{d}(\mathrm{A}) = 3(\kappa_2 - \kappa_1)$ as (A) $\to$ 0; and the limit of $\mathrm{d}\bar{\nu}/\mathrm{d}(\mathrm{A}) = 4\kappa_1$ as (A) $\to$ 0. The ratio of these two limits

is the limiting slope defined in 7. Similarly eq. 8 may be derived by differentiating ln Q and $\bar{\nu}$ with respect to $(A)^{-1}$, as (A) becomes infinite and $\bar{\nu}$ approaches 4. This gives the limit of $d \ln Q/d(A)^{-1} = 3(\kappa_3 - \kappa_4)/\kappa_3\kappa_4$ as $(A)^{-1} \rightarrow 0$; and the limit of $d\bar{\nu}/d(A)^{-1} = -4/\kappa_4$. The ratio of these two limiting slopes gives the slope defined in 8.

The definition of the function Q is readily generalized for the case in which there are N sites available for binding of the ligand. In eq. 6a, k_1/N is written instead of $k_1/4$. Equation 6b is unchanged, except that we write k_N and κ_N, respectively, instead of k_4 and κ_4. Equations 7 and 8 become, respectively

$$\lim_{\bar{\nu}\rightarrow 0} \frac{d \ln Q}{d\bar{\nu}} = (N - 1)(\kappa_2 - \kappa_1)/N\kappa_1 \qquad (7a)$$

and

$$\lim_{\bar{\nu}\rightarrow N} \frac{d \ln Q}{d\bar{\nu}} = (N - 1)(\kappa_N - \kappa_{N-1})/N\kappa_{N-1} \qquad (8a)$$

To determine the limiting slopes defined by eqs. 7 and 8, data of high precision in the neighborhood of $\bar{\nu} = 0$ and $\bar{\nu} = 4$, respectively, are required. Experimentally it is in just these regions that accurate values are most difficult to obtain. The values of the κ's estimated from these slopes, therefore, are to be regarded as preliminary and subject to further adjustment, in order to give the best fit to the experimental data over the whole range covered by the measurements. This method of analysis, however, gives preliminary values of the constants with much less labor than Bjerrum's procedure, and the final values are obtained without much further work.

In the limiting case of a set of N binding sites which are all equivalent and independent in their binding affinity for the ligand A—that is, in the case for which all the κ's are identical—the function Q is independent of (A) and equal to κ_1. This is immediately apparent from eq. 5. For such a system, a plot of the type shown in Figs. 1 and 2 would be a horizontal straight line. The next simplest case to that in which all the κ's are equal is that in which $\kappa_{\nu+1}/\kappa_\nu$ is a constant independent of ν, for all values of ν from 1 to N. This constant may be greater or less than unity, depending on whether the interactions are positive or negative. It is equal to $1/X^2$, where X^2 is denoted by Bjerrum[4] as a "spreading factor" relating the successive constants in such cases. Computation shows that, if this relation holds, log Q is not a strictly linear function of $\bar{\nu}$, although it does not deviate far from linearity. Calculations were made for a hypothetical system for which $\kappa_2/\kappa_1 = \kappa_3/\kappa_2 = \kappa_4/\kappa_3 = 0.40$. This gives a curve for log Q as a function of $\bar{\nu}$ which is fairly close to the experimental data for the copper–imidazole system, through which we have drawn straight lines in Fig. 2. If, for the hypothetical system mentioned above, a straight line is drawn between log Q = log κ_1, at $\bar{\nu} = 0$, and log Q = log κ_4, at $\bar{\nu} = 4$, this line intersects the computed curve at the mid-point, $\bar{\nu} = 2$. To the left of the mid-point, the curve lies above the straight line, to the right, below it, the mid-point being a center of symmetry for the curve. (Such symmetry is to be expected for any system involving N association constants, if they are related by a constant spreading factor.) The maximum deviation between the curve and the straight line is approximately 0.06 in log Q, and occurs at or near $\bar{\nu} = 1$ and $\bar{\nu} = 3$.

We have not explored the nature of the curves to be expected in more complicated cases, when the κ's are no longer related by a constant spreading factor. It should be clearly understood, however, that eqs. 6a, 6b, 7 and 8 hold quite generally for a system containing 4 equivalent sites for the binding of ligands; and 7a and 8a, with the general analogs of 6a and 6b, for a system containing N equivalent sites, regardless of the relative values for the successive association constants. The papers of Scatchard[11] and Wyman,[12] and chap. 20 of ref. 10 may be consulted with respect to the formulation of concepts closely related to the method presented here.

If the binding sites are all equivalent, but there is interaction between the bound ligand molecules attached to the same binding center—as in the experiments reported here—the slope of the curve shows the character of the interactions, as indicated by the discussion above. It is obvious from Figs. 1 and 2 that there is a striking difference between the copper–imidazole and the zinc–imidazole systems. The slopes of the curves for the copper system are large and negative, whereas for zinc they are markedly positive. Correspondingly the successive values of κ, from κ_1 to κ_4, progressively decrease for copper and increase for zinc. The first imidazole molecule taken on is bound much more strongly by the cupric ion than by the zinc ion. However, the binding of the first imidazole by copper decreases the affinity for imidazole of the other three available ligand positions around the cupric ion; whereas for zinc the binding of one imidazole molecule enhances the tendency to bind others. In other words, the copper–imidazole system shows negative interactions between the imidazoles; the zinc–imidazole system shows positive interactions. The slopes of the curves in Figs. 1 and 2 indicate immediately the character of the interactions between the successively bound molecules. The method also reveals clearly the influence of errors in the data which are much less apparent in the plot of the "formation function" as given by Bjerrum. It is obvious that there is some scatter in the data of Figs. 1 and 2. Some of the points at the extreme ends of the curves have been disregarded in drawing the graphs.

The successive values of log κ obtained from the data in Tables I and II are given in Table III. Their estimated accuracy is of the order of ± 0.05. The values for zinc at 24° are the least certain.

TABLE III

CALCULATED INTRINSIC ASSOCIATION CONSTANTS[a] FOR Cu^{++} AND Zn^{++} WITH IMIDAZOLE

	Cu^{++}(4.5°)	Zn^{++}(4.5°)	Cu^{++}(22.5°)	Zn^{++}(24°)
log κ_1	4.00	2.16	3.76	1.98
log κ_2	3.63	2.32	3.39	2.19
log κ_3	3.27	2.53	3.03	2.41
log κ_4	2.90	2.80	2.66	2.62

[a] Note that these intrinsic constants are not identical with the constants k_1, k_2, k_3, k_4 appearing in equations 4–6 inclusive, but are related with them by the equations previously given.

(11) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949).

(12) J. Wyman, *Advances in Protein Chem.*, **4**, 407 (1948); see especially p. 436 ff.

The values of log κ_2 and log κ_3 for the zinc interaction at 4.5° are difficult to arrive at with certainty. In our earlier calculations by the laborious Bjerrum method the values 2.22 and 2.56, respectively, were obtained after ten successive approximations. The constants are so crowded together that it is impossible to distinguish which set of constants is more nearly correct. The Bjerrum method yielded precisely the same values of log κ_1 and log κ_4 as are shown in Table III. A marked advantage of the method of Scatchard over that of Bjerrum is that it yields κ_1 and κ_4 directly without the necessity of repeated approximations in which, for instance, the value assigned to κ_2 may influence the final value chosen for κ_1. For some purposes, κ_1 is the constant which must be known most accurately.[2]

Since the curves for the copper–imidazole interaction in Fig. 2 are parallel and the curve for 4.5° lies above that for 22.5°, it follows that the standard heats of reaction for each successive step are approximately equal and negative. The value of ΔH° under the conditions of this study was calculated to be roughly −2200 cal./mole. The combination of this value with the free energy changes derived from the constants in Table III gave values for the successive standard entropy changes of roughly 13, 10, 6 and 3 cal. deg.$^{-1}$ mole^{-1}, respectively. The values are sufficiently reliable to show that the entropy changes are positive and decrease with successive association reactions, but a more detailed interpretation would not be justified. The heats of reaction for zinc–imidazole appear to be of similar magnitude to those for copper–imidazole; obviously, considering the scatter of the points, the measurements are not reliable enough to be useful for the calculation of entropy changes.

Evidence that the Acidity of the Aqueous Copper and Zinc Ions, and of Bound Imidazole Molecules, Can Be Neglected in the Calculation of $\bar{\nu}$

In the steps leading to the calculation of it has been assumed that all the hydroxyl ion added in the form of sodium hydroxide is used for the removal of protons from the imidazolium ion, except for the negligible amount which is represented by the increase of free hydroxyl ion concentration during the titration. However, this assumption neglects the fact that the hydrated cupric or zinc ion is also an acid and that a small fraction of the added hydroxyl ion serves to convert the metallic ion into its hydroxide. Our discussion up to this point has involved the assumption that this effect is negligible by comparison with the conversion of ImH^+ to Im. Quantitative data appear to justify this assumption.

In the case of the copper ion, detailed studies of hydroxide formation have been made by Pedersen[13] who explained his findings on the basis of three reactions

$$Cu^{++} \rightleftharpoons CuOH^+ + H^+ \quad (A)$$

$$2Cu^{++} \rightleftharpoons Cu_2OH^{+++} + H^+ \quad (B)$$

$$2Cu^{++} \rightleftharpoons Cu_2O^{++} + 2H^+ \quad (C)$$

We denote the equilibrium constants for these reactions as k_A, k_B and k_C, respectively. (Pedersen denoted them as k_1, k_2 and k_3).

$$k_A = \frac{(CuOH^+)(H^+)}{(Cu^{++})} = 10^{-8.26} \quad (9)$$

$$k_B = \frac{(Cu_2OH^{+++})(H^+)}{(Cu^{++})^2} = 10^{-6.56} \quad (10)$$

(13) K. J. Pedersen, *Kgl. Danske Videnskab. Selskab. Mat.-fys Medd.*, **20**, No. 7 (1943).

$$k_C = \frac{(Cu_2O^{++})(H^+)^2}{(Cu^{++})^2} = 10^{-11.10} \quad (11)$$

The values indicated for these three constants are those which Pedersen reported for the ionic strength of 0.16 which was used in our experiments.

The order of magnitude of these constants indicates that it is a reasonable first approximation to calculate the total concentration of copper in all forms as if it were given by the sum of the free Cu^{++} and of the imidazole complexes $CuIm^{++}$ to $CuIm_4^{++}$. The concentrations of $CuOH^+$, Cu_2OH^{+++} and Cu_2O^{++} can then be evaluated from the measured values of (H^+) and the estimated values of (Cu^{++}). Using the first approximation indicated above, the relation between the total stoichiometric concentration of copper, T_{Cu} and Cu^{++} ion concentration is given by the relation

$$T_{Cu} = (Cu^{++})(1 + k_1(A) + k_1k_2(A)^2 + k_1k_2k_3(A)^3 + k_1k_2k_3k_4(A)^4) \quad (12)$$

where the k values are the imidazole association constants previously defined, and employed in equations 4, 5 and 6.

As an example we may consider one of the solutions at 22.5° listed in Table II, at pH 4.52 and $\nu = 1.53$. Since $T_{Cu} = 0.01$, we may calculate from eq. 12 and the data of Table III that $(Cu^{++}) = 10^{-3.6}$. From eq. 9 we then obtain $(CuOH^+) = 10^{-7.3}$, which is entirely negligible in the calculation of $\bar{\nu}$, as it was evaluated by the procedures previously outlined. It is seen from eqs. 10 and 11 that the concentrations of Cu_2OH^{+++} and of Cu_2O^{++} are still lower than that of $CuOH^+$. Similar calculations can be used to show that the concentrations of these ions are negligible at all the other points on the curve.

This treatment neglects the acidity of the bound water in the intermediate Cu–imidazole complexes, which can be denoted by such formulas as $Cu(Im)_2(H_2O)_2^{++}$, assuming that water molecules occupy the coördination positions around the copper not occupied by imidazoles. On statistical grounds alone, such ions should be weaker acids than $Cu(H_2O)_4^{++}$. The only experimental evidence on this point of which we are aware is in the study of Prue and Schwarzenbach[14] on the copper complex of diethylenetriamine ("den"). Their titration curves indicated the acid ionization $Cu(den)^{++} \rightleftarrows Cu(den)(OH)^+ + H^+$, with a value of k near $10^{-9.5}$, or about 20 times weaker than the corresponding reaction for Cu^{++} (see eq. 9). This is considerably greater than the decrease by a factor of 3 which would be expected on purely statistical grounds. Even if the effect of imidazole is not so large as that of "den," the acidity of these intermediate complexes should be negligible for the purposes of our calculations.

There appears to be no study on the acidity of aqueous zinc ions as detailed as that of Pedersen for copper. However, Prytz,[15] in a careful piece of work, reported the equilibrium constant

$$(ZnOH^+)(H^+)/(Zn^{++}) = 10^{-9.0}$$

Since this is considerably smaller than the corresponding value for copper (eq. 9) calculation shows that the effects of the acidity of the zinc ion are negligible in the zinc–imidazole system, even though the association constants of zinc for imidazole are less than those of cupric copper.

It is probable that an imidazole molecule, when linked to a metallic ion, is an acid of appreciable strength, owing to resonance which strengthens the acidity of the =NH group not bound to the metallic ion. The acidity of imidazole complexes of ferrous iron has been discussed by Coryell and Pauling,[16] in connection with their consideration of the heme-linked acid groups in hemoglobin. They concluded that in such structures acid pK values of the order of 7 might be found. However, it appears unlikely from our measurements that the acid pK values of the copper and zinc imidazole derivatives can be as low as this. The measurements made with varying concentrations of total copper and total imidazole, recorded in the last part of Table II, gave no indication of discrepancies requiring explanation along these lines. The value of $\bar{\nu}$ appeared to be a function only of the calculated concentration of free imidazole and not of the pH or of the total copper concentration, It was, there-

(14) J. E. Prue and G. Schwarzenbach, *Helv. Chim. Acta*, **33**, 985 (1950).

(15) M. Prytz, *Z. anorg. allgem. Chem.*, **200**, 133 (1931).

(16) C. D. Coryell and L. Pauling, *J. Biol. Chem.*, **132**, 769 (1940).

fore, concluded that the neglect of the acidity of imidazole bound to metallic ions was justifiable in the computation of $\bar{\nu}$ under the conditions we have employed.

Absorption Spectra of the Copper Complexes of Imidazole and Histidine

Imidazole reacts with copper to give an intense color which varies from a greenish blue, when the ratio of imidazole to Cu^{++} is low, to a deep blue-purple when the ratio is so large that the predominant complex is $Cu(Im)_4^{++}$. The color changes were particularly striking when cupric chloride was used as the source of copper ion; however, solutions of the nitrate were almost always employed in the quantitative measurements, since the nitrate ion has far less tendency than chloride to bind to Cu^{++}.

Absorption Spectra in the Visible.—The absorption spectra in the visible for varying ratios of imidazole to total copper are shown in Fig. 3. All readings were made against blanks containing copper nitrate at the same total copper concentration as in the solution containing imidazole. All values of extinction coefficient are given for a one-cm. path length, in terms of the total copper concentration in moles per liter of solution. The absorption shown in the bottom curve of Fig. 3, when the molar ratio of imidazole to Cu^{++} is 0.4, is almost entirely due to Cu^{++} and to the complex $CuIm^{++}$; approximately 4/10 of the total copper being in the form of this complex and 6/10 in the form of free cupric ion. The proportion of the higher complexes is very small. The absorption maximum is at approximately 690 mμ; the observed extinction coefficient at the maximum is 4.2; this represents the excess extinction in the solution containing imidazole above that for a solution containing the same total copper concentration in the form Cu^{++}. Klotz[17] has reported an absorption spectrum for cupric ion which gives $\epsilon = 6.0$ at 690 mμ. From these values of extinction coefficients we may calculate the molar extinction coefficient for $Cu(Im)^{++}$ as approximately 16.5 l. $mole^{-1}$ $cm.^{-1}$.

As the molar ratio of imidazole to copper increases, the extinction increases also and the absorption maximum is displaced to shorter wave lengths. In the uppermost curve of Fig. 3 it may be calculated, from the association constants given earlier in this paper, that at least 95% of all the copper is in the form $Cu(Im)_4^{++}$. Hence the observed extinction coefficient of 53 at 590 mμ may be taken as the molar extinction coefficient for $Cu(Im)_4^{++}$ with a probable error ±2. The intermediate curve in Fig. 3 shows an intermediate state of the absorption spectrum. A large number of other measurements not reported here gave results which were fully concordant with those shown. No attempt has been made, however, to calculate the absorption spectra of all the individual copper imidazole complexes as Bjerrum[4] has done for the ammonia complexes of copper and nickel.

Absorption in the Ultraviolet.—The ultraviolet absorption of free imidazole is practically negligible in the wave length region above 240 mμ and the zinc imidazole complexes also show no significant absorption in this region. The complexes of copper and imidazole, however, show marked absorption in the region between 240 and 290 mμ, the extinction coefficients rising as the ratio of imidazole to copper increases, and the position of the maxima being displaced progressively in the direction of longer wave lengths. The wave length of the absorption maximum, therefore, shifts in the opposite direction from that observed in the visible. Examples of the spectra are shown in Fig. 4 for three different ratios of imidazole to copper. It will be observed that in the uppermost curve, for a solution in which the copper is present almost entirely in the form $Cu(Im)_4^{++}$, an absorption maximum is obtained at approximately 278 mμ.

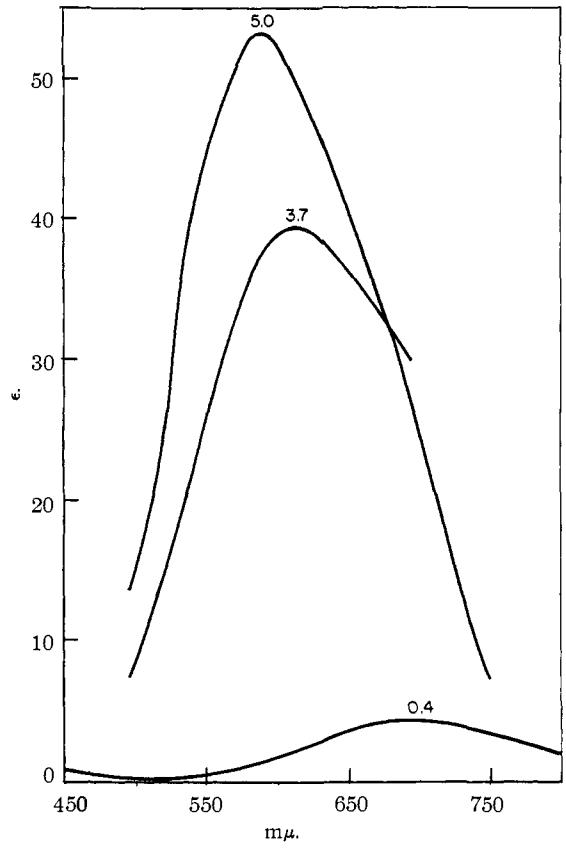

Fig. 3.—Absorption spectra of copper–imidazole complexes in the visible region of the spectrum. The number above each curve indicates the molar ratio of total imidazole to total copper in the system.

These absorption bands in the ultraviolet are of particular interest because such absorption is apparently not exhibited by complexes of copper with amino groups, which show strong absorption in the visible. A spectrum of the $Cu(NH_3)_4^{++}$ complex showed progressively decreasing absorption from 230 to 280 mμ, except for a small maximum near 235 mμ. The value of ϵ decreased from approximately 60 at 235 mμ to 20 at 280 mμ. Thus the absolute value of the extinction coefficient was very small over the whole range compared to that for $Cu(Im)_4^{++}$ and there was no evidence whatever of a maximum in the curve above 235 mμ.

It is thus possible to detect complex formation between imidazole groups and copper ions, by

(17) I. M. Klotz, L. L. Faller and J. M. Urquhart, *J. Phys. Colloid Chem.*, **54**, 18 (1950).

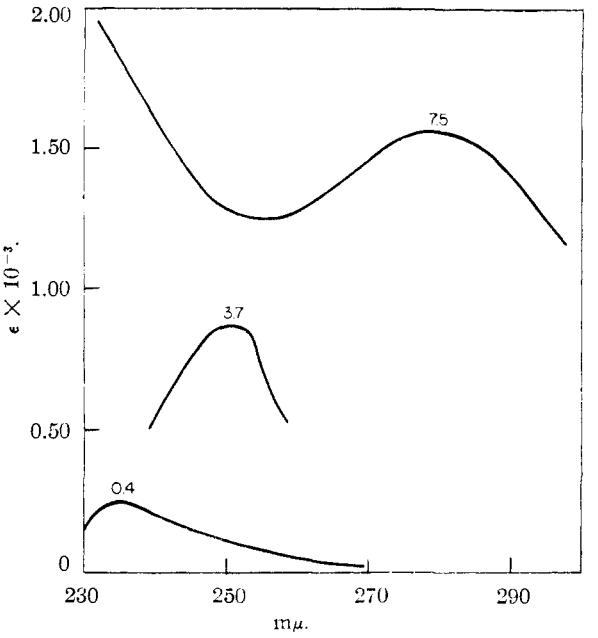

Fig. 4.—Absorption spectra of copper-imidazole complexes in the ultraviolet. The number above each curve indicates the molar ratio of total imidazole to total copper in the system.

means of the absorption spectrum, in solutions of compounds containing both amino and imidazole groups. The absorption spectra of copper complexes of histidine in the ultraviolet (Fig. 5) illustrate such a case. Curve III shows the absorption spectrum of histidine without copper. Curve I shows the copper–histidine complex at a molar ratio histidine:Cu^{++} of 75:1, and Curve II represents the difference between I and III. There is a distinct absorption maximum in the resulting curve between 250 and 255 mμ. Comparison with Fig. 4 indicates that such a maximum would be expected if two imidazole groups of histidine were interacting with copper. It seems probable that the copper–histidine complex involves two histidine molecules bound to the four ligand positions, each histidine being bound through its amino and its imidazole group. It is, of course, theoretically possible that the carboxyl groups of the histidine molecules are also involved. For comparison, the absorption spectrum of the copper glycine complex was determined using the same molar amounts of amino acid and copper nitrate as in the histidine study and a similar set of curves was constructed. This showed an absorption maximum at 235 mμ, practically identical with that found in the copper ammonia complex.

Maley and Mellor[18] have already drawn the conclusion from titration studies that the imidazole group of histidine is important for its binding to cupric ion. Comparison of the values for the histidine association constants (for copper, log (k_1k_2) = 18.33, and for zinc, 12.88) shows that these are considerably higher than the equivalent constants for glycine (15.42 and 9.72, respectively).

In view of the fact that the constants for imidazole binding are almost identical with the binding constants for $-NH_2$ and related groups,[4,6] and since the carboxyl binding power is considerably less,[6] we may reasonably conclude that the increased binding capacity of histidine is due to its ability to bind through the imidazole and amino groups, rather than through carboxyl groups.[19]

(18) L. E. Maley and D. P. Mellor, *Australian J. Sci. Research*, **2A**, 579 (1949).

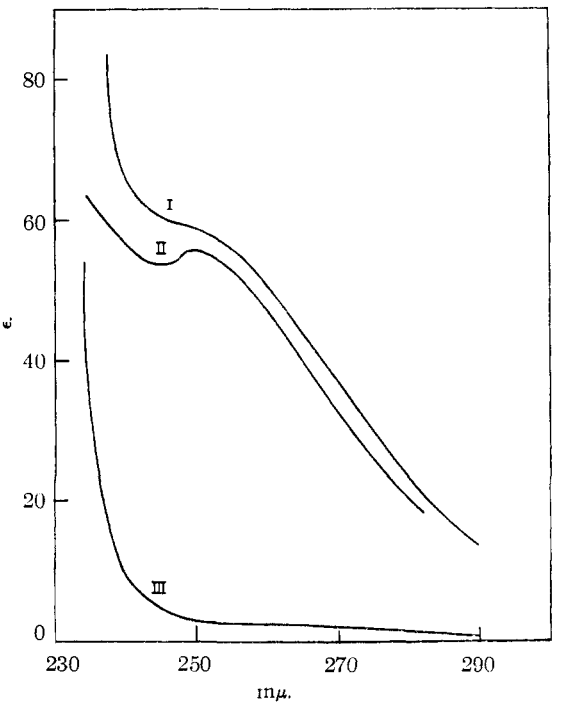

Fig. 5.—Absorption spectra of copper–histidine complexes: curve I represents absorption spectrum of the complex at a molar ratio of histidine–copper of 75:1; curve III is the absorption spectrum of histidine alone at the same concentration; curve II is the difference spectrum between curve I and curve III.

Crystalline Complexes

In the course of these studies, some data were obtained for the approximate solubilities of the complexes. It was found, for example, that the solubility of the salt $(CuIm_4^{++})(Cl^-)_2$ was of the order of 0.5 *M*. By adding various amounts of acids and thereby changing the ratio of copper to basic imidazole in solution, it was found possible to precipitate several different types of crystals in the presence of $CuCl_2$. These crystals ranged in color from the green of the complexes containing small amounts of imidazole to blue-violet plates which presumably represented the higher complexes.

In some of the studies on zinc complexes concentrated solutions were prepared containing approximately 2 *M* imidazolium ion, 2 *M* imidazole and 4 *M* zinc chloride. The solution was thus approximately 2 *M* in the monocoördinated complex. Shaking of this mixture led to crystal formation although a super-saturated solution could be produced by slow cooling. An analysis of the crystals indicated the presence of two imidazole molecules and two chloride ions for each zinc ion present. The crystals were soluble with difficulty in water but addition of a small amount of acid facilitated solution. The crystals were transparent needles which could, with care, be grown to fairly large dimensions. They were strongly birefringent and melted sharply at 161°.

(19) This conclusion was drawn by Maley and Mellor on the basis of their comparison of the binding constants of histidine with those of other amino acids; the tacit assumption was made by them that imidazole binding was approximately equivalent to amino binding, an assumption which is shown to be quite accurate.

Discussion

The evidence given here shows that the maximum coördinate number of cupric and zinc ions for imidazole molecules is 4, a finding completely in accord with the evidence derived with other complexes of these ions. Whereas the first imidazole molecule attached to Cu^{++} is bound almost a hundred times as strongly as the first imidazole attached to Zn^{++} (see Table III), the interactions between the successively bound molecules are negative in the case of copper, positive in the case of zinc, as previously pointed out. The difference between copper and zinc in this respect is analogous to that found by Bjerrum for the ammonia complexes of these two ions (see reference 4, Table 4, on page 57, and Table 8, on p. 71 of Bjerrum's monograph).

The stereochemical evidence from crystallographic studies shows that the tetracoördinated complexes of zinc are generally arranged in tetrahedral configuration around the metallic ions, while those of cupric copper are planar.[20] The planar configuration of the ion $Cu(Im)_4^{++}$ would impose a very close approach of some of the atoms in adjoining imidazole rings around the central copper ion, if the plane of all the imidazole groups were to coincide with the plane of the four Cu–N bonds. Much of the steric hindrance could be avoided by rotation of the planes of some of the imidazole groups around these bonds. However, if there is any significant amount of single bond-double bond resonance in the Cu–N bond, this would tend to stabilize the completely planar configuration. The steric obstacles to the stability of this configuration may largely explain the consistently decreasing values of the successive association constants. On the other hand, the formation of the zinc imidazole complexes with their tetrahedral coördination should not be impeded by any steric hindrance of comparable magnitude. This in itself provides no explanation of the observed fact that the zinc imidazole association constants, after correction for the statistical effect, increase progressively from κ_1 to κ_4. It does, however, indicate the absence of the sort of steric interference which would lead to marked negative interactions.

It is interesting to contrast the cases of copper–imidazole in which $\kappa_1 > \kappa_2 > \kappa_3 > \kappa_4$ and of zinc–imidazole in which $\kappa_1 < \kappa_2 < \kappa_3 < \kappa_4$ with that of cadmium in which the values of κ decrease gradually. Tanford and Wagner[9] reported for cadmium–imidazole at 25° the following values of log k_1, k_2, k_3 and k_4: 2.80, 2.10, 1.55 and 1.13. When converted to log κ values, these are 2.20, 1.92, 1.73 and 1.73, respectively.

It has already been shown recently[2] that the binding of zinc ions by human serum albumin is described by an association constant with a value of log k = 2.82, after correction for charge effects. Evidence was given, based on the number of zinc binding groups in the albumin molecule and on other grounds, for believing that these groups were imidazole groups of histidine residues. Indeed, the agreement with the value of log k_1 = log κ_1 + log 4 = 2.76 for the zinc imidazole complex at 4.5°, as reported here, is extraordinarily good. There is every reason to believe that the imidazole groups found in other proteins play an important part in the binding of zinc and other divalent cations by these large molecules. The association constant for zinc and imidazole groups is approximately the same as for zinc and amino groups. However, since the association constant for imidazole with hydrogen ions is lower than that for amino groups by a factor of the order of 1000, the imidazole groups of proteins are far more likely under physiological conditions to be available for binding with metallic ions.

Acknowledgment.—We are deeply indebted to the criticisms and suggestions of Prof. George Scatchard, who developed the method for evaluating the association constants shown in Figs. 1 and 2 and discussed in the accompanying text.

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(20) See the discussion by Bjerrum (ref. 4) especially pages 84–113 inclusive; also A. F. Wells, "Structural Inorganic Chemistry," Second Ed., Oxford, 1950, pp. 316 and 617 ff.